Oral ecosystem modulation and bacterial pneumonia

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Joshua Twigg

Oral and Biomedical Sciences

School of Dentistry

Cardiff University

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Acknowledgements

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Abstract

Bacterial pneumonia affects a disproportionate number of elderly in the UK, with substantial morbidity and mortality. Mounting evidence implicates removable dentures as a potential nidus for respiratory pathogens to form a pathogenic reservoir which could seed colonisation and infection of respiratory tissues in susceptible individuals. However, research evaluating the denture-associated microbiome in patients with an active pneumonia diagnosis is lacking. This research had two primary aims. Firstly, unrestricted characterisation of denture-associated bacterial communities in pneumonia patients and long-term care home residents, through 16S rRNA gene metataxonomic sequencing. Secondly, development of a model denture acrylic-associated biofilm incorporating clinically relevant respiratory pathogens, to allow exploration of inter-microbial and host-microbial interactions in infection and testing of novel anti-biofilm strategies.

There were significant shifts observed in species composition, diversity and richness in denture-associated microbiota of pneumonia patients. Importantly, the relative abundance of putative respiratory pathogens was significantly increased in pneumonia patients compared with respiratorily healthy care home residents. The magnitude of this increase was approximately tripled in denture-associated bacterial communities compared with other oral sites examined.

An *in vitro* denture-acrylic biofilm model was developed incorporating the respiratory pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This model was extensively characterised using a range of techniques and showed excellent scalability to high throughput applications. Subsequent infection of epithelial tissue models revealed the role of *P. aeruginosa* in instigating tissue invasion and disruption. A potential novel function for the pseudomonal aprA gene, encoding alkaline protease was suggested; providing a competitive advantage to this microorganism in mixed-species infections. A range of novel antimicrobial compounds was screened for biocidal activity and successfully incorporated into silicone biomaterials. However, antimicrobial activity of several tested compounds was abated in biomaterials. Highly controlled microwave energy delivery using a cavity resonator showed considerable promise as a denture sterilisation modality.

This work was novel in several aspects. The *in vitro* model was the first model of denture-associated biofilms to incorporate respiratory pathogens, and subsequent molecular characterisation revealed the biogeography of microorganisms within the biofilm and in tissue infections. The models were subsequently employed in a high throughput screening assay wherein novel antimicrobial compounds and antibiofilm methods were tested. The clinical study was the first to examine the denture-associated oral microbiome in pneumonia patients, with novel insights into changes in bacterial composition associated with respiratory infection.

CHAPTER 1: INTRODUCTION

1.1: Microbial biofilms

Biofilms can be defined as "aggregates of microorganisms in which microbial cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface" (Vert et al. 2012). Biofilms are the preferred form of microbial growth and represent a ubiquitous and highly adaptable mode of life, permitting the colonisation of an unparalleled range of ecological niches (Donlan 2002). The biofilm lifestyle confers a survival advantage to microorganisms compared with a planktonic mode of growth and permits greater microbial diversity through modulation of nutrient and metabolite gradients, oxygen tension and pH to produce unique micro-environments (Stewart and Franklin 2008). In humans and all higher animals, microbes exist on all body surfaces, and can form biofilms on a number of host tissues (Cho and Blaser 2012). During health, a number of highly regulated homeostatic mechanisms maintain a stable population of microbial species, which are beneficial or neutral to the host, developed through co-evolution. Such microbial communities are considered "commensal". However, because of their protective features, microbial biofilms are the predominant mechanism by which disease pathogenesis, transmission and resistance to both host defences and therapeutic intervention is enabled (Lewis 2001).

1.1.1: The developmental model of biofilm formation

The establishment of microbial biofilms has been studied for several decades (O'Toole et al. 2000). From early observational data which enabled little insight into complex and varied biofilm architectures, advances in imaging techniques, microelectrodes and molecular methods have opened a window into the structural intricacy, micro-environmental heterogeneity and hierarchical genetic control of the biofilm lifecycle (Azeredo et al. 2017).

While far from conclusive, and not without competing explanations, the developmental model of biofilm formation (Figure 1) is a useful framework for examining the events which lead to microbial biofilm assembly (O'Toole et al. 2000; Monds and O'Toole 2008). In this model, biofilms form in discreet, ordered stages: reversible attachment of microbes to a surface, then "irreversible" adherence mediated by specific adhesive proteins (adhesins). Following commitment to surface-adherent growth, clonal growth of microcolonies and production of extracellular polymeric substance (EPS) ensues. During subsequent maturation of the biofilm, the majority of structural organisation is generated, alongside a number of important

genotypic and phenotypic changes, followed finally by dispersal of planktonic microorganisms to colonise new areas.
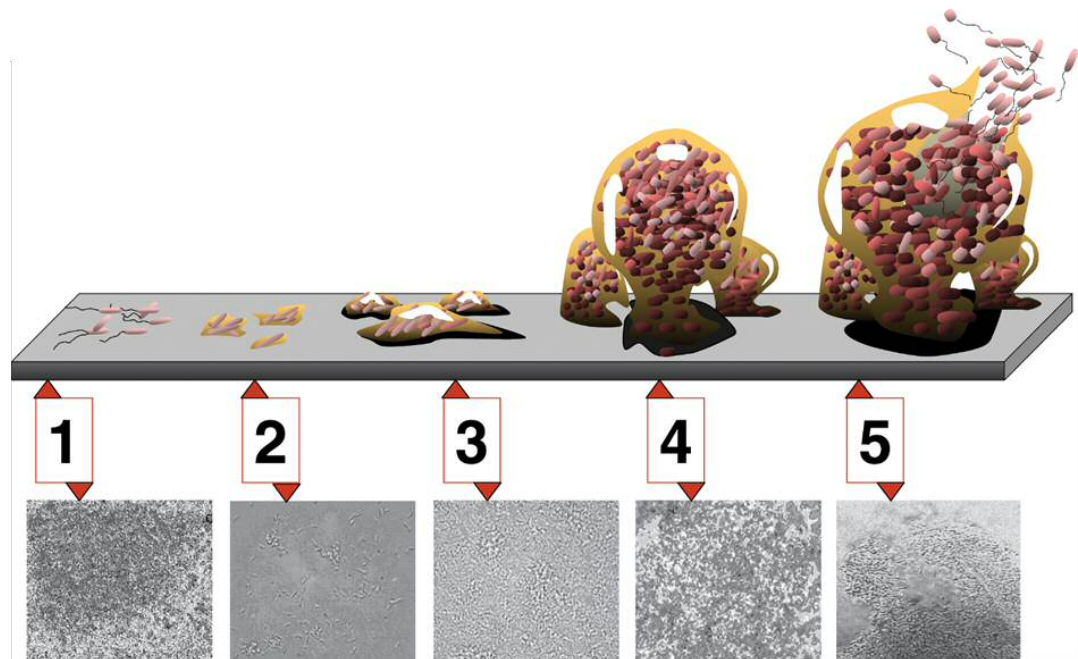
Figure 1.1.1: Developmental model of biofilm formation.
The upper segment provides a conceptualisation of the stages of biofilm development, while the lower segment is composed of corresponding photomicrographs of *Pseudomonas aeruginosa* biofilm development.
1) Initial ("reversible") attachment of microbes to a surface
2) Adhesion ("irreversible") of cells by expression of substrate-specific adhesins
3) Growth of clonal microcolonies with deposition of EPS marks the onset of biofilm formation
4) Maturation of biofilm leading to complex structure and a climax community
5) Dispersal of planktonic cells from the biofilm surface
(Image from Monroe 2007)

1.1.1.1: Attachment

The first stage in colonisation of a surface is, unsurprisingly, attachment of cells from bulk liquid phase to the surface, although a notable and important exception to this is *P. aeruginosa* in the cystic fibrosis lung, where microbial biofilms are found in the mucus layer rather than attached to epithelium (Moreau-Marquis et al. 2008). On dental surfaces, preconditioning first occurs through an adsorbed glycoprotein pellicle (Hannig et al. 2004). Components from saliva are the predominant source of this acquired pellicle, including mucins, statherins and proline rich proteins (PRPs). Indeed, saliva has an important role in coordinating the pattern of initial attachment of microbes to dental surfaces to maintain a healthy "commensal" oral microbiota (Siqueira et al. 2012).

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The attachment of microorganisms to surfaces occurs in a stochastic manner. However, a number of factors other than the presence of an acquired pellicle have been found to influence patterns of surface colonisation. A number of studies have demonstrated an association between growth medium, cation concentration, nutrient sources, pH, temperature and proclivity of microbes to attach to a surface. Marshall et al. (1971) applied the Derjaguin, Landau, Verwey and Overbeek (DLVO) theory of colloidal stability to model the interactions of non-specific forces which may govern, at least in part, behaviour of negatively charged cell surfaces and substrates (Figure 1.2). This model is supported by work from Rijnaarts et al. (1999) which demonstrated enhanced binding affinity between microbes and substrate following increases in the ionic strength of liquid medium.
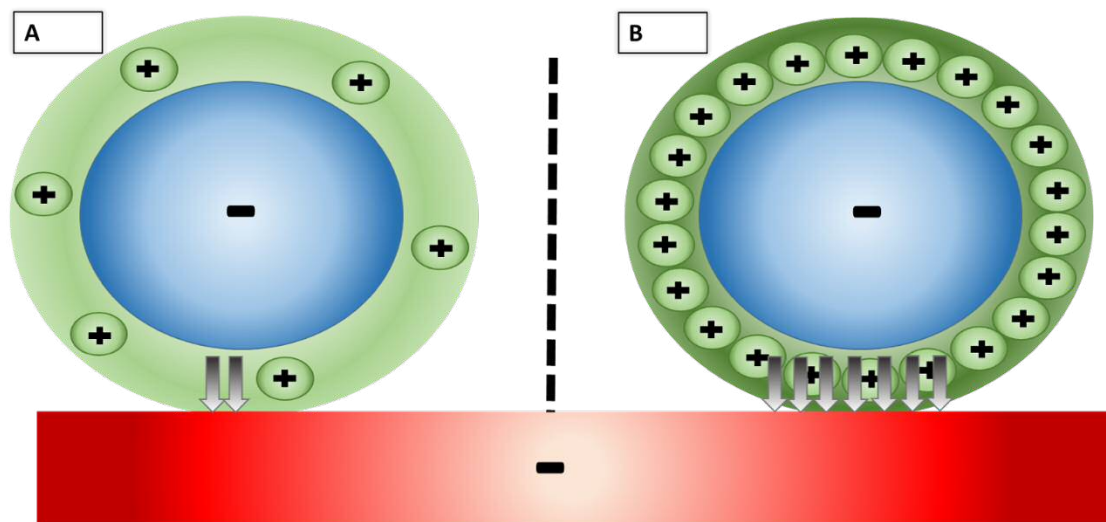
Figure 1.1.1.1: A schematic of the principle electrostatic interactions that influence microbial attachment

DLVO theory describes the principle forces thought to govern the stochastic attachment of microbial cells to surfaces. Negatively charged microbial surfaces (blue) interact with substrate surfaces (red) by weak repulsive electrostatic forces. An interpolation of these forces by cations in growth medium (green) may provide a counter-ion bridge between the two surfaces.

A) At low concentrations of ions, there is minimal mediation of repulsive electronegative surface charges, resulting in weak or no attraction.

B) Stronger cation concentrations in growth medium provides a counter-ion screen, resulting in strong electrostatic interactions between surfaces, and a more stable attraction.

DLVO theory provides a helpful framework to describe the interaction of microbial surfaces with electronegative substrates. However, because it is derived from a model of uniform particulate behaviour, it lacks the complexity to account for certain cell attributes which may contribute to more variable biological responses.

While protein-mediated adhesion and hydrophobic interactions are conventionally considered to feature more prominently in the "irreversible" adhesion stage;

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variations in growth conditions can lead to altered patterns of constitutive surface protein expression, which will in turn alter surface charge (McEldowney and Fletcher 1986; Goulter et al. 2009). Indeed, the heterogeneous topographies of cell surfaces, which present a rich and varied configuration of glycoproteins, lectins and other components, means that nano-scale interactions may differ entirely from bulk cell behaviour due to electronegative membranes or cell wall components such as lipopolysaccharide (LPS) and peptidoglycan (PG).

1.1.1.2: Adherence

In contrast to the non-specific and stochastic attachment of microbes to surfaces, adherence is considered to be an active commitment to a surface lifestyle, with production of specific adhesins to anchor cells to the underlying substrate (Dunne 2002). The ligand-receptor interactions which mediate adherence may be both species and substrate-specific (An and Friedman 1998; Katsikogianni and Missirlis 2004). In addition, many of the surface molecules involved are multifunctional, and may have roles in cell-cell adherence, either intraspecies, such as the inter-cellular adherins of *Staphylococcus aureus* (Foster et al. 2014), interspecies or even interkingdom, such as sspA and sspB of *Streptococcus gordonii*, which can bind both oral mucosal surface components and promote adherence of *Candida albicans* to the bacteria (Wright et al. 2013).

1.1.1.3: Growth and maturation

Adhesion is an active cellular process, thus is metabolically demanding and requires allocation of resources (Beceiro et al. 2013). However, once stable attachments are formed, microbes are able to repurpose efforts to propagation by clonal growth to produce microcolonies. Additional cells may be encouraged to adhere to surface components of microbes, adding to growth and increasing colony diversity (Rickard et al. 2003). It is at this juncture that the production of EPS may begin, marking the true beginnings of a biofilm. This extracellular matrix material is critical in affording spatial structure and protection, as well as providing a cache of nutrients, enzymes and a fluid reservoir. The EPS matrix also provides a readily adherent substrate which permits further recruitment of a range of microbes to the nascent biofilm (Flemming et al. 2007).

Early morphological examinations of biofilms by electron microscopy (EM) gave the misleading notion that these were merely densely stacked piles of cells with no real structure or organisation. This was due to artefacts generated in the preparation of biofilms for imaging (Surman et al. 1996). However, more recent advances in

imaging, most notably the advent of confocal laser scanning microscopy (CLSM), has provided cross-sectional observation of relatively unperturbed biofilms *in situ*. Such techniques have revealed structural organisation and coordination within microbial biofilm communities, including water and waste channels, complex topographies and spatial order (Reichhardt and Parsek 2019).

Molecular techniques have allowed researchers to gain insight into the genetic control of biofilm features and have revealed the presence of cell-cell signalling, termed quorum sensing (QS), which allows microbes to sense their environment and communicate in a cooperative manner (Wolska et al. 2016). In addition, microelectrode studies have revealed the diversity of microniches which result from oxygen, nutrient and pH gradients (Stewart and Franklin 2008). It is the presence of so many environments within biofilms which results in the startling number of microbial species which can form such climax communities, despite an external environment which may not favour their survival. In *in vitro* experiments the use of growth medium drastically alters these nutrient gradients, as media is typically much more nutrient rich than physiological environments. Similarly, the choice of biofilm development method (such as static batch models compared with flow-based assays), incubation atmosphere and frequency of medium replenishment all provide strong environmental changes with consequent impact on biofilm structure, viability and composition (Lebeaux et al. 2013).

1.1.1.4: Dispersal

An important feature of biofilms in relation to human diseases is the process by which planktonic cells may detach and disperse to colonise new sites (Kaplan 2010). The nature of biofilm dispersal is largely unknown. Experimental evidence suggests that a transition from laminar flow to static culture conditions typically results in initiation of dispersal events. It has been proposed that this may indicate a starvation response similar to that seen in fruiting bodies of *Myxococcus xanthus*, due to depletion of nutrients in the growth medium under batch culture conditions (Hunt et al. 2004). The potential for aspiration of planktonic cells or fragmented biofilm from the oral cavity is thought to be the primary mechanism linking the oral microbiome to respiratory infection in humans (Kaplan 2010).

1.1.2: Biofilms in health and disease - a brief overview

While biofilms are a ubiquitous mode of microbial life and provide pivotal support to most ecosystems and biological processes, they are associated with a wide range of human infectious diseases (Hall-Stoodley et al. 2004). An important colonisation site

in health is the aerodigestive tract, where microbes benefit the wellbeing of their human hosts by a number of means. The most well-characterised example of host-microbial synergy is the role of the gut microbiome in digestion (Shreiner et al. 2015). A secondary means of maintaining health relates to the stability and resistance to colonisation by atypical, potentially pathogenic microorganisms of the oral microbiome (Marsh 2006). It is likely that commensal microorganisms associated with health are selected in host tissues through co-evolution to procure a symbiotic relationship, which provides such species with a competitive advantage in adhering to and surviving on human tissue surfaces (Rosenberg and Zilber-Rosenberg 2011). However, a number of factors may alter the host-microbiome relationship, leading either to the invasion of the commensal microbiome by atypical, pathogenic species, or changes within the commensal microflora in response to an altered host environment which causes the emergence of virulence and can initiate infectious disease. Much of the biomedical literature focuses on the role of such pathogen-harbouring microbial biofilms in human disease, particularly with regard to healthcare device-associated infections (Donlan 2001; Deva et al. 2013; Percival et al. 2015). Previous work from our group has examined the role of pathogenic biofilms in urinary catheter-related infection (Malic et al. 2014), colonisation of endotracheal tubes by oral biofilms containing putative respiratory pathogens which could seed respiratory infection (Sands et al. 2017) and the role of denture-associated biofilms in the pathogenesis of denture stomatitis (Morse et al. 2019).

An alternative model of biofilm-related pathogenicity has been described in the dental literature, termed the "keystone pathogen" hypothesis (Hajishengallis et al. 2012). This concept is derived from observations of enhanced virulent traits of normal oral microorganisms, typically considered to be commensal, in response to a low-level "keystone" species (posited to be *P. gingivalis* by the authors). This is thought to be primarily mediated through the ability of *P. gingivalis* to modulate the local host environment through invasion and persistence within gingival epithelial cells; with concomitant inhibition of the innate immune response by degrading IL-8, and subversion of leukocyte killing through gingipain production (Imamura 2003; Darveau 2010). These actions can drive dysbiotic changes in the gingival microbiome, with unrestricted growth of other pathogenic species normally kept in check by normal host immunity. Additionally, there is emerging evidence that specific interspecies interactions such as between *P. gingivalis* and *S. gordonii* leads to enhanced virulence and periodontal disease severity compared to either species alone in a murine infection model (Daep et al. 2011). While the mechanisms behind such interactions are poorly understood, these observations challenge the traditional

paradigm of pathogenicity and virulence; underpinning the importance of metagenome- and transcriptome- focussed studies in disentangling the complex mechanisms that lead to microbial disease (Duran-Pinedo and Frias-Lopez 2015). It should be noted that while the microbiome proper includes all microbes within an environment: viruses, archaeae, bacteria, fungi and protozoa; the term microbiome in this thesis will refer only to the bacterial microbiome, unless otherwise specified.

1.1.3: Challenges in biofilm eradication

The formation of biofilms by microbes is a complex and metabolically costly process which temporarily reduces growth and reproduction of the constituent microorganisms. However, there are a number of long-term survival advantages afforded to biofilm-associated life which far outweigh this immediate detriment. Many such advantages are manifest in the difficulties encountered in eradicating microbial biofilms, summarised in Figure 1.1.3. A notable example is provided by the work of Ceri et al. (1999) who found that the concentration of a range of antimicrobials required to eradicate *S. aureus, P. aeruginosa* and *E. coli* in biofilm form was increased up to 1000-fold compared to these strains in planktonic form. While there are a number of industrial and environmental situations in which biofilm eradication poses a substantial challenge, for brevity only biofilms relevant to medical infections shall be considered in this section.
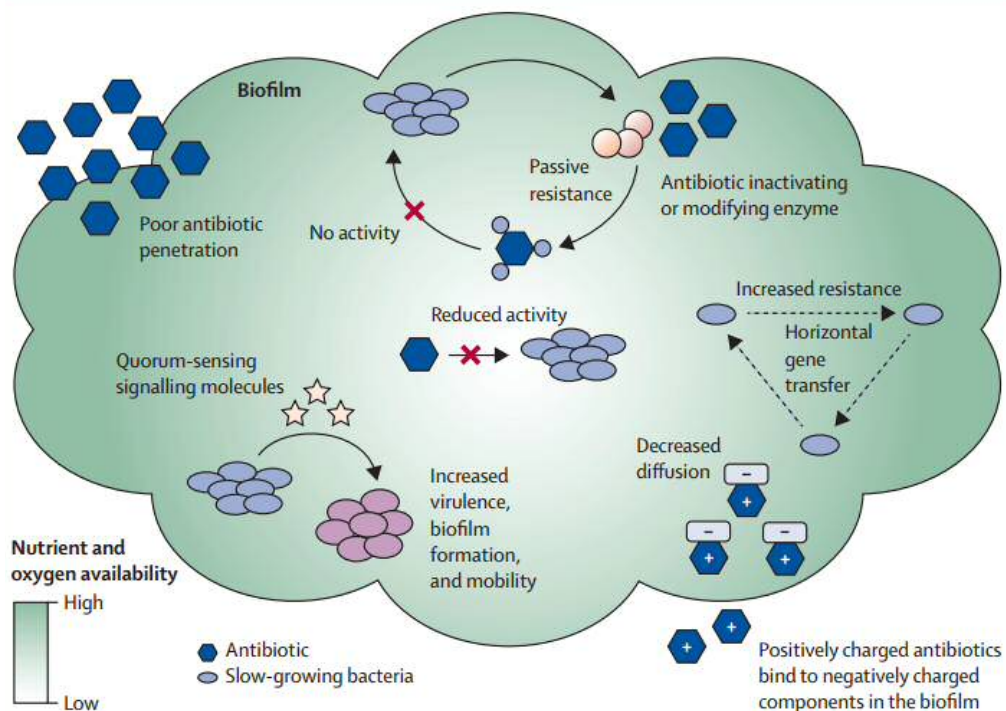
Figure 1.1.3: Potential mechanisms of microbial resistance to antimicrobial agents related to biofilm formation.

(Image from Sherrard et al. 2014)

1.1.3.1: Extracellular polymeric substances (EPS)

The hallmark feature that separates true microbial biofilms from mere aggregates of cells, is the production of extracellular polymeric substances (EPS). The specific components of this encompassing matrix differ markedly between microbial species and the external environment (Flemming et al. 2007). Frequently, a combination of polysaccharides, proteins (including extracellular enzymes) and extracellular DNA (eDNA) are present. The functions of the EPS matrix are multifaceted; EPS gives form and structure to biofilms, enhancing adhesion while affording protection to microbial cells from physical disruption, dehydration, damage from ultraviolet light, predation and host immune cells. The antigen profile of microbes in biofilms is markedly different compared with their planktonic counterparts is typically reduced, making biofilm populations a potentially less immunogenic stimulus (Sanchez et al. 2011).

EPS confers a number of important physico-chemical properties to biofilms. Many EPS components are involved in the adhesion of microbes to surfaces and each other in the initial colonisation process, for instance, polysaccharide intercellular adhesin (PIA) serves a fundamental role in the adhesion of many strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* to a range of surfaces, and also promotes biofilm formation through enhancing cell-cell interactions (Formosa-Dague et al. 2016). Similarly, lectins produced by *Pseudomonas aeruginosa* (LecA and LecB) are important mediators of biofilm formation, primarily by stabilising exopolysaccharide chains (Tielker et al. 2005; Diggle et al. 2006).

Many EPS components are charged and can therefore sequester inorganic ions and water. This provides protection against desiccation. In fact, enhanced EPS production has been observed in response to dehydration in soil-residing microbes, underscoring its importance in trapping water (Roberson and Firestone 1992). The hydrated environment of cross-linked polysaccharide and protein chains results in viscoelastic behaviour. Both EPS production and the degree of cross-linking are enhanced in conditions of increased shear flow (Stoodley et al. 2002). Additionally, electrostatically repellent interactions between certain EPS components provides resistance to compression and collapse (Ehret and Böl 2013). The combination of enhanced adhesion and physical robustness results in protection of biofilm-associated microorganisms from mechanical disruption and removal from surfaces (Peterson et al. 2015).

A further consequence of trapped cations is the restricted permeability of EPS to charged antimicrobials, such as chlorhexidine, or aminoglycoside antibiotics.

The impact of EPS on *P. aeruginosa* biofilms has been studied extensively, due to the frequent involvement of this species in multi-drug resistant infections. Biofilm penetration by the cationic aminoglycoside antibiotic tobramycin was studied by Tseng et al. (2013), who observed penetration of fluorescent-labelled tobramycin in mucoid (alginate-producing) strains was limited in a concentration-dependent manner. Adding metal cations at sub-MIC levels markedly improved tobramycin biofilm penetration, supporting the notion that the primary mechanism of diffusion limitation was electrostatic interaction with matrix components.

Similar results were found in studies of chlorhexidine penetration into a mixed-species oral biofilm model using confocal laser scanning microscopy (CLSM) to observe uptake of the fluorescent dye Calcein AM (CAM) indirectly via increased membrane permeability, which was inferred to indicate cell death (Takenaka et al. 2008). The authors selected a biofilm region from the corner of a flow-cell device to analyse antimicrobial action. The corner of this device was selected because it allowed for greater accumulation of biomass, and the decreased flow-rates limited diffusive transport of test agents within the biofilm. It was observed that the deepest regions of the biofilm (100 µm) were apparently entirely unaffected by chlorhexidine. Interestingly, the same study demonstrated that biofilm penetration by a biotene mouthrinse, which contains a number of ionic salivary antimicrobial peptides, was not hindered from reaching the deepest regions of the biofilm. This may be due to the incorporation of non-ionic surfactants such as poloxamer 407 in the formulation of this product. Similar formulation strategies may offer a potential solution to the limited diffusion of chlorhexidine and other locally-delivered, charged antimicrobials.

In addition to these constituents, EPS can act as an extracellular store of both ions and nutrients, protecting the inhabitant microorganisms from starvation. In some cases, EPS exopolysaccharides such as dextrans and levans produced by oral streptococci are themselves a nutrient reserve for times of privation (Colby and Russell 1997). To enable metabolism of matrix-associated nutrients, the EPS also contains numerous extracellular enzymes. Importantly, these may also include enzymes capable of inactivating some antimicrobials, such as β-lactamases produced by a wide range of microorganisms in response to exposure to penicillins (Ciofu et al. 2000).

1.1.3.2: Altered environmental niches

Mature biofilms may be several millimetres thick, with an intricate array of nutrient, gaseous and metabolite gradients. This leads to a highly diverse milieu, the full extent of which is only now becoming appreciated, that permits the coexistence of many

divergent species within a biofilm (Stewart and Franklin 2008). An obvious consequence of biofilm formation is the accumulation of a high number of microorganisms, which can be densely packed. This could result in exhaustion of an antimicrobial agent before the entire population is eradicated, although this is rarely a problem in reality. However, a number of environmental factors beyond this simple concentration-population mismatch have been proposed to explain the stark differences in antimicrobial tolerance of microbial biofilms compared with their planktonic counterparts.

EPS provides structural features which control nutrient delivery to areas of biofilms, such as the archetypal mushroom-like colonies formed by *P. aeruginosa* biofilms, with water channels coursing between 'stalks', supplying nutrients and permitting removal of waste metabolic by-products. The selective permeability of the EPS results in gradients of gases, nutrients and metabolites, which can help support complex polymicrobial communities, with many constituent species that would otherwise be unable to survive (Flemming and Wingender 2010). A pertinent example of this is seen in the work of Welch et al. (2016), where the presence of obligate anaerobes such as *Actinomyces* and *Prevotella* species is found in supragingival plaque, despite an oxygenated external environment (Figure 1.1.3.2A-D). The aerobic metabolic activity of superficial species results in an increasingly anoxic environment in deeper regions of biofilm, with an intermediate region that may contain small numbers of aerobes and anaerobes but is dominated by facultative aerobes including streptococci and lactobacilli, because of the selective advantage their metabolic flexibility confers. There are, however, apparent exceptions to this rule. *Porphyromonas* species can be seen to localise to the superficial regions of the biofilm in Figure 1.1.3.2E despite higher oxygen tension in these areas. The authors speculate that lesser-known species of *Porphyromonas e.g. P. catoniae* and *P. pasteri*, with at least some aerobic tolerance might have been identified by the probes, rather than *P. gingivalis*, which is frequently isolated in subgingival, but rarely isolated from supragingival plaque. This explanation partly addresses this unexpected finding, but it seems likely that specific binding sites and species-specific metabolic synergy are important factors in permitting *Porphyromonas* species to out-compete other aerobes in this micro-environment.
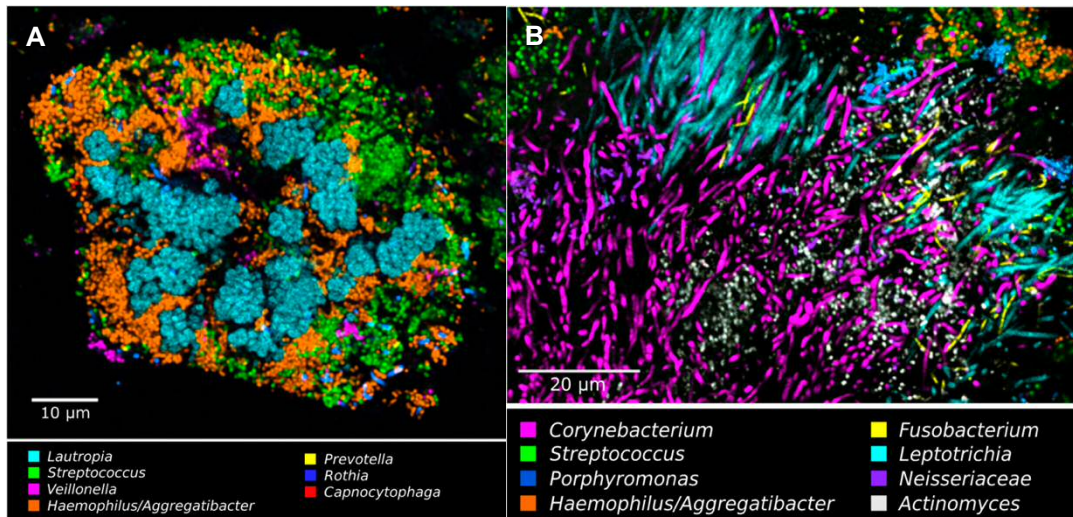
Figure 1.1.3.2: Photomicrographic projections of *ex vivo* supragingival plaque samples imaged using Combinatorial Labelling and Spectral Imaging FISH (CLASI-FISH).

A)   Superimposition of multispecies plaque biofilm showing presence of *Prevotella* species superficially located in plaque biofilm

B)   Superimposition of multispecies plaque biofilm showing superficial location of *Porphyromonas* species and substantial *Actinomyces* species throughout biofilm

 (Images from Welch et al. 2016)

Similarly, waste-products, such as lactate from superficial aerobes which would be toxic in high concentrations, are a source of nutrients to underlying fermenters like *Veillonella* species (Takahashi 2015). Biofilm communities are replete with such synergistic relationships between diverse species. Similar synergism is exhibited following challenge with some antimicrobials. It has been found that mixed biofilms of *Streptococcus mutans* and *Veillonella parvula* are able to survive treatment with chlorhexidine in much greater numbers than single-species biofilms of either species, using a viable colony plate-count method (Kara et al. 2006). Similarly, mixed biofilms of *S. aureus* and *P. aeruginosa* can tolerate a number of antimicrobials which are normally effective against one or both species (DeLeon et al. 2014). The mechanisms involved in such interactions are poorly understood.

1.1.3.3: Metabolic changes

Alterations in microbial metabolism within biofilms are primarily derived from two factors. Firstly, environmental changes to generate divergent microniches within biofilms lead to concomitant variations in metabolic pathways (detailed in the above section). Secondly, there are inherent changes in microbial metabolism associated with biofilm formation, which has led to the concept of the 'biofilm lifestyle'. This may simply reflect the environmental conditions within the biofilm, with a relatively high density of microbes and scarcity of space and resources. Consequently, most microbes, particularly in the deeper regions of a biofilm, exhibit marked reductions in

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metabolic and biosynthetic activity (Anderson and O'Toole 2008). The majority of antimicrobials target biosynthetic pathways in active and reproducing cells, such as the ß-lactam, macrolide and aminoglycoside classes. Several studies have found starved planktonic cells are able to tolerate such antimicrobials, while chemotherapeutics which exert their effects by alternative mechanisms, such as lipoglycopeptides and imidazole derivatives, retain effectiveness (Hurdle et al. 2011). Some metabolites can act as signalling molecules, such as the autoinducer 2 (AI2) quorum sensing molecules produced by the LuxS enzyme as a by-product of the methyl cycle (Whitely et al. 2017). AI2 is a universal interspecies and interkingdom signal, which is highly conserved across bacterial and archaeal species, in addition to many eukaryotes. AI2 and the luxS pathway were initially identified in *Vibrio harveyi* because of their role in regulating bioluminescence in this species. However, AI2 is produced by a wide range of species and can influence biofilm formation and interactions between oral bacteria, including *S. gordonii, S. mutans* and *P. gingivalis* (McNab et al 2003; Yoshida et al. 2005). Knockout studies have demonstrated both altered biofilm architecture and abrogation of co-aggregation between such species (Wang et al. 2017).

1.1.3.4: Microbial interactions

Within biofilm communities, microbial populations are organised in close proximity, through both specific receptor-mediated co-localisation, and ecological phenomena governed by limitations in space and resources within a given microniche (Burmølle et al. 2014). Microorganisms also interact dynamically with their host environment. The net impact of the microbial community contributes to maintenance of health for much of life, but changes in either the host environment or microbial population may be of detriment to the host and result in harm. When the interactions between host and microbe, or within microbial communities are beneficial to both organisms, as has been recently observed in polymicrobial biofilms containing both *F. nucleatum* and *P. gingivalis* (El-Awady et al. 2019), this is termed a 'synergistic' relationship. Here the authors noted enhanced biofilm formation and proliferation by both species when grown together compared to either species alone. More frequently documented relationships entail benefit to one organism from the relationship, such as microbial colonisation of host surfaces facilitating the acquisition nutrients, shelter and a suitable habitat; while the corresponding organism (typically the host) receives negligible benefit or harm. Such interactions are considered 'commensal'. The majority of host-microbe interactions are typically considered commensal in nature. In some instances, the interaction between organisms results in harm to one, usually

to the benefit of the other. Microorganisms which are considered harmful to human health are termed 'pathogens'. Many historically significant infectious diseases which drove dramatic advances in medical microbiology are examples of classical pathogenic relationships. Of particular relevance is the influence of any number of possible bacterial respiratory pathogens in the development of pneumonia.

Outside of the interactions outlined above, further classical ecological relationships can be considered relevant to microbial populations. One such relationship of considerable importance is the role of competition in the establishment of a stable oral microbiome in health, and the influence of environmental perturbations in destabilising established communities (Sultan et al. 2018). If a disturbance is of enough magnitude, this could drive changes in the composition of microbial communities towards more pathogenic populations or functional behaviours, termed dysbiosis. This 'ecological catastrophe' (Marsh 2003) will be explored in greater detail in later sections.

More recently, a novel means of microbial interactions has emerged, the importance of which is as yet not fully understood, but increasingly appears likely to be substantial: Quorum Sensing (QS). First formally identified through the luxI and luxR luminescence genes of *Vibrio fischeri*, which respectively coordinate the production a homoserine lactone molecule and its autoresponsive transcriptional activator, it was not until the late 1990s that similar pathways and molecules were shown to serve key functions in important pathogens such as *S. pneumoniae* (Stroeher et al. 2003; Trappetti et al. 2011) and *S. aureus* (Cheung et al. 2004; Qazi et al. 2006). These small molecules all shared common structures and were able to readily diffuse throughout a biofilm, and through a number of experiments on *Vibrio* species were implicated in the sensing of population density. A resultant behaviour thought to be regulated through QS is the production of 'public goods'; extracellular substances that benefit not only the producer, but its nearby neighbours (Whitely et al. 2017). Further work has suggested that such behaviour only occurs in the presence of sufficient nutrient surfeit (Dandekar et al. 2012), which not only promotes survival of the 'altruistic' microbial cell, but also serves to regulate the proportion of passive beneficiaries within a population (sometimes referred to as 'cheaters'). Importantly, it has been shown that QS is not limited to intra-species relationships, but can affect behaviour between different species, or even result in inter-kingdom interactions. The most well-established example of this is found in the interactions between the *P. aeruginosa* QS pathways lasI and rhlI, which govern the genetic transcription of a number of virulence factors, and *C. albicans* QS molecule farnesol, which governs

morphological switching of this microbe from the yeast to hyphal form (Fourie et al. 2016).

Not only has experimental evidence shown that each microorganism is able to sense the QS molecules of the other, but these molecules appear to have been 'weaponised' to influence behaviours of each species. *P. aeruginosa* adhesion to candidal hyphae appears to be QS dependent from knockout studies and may be implicated in the production of a number of virulence factors which enable killing of candidal cells. Similarly, *C. albicans* behaviour changes in the presence of pseudomonal QS molecules in a concentration-dependent manner, marked by switching from hyphal to yeast form. The candidal QS molecule farnesol which regulates this behaviour has also been found to inhibit the production of virulence factors by *P. aeruginosa.* While such behaviours point towards mechanisms aimed at promoting survival of one species over the other, the ecological context of these traits remains unclear. There remains the possibility of further interactions and pathways as yet undiscovered which may change our understanding of microbial QS.

1.1.3.5: Mobile genetic elements and transferred resistance

Microbes are capable of 'horizontally' transferring genomic DNA through three primary mechanisms; transformation, conjugation and transduction (Frost et al. 2005). Transformation was first discovered in the respiratory pathogen *S. pneumoniae* in 1928 (Griffith), although it was not until work by Avery et al. (1944) that DNA was identified as the key molecule which was internalised through this process. The uptake of extracellular, free DNA from the environment requires the recipient microbe to be capable of binding, processing and internalising in single-stranded form, followed by integration of this DNA with chromosomal genomic DNA. This state, termed 'competence' is not constitutively present in the majority of microorganisms, and may in fact be regulated in part by QS (Shanker and Federle 2017). Extracellular DNA may be present in the environment due to lysis of cells in death, or actively excreted DNA either as a constitutive process during growth or in response to specific stimuli. At present, experimental evidence exists for a combination of all the above sources contributing to the environmental pool of extracellular DNA (Lorenz and Wackernagel 1994).

Conjugation involves direct cell-cell transfer of genetic material, in a process analogous to sexual reproduction in Eukaryotes. Most pertinent to the field of infectious diseases has been the finding that microbes are able to transfer genes encoding antimicrobial resistance traits (Llosa et al. 2002). This process appears to be dependent upon Type IV pili for both Gram-positive and Gram-negative

microorganisms. Despite considerable heterogeneity in protein structure and specific mechanisms across different bacterial species, the fundamental pathways appear to be highly conserved, suggesting that conjugation confers an important evolutionary advantage (Lawley et al. 2004; Goessweiner-Mohr et al. 2015). This notion is reinforced by the complexity and energy cost of coordinating detection of cell-cell contact (a feature unique to conjugation among microbial horizontal gene transfer (HGT) mechanisms), with subsequent cleavage, replication and packaging of DNA into a circular plasmid form, 'unzipping' of double-stranded DNA with transport and secretion of one strand, then replication of the complementary strand by both donor and recipient cells prior to integration with chromosomal DNA (Griffiths et al. 2000). The potential to increase genetic diversity among a bacterial population or potentially exchange genes which confer survival between individual cells is clear, although the regulation of the processes which drive conjugation are yet to be fully understood.

The most recently discovered means of bacterial HGT, transduction, requires viral vectors for genetic material in the form of bacteriophages. Three primary forms of transduction have been identified at present (Van Hoek et al. 2011): generalised, which involves the essentially random, erroneous incorporation of bacterial DNA into viral capsids; specialised, wherein entire genes adjacent to the phage genome position in the infected bacterial chromosome are restricted and packaged into viral capsids; and lateral transduction, which has been shown to exist in *S. aureus* only to date, and can involve a substantial number of genes more distant to the phage DNA in the bacterial chromosome (Chen et al. 2018).

All methods of HGT can lead to acquisition of antimicrobial resistance by microbes. However, more recently a growing appreciation for the role of transduction in both antimicrobial resistance and virulence genes has emerged. A recent report documented the potential for Gram negative bacilli in the oral microbiome to act as a reservoir of antimicrobial resistance, in addition to harbouring greater pathogenic potential for both oral and systemic disease (Dupin et al. 2015).

1.1.3.6: Persister cells - a phenotypic aberration

Persister cells present a unique challenge to medical microbiology and may provide one of the primary mechanisms behind the recalcitrance of biofilm-associated infections to antimicrobial chemotherapy (Lewis 2010). The available evidence suggests that within a given microbial population, a small proportion of cells will enter an ostensibly dormant state, which renders them tolerant to the majority of antimicrobials (the notable exception being fluoroquinolones, which possess the additional advantage of generally demonstrating good penetration through biofilm

matrix). Despite extensive research aiming to establish predictive factors for persister formation, and the genetic control thereof, efforts thus far have largely been fruitless in offering meaningful opportunities to overcome the problem of persister cells (Lewis 2007). Regulation of persister formation can be linked to specific genes, such as *tisB* in *E. coli*, but while knockout studies have shown elimination of this gene reduces persister formation substantially, the high level of redundancy in persister regulation means it is unlikely that a convenient therapeutic target will be identified that can reliably abrogate the formation of persister cells (Lewis 2010). Indeed, a number of current antibiotics have been shown to increase the number of persisters in a microbial population, likely through induction of the general 'SOS' stress response pathways. In this manner, conventional antimicrobial therapy may actually act as a selective pressure for persister cell formation (Phillips et al. 1987; Miller et al. 2004). The problem of persister cells is compounded massively in the context of polymicrobial biofilm-mediated infections. When encapsulated in the EPS matrix, antigenic cell material is shielded from immune surveillance, and cells are effectively sequestered from phagocytosis (Vuong et al. 2004). In addition, many of the microbial interactions outlined in section 1.1.3.4 favour persister cell survival, as 'altruistic' actions by surrounding cells may better facilitate survival of essentially dormant, passive cells. The biofilm environment itself is conducive to persister formation: mature biofilms contain a population primarily in the stationary growth phase, and nutrient privation, population density and waste product accumulation all provide a chronic, high-stress environment which may drive persister formation through the 'SOS' response (Nguyen et al. 2011). Furthermore, the biofilm matrix can slow the penetration of a number of antimicrobials or may contain extra-cellular enzymes which can inactivate antimicrobial agents (Olsen 2015). This results in lower concentrations of antimicrobial which take longer to reach a bactericidal concentration and therefore 'buys time' for persister formation to occur as a response to increasing, but non-lethal, stress levels. When antimicrobial levels fall following treatment, biofilm matrix and a small number of surviving persister cells will now be in a relatively low-stress environment, as competition is reduced due to massively decreased population density. The same gene-switching processes that result in persister formation may now reverse, and these surviving cells can repopulate the biofilm (Balaban et al. 2004). It is important to note that while the above narrative is helpful to understand the potential mechanisms by which persister cells contribute to biofilm recalcitrance, the switching from active to dormant phenotype appears to be almost entirely stochastic, with the likelihood of either state dependent on the net balance of surrounding stressors.

Importantly, persister cells have been identified as contributing to the recalcitrance of respiratory infection to antimicrobial therapy by *P. aeruginosa* in cystic fibrosis patients (Mulcahy et al. 2010). This finding was present despite no inherent increase in antimicrobial resistance of clinical isolates. *Staphylococcus aureus* in the stationary growth phase has been found to produce an exceptionally high number of persister cells, with the entire population demonstrating substantial increases in antimicrobial tolerance (Keren et al. 2004). This has proven pivotal in recalcitrant *S. aureus* infections failing to respond to vancomycin and linezolid despite no resistance to either antibiotic being detected (Welsh et al. 2011). Promisingly, combination of antibiotic treatment and chemotherapeutics which activate relevant antimicrobial targets have been found to eradicate biofilms in chronic wounds (Conlon et al. 2013). Although only isolated cases of such treatment have been reported at present, bespoke, targeted combination therapies formulated on precise understanding of the mechanisms in persister formation offer an important last line of defence against highly recalcitrant, biofilm-mediated infections.

1.2: The Oral Ecosystem

In response to the inception of the field of microbial ecology, our view of the oral cavity includes an appreciation for the range of highly diverse and dynamic environmental niches, in a state of dynamic equilibrium with the microbial communities which populate them. Any change to either environment or microbial populations may cause extensive and potentially irreversible sequelae to both host and microbiome.

1.2.1: The diverse habitats of the oral cavity

The oral cavity is an anatomical space bounded by the lips and cheeks externally, and the pillars of fauces at the junction of the oropharynx internally (Berkovitz et al. 2017). In reality, it is continuous with the oropharynx, which connects the oral cavity with the digestive and respiratory tracts. The continuity between the external environment and the respiratory and digestive tissues makes the oral cavity a critical interface in the body. Indeed, it is difficult to imagine a scenario in which the oral cavity is not at least passively involved in the pathogenesis of respiratory infections by acting as a conduit, with emerging evidence suggesting that ecological shifts within the oral cavity and dysbiotic changes in the oral microbiome may directly seed respiratory infection, as outlined in later sections. There are a number of environments in the oral cavity which are unique, and substantial contrast between different niches available to microbial communities therein (Figures 1.2.1A & B).

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Here, the total number of unique species-level OTUs is plotted against number of sequencing reads as a rarefaction curve (Figure 1.2.1A). The steepness of the rarefaction curve relates to diversity, with saliva having the most diverse microbiota. Of the specific oral sites, the tongue shows greatest diversity. Figure 1.2.1B demonstrates the relative abundance of different genera across oral sites. Of note, Candida is not included in this analysis, but is commonly found on mucosal surfaces, particularly the palatal mucosa of denture-wearers.
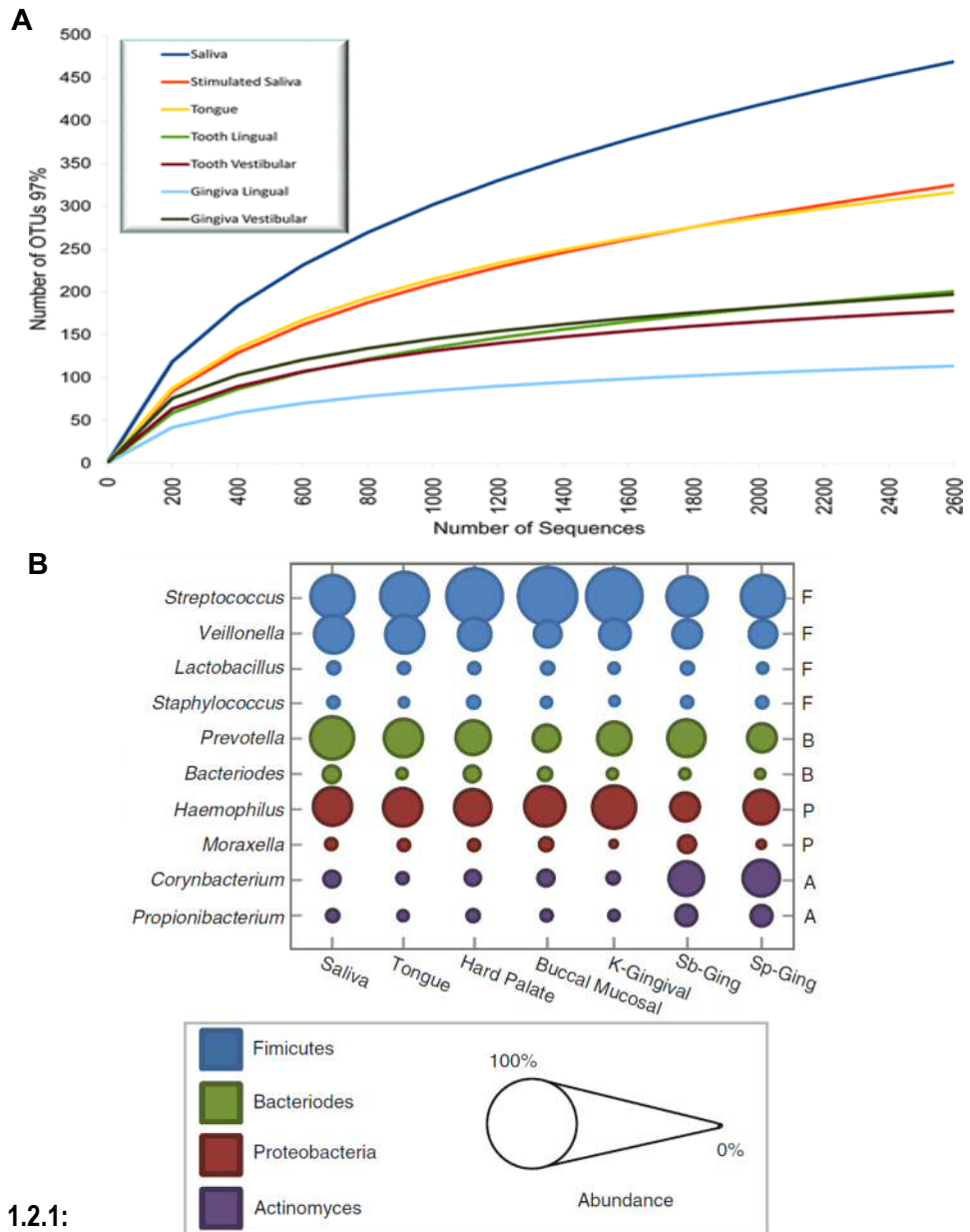
Figure 1.2.1:

A) Phylogenetic diversity of bacterial samples from one individual at different oral sites indicated by rarefaction curves.

 (Image from Simon-Soro et al. 2013)

B) Relative abundance of selected bacteria in different ecological niches within the oral cavity. (Kerr and Tribble, 2015).

1.2.1.1: Non-keratinised, shedding surfaces

The soft tissues of the oral cavity are lined with mucosa; a membrane comprising stratified squamous epithelial cells overlying a fibroblast rich connective tissue layer called the lamina propria. A feature of all oral mucosa is the maturation of epithelial cells from proliferation in the deepest basal layer to most superficial layer, with morphological changes reflective of tissue function accumulating throughout the maturation process (Figure 1.2.1.1). The oral mucosa can be divided based on anatomical, functional or histological differences. However, in reality these three conceptual fields are intimately intertwined in biology. The broadest subdivision of oral mucosal surfaces is to differentiate keratinised and non-keratinised surfaces.

The flattened surface cells of non-keratinised epithelium develop cytoplasmic filaments which lend elasticity, while losing many of the organelles seen in deeper layers (Berkovitz et al. 2017). All mucosal surfaces are continuously shed and replenished by cells maturing from the underlying layers. This process is termed desquamation. The mechanisms underpinning desquamation remain unclear. However, the process is an important facet of innate immunity – by continuous shedding of the mucosal surface, colonisation and establishment of mature microbial biofilms is impaired. By surface area, non-keratinised mucosa forms the majority of the oral cavity lining (Nanci et al. 2017). It lines the lips, soft palate, floor of mouth, ventral tongue, buccal tissues and non-attached portion of the alveolar gingivae. Non-keratinised mucosa comes into contact with polished denture surfaces, but typically has minimal contact area with the denture-fit surface. As the polished denture surfaces tend to be smoother and less porous, exposed to saliva and masticatory friction, these sites are less likely to accumulate substantial biofilm outside of very poor denture hygiene or dentures which are in disrepair, the influence of dentures on the non-keratinised tissues is likely lower than in the denture-bearing mucosa. However, these surfaces may still provide relatively sheltered niches that allow substantial pathogenic bioburden to accumulate, such as the reflection of the sulci in individuals who do not remove their dentures.
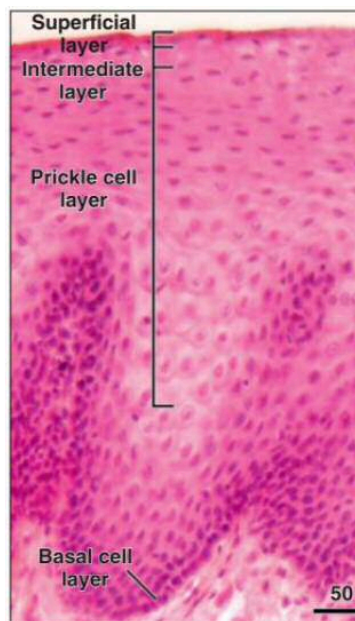
Figure 1.2.1.1: Maturation of the non-keratinised epithelial tissues.
The basal layer (lowest on image) lies at the lamina propria, at the deepest part of the epithelium. This cell layer is highly proliferative and provides the cells which make up the overlying layers. As the epithelial cells travel superficially through continued replacement by proliferation of the basal layer, they undergo the process of maturation. As maturation progresses there are distinctive morphological changes which allow the broad subdivision of epithelial tissue into layers. With each superseding layer, there is a progressive flattening of the cells, as much of the organelle content is lost, although nuclei are retained. In the superficial layer, cells have a flattened appearance and are called 'squames'. (Nanci 2017, © Elsevier 2018)

1.2.1.2: Keratinised, shedding surfaces

The remaining oral surfaces, namely the vermillion border of the lips, attached gingiva, dorsal tongue and hard palate, are covered by keratinised mucosa. While typically the mucosa of the vermillion border of the lip and dorsum of the tongue are termed specialised mucosa due to their atypical structures and more specialised functional roles (Berkovitz et al. 2017, Nanci et al. 2017); these and the other keratinised mucosal sites have a clear role in masticatory function. Thus, all oral keratinised mucosa can be considered a form of masticatory mucosa without this being mutually exclusive with other, specialised functions.

While keratinised mucosa shares a number of morphological similarities with non-keratinised mucosa, such as the relationship of the epithelial layer with the underlying lamina propria, and the maturation process from basal layer to superficial layer, a number of important differences emerge during maturation of the epithelial cells (Figure 1.2.1.2). The morphological changes that occur during maturation are considerably more marked in keratinised epithelium, with considerable accumulation of tonofilaments and keratohyalin granules in the intermediate cell layers, coupled with flattening of the cells and reduction in organelle content. The granules contain profilaggrin, which is released extracellularly and self-assemble into a densely cross-linked matrix which act as a barrier between cells. In the superficial layer, epithelial cells lose all normal ultrastructural features, with total absence of organelles. They are filled entirely with tonofilaments and filaggrin matrix, the combination of which are called keratin, eponymous for this cell type (Nanci et al. 2017). The thickness of the

keratinised layer varies in different areas and can thicken in response to stimuli such as chronic trauma, termed hyperkeratosis (Rubin 1949).

Critically, the keratinised epithelium of the hard palate and tongue have macroscopically complex topographies, due to the presence of the palatal rugae and taste buds. In the case of the dorsal tongue, microscopic invaginations also exist between the specialised papillae. This results in highly heterogeneous environments, may lead to relatively sheltered micro-niches which could shield denizen microbes from salivary cleansing. During masticatory function, these surfaces are typically exposed to frictional forces, which accelerate the normal desquamative processes and can disrupt local microbial communities. The impact of removable dental prostheses on shielding the palatal microbiota from such effects is unclear, but certainly these may promote areas of stagnation, in addition to acting as a vector for environmental, extraoral microbes to be acquired and transported directly to colonise the palatal mucosa during prolonged intraoral contact times. Certainly, despite constant desquamation, the dorsal tongue microbiome appears to be more diverse in composition than most other oral sites, which reflects the complex environment offered by this site (Simon-Soro et al. 2013).

The gingival tissues, because of their intimate relationship with the teeth, are best considered separately.
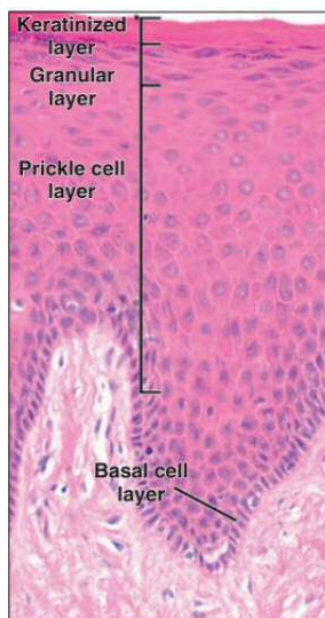
Figure 1.2.1.2: Maturation of the keratinised epithelial tissues follows a similar process to the non-keratinised tissues.
The progressive changes in cell morphology are more pronounced however, as all organelle content and even cell nuclei are lost and replaced with densely packed fibrillar material. At the most superficial layer, the tight-junctional attachment of cells forms an impermeable barrier which makes differentiating individual cells difficult. In some instances, nuclei are retained, although in a dense, compacted (pyknotic) form. Epithelial tissue with such cells is referred to as 'parakeratinised', and can be considered as an intermediate tissue type interposing non-keratinised and keratinised tissues. (Nanci 2017 © Elsevier 2018)

1.2.1.3: The dental tissues

The dental tissues make the oral cavity a unique site in the body; where hard, non-shedding tissues are directly connected to the epithelial tissues of the gingivae and

exposed to the external environment. The outer tooth surface exposed to the mouth is formed by enamel, a highly mineralised tissue which is composed of approximately 96% inorganic hydroxyapatite crystals, which proffer the hard-wearing resilience needed for mastication (Berkovitz et al. 2017). Deep to the enamel is dentine, a tubular tissue which is approximately 70% hydroxyapatite mineral, with the remaining 30% comprising mainly collagen protein. The porous nature of dentine makes this tissue essentially continuous with the deepest tooth tissue; the pulp. This soft, vital tissue contains neurovascular bundles and cells responsible for dentine formation, maintenance and repairs. The portion of the tooth not exposed to the mouth in health, but instead anchored to the supporting alveolar bone of the jaws by the specialised connective tissue fibres of the periodontal ligament is termed the root. This differs from the coronal tooth in that the outer tissue is comprised of cementum, which is approximately 50% mineral content, 50% collagen. The dentinal-periodontal complex is illustrated in Figure 1.2.1.3A.
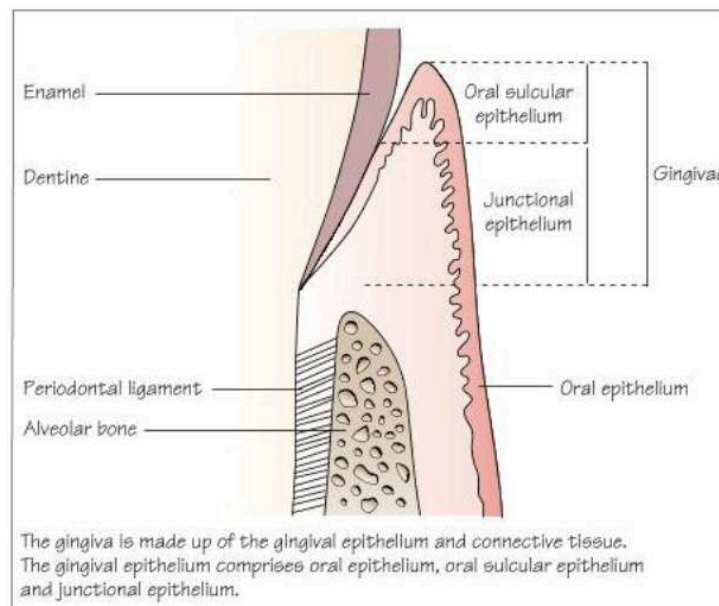
Figure 1.2.1.3A: Basic anatomy of the dentinal-periodontal complex (longitudinal sectional view)
Of note, the cementum is not indicated, but is represented by that part of the tooth surface into which the periodontal fibres are anchored. The relationship between cemento-enamel junction, approximately coincident with the apical attachment of the junctional epithelium can also be appreciated.
(Image from Clerehugh et al. 2013)

The tooth root is anchored to bone by a specialised connective tissue called the periodontal ligament, which contains collagen fibre bundles traversing the 0.15 – 0.38 mm space from alveolar bone to cementum tissue (Nansi 2017). Coronal to the level of the alveolar bone crest, similar fibres extend from tooth to the free gingivae,

contributing to gingival architecture. In addition to collagen, various elastic fibres, fibroblasts, neurovascular bundles are contained within a glycosaminoglycan 'ground substance' matrix (Berkovitz et al. 2017).

The gingivae form a mucosal 'collar' to surround each tooth. In health, the gingival mucosa attaches to the cementum immediately below the border of cementum and enamel. There is a protuberance of gingival tissue coronal to this attachment, of approximately 2-3 mm, which is termed free gingiva in reference to the absence of direct attachment to adjacent hard tissues. This results in a space between the tooth-facing gingival epithelium and the coronal enamel, termed the gingival sulcus. The portion of the gingiva that attaches to the cementum is termed the junctional epithelium. It ranges from 1 – approximately 30 cells in thickness from the most apical to coronal extent respectively. Unlike most epithelial tissues, this specialised region consists only of a germinative layer of cuboidal cells and a layer resembling the stratum spinosum in thicker portions. This epithelium is highly permeable, which permits the diapedesis of leukocytes critical to the maintenance of this vulnerable tissue (Bosshardt and Lang 2005).

The gingival sulcus is a key site in the aetiopathogenesis of periodontal disease, which is one of the most prevalent microbial diseases worldwide (Peterson and Ogawa 2012). The tooth, offering a non-shedding surface, is readily colonised by microbes. The sequence of colonisation is to some extent determined by the presence of a coating of salivary glycoproteins, termed the salivary pellicle, which occurs essentially instantaneously on the clean tooth surface (Kerr and Tribble 2015). The role of saliva in orchestrating colonisation and in providing antimicrobial actions will be detailed in Section 1.2.2.4.

Typically, the community dynamics of early dental biofilms are predominated by commensal early colonisers, with a lower number of strict anaerobes and putative pathogenic species which occurs within the first few minutes of exposure of the dental surface (Marsh 2000). If allowed to mature over several hours to days however, biofilm diversity increases, as microniches develop within the biofilm architecture. As the biofilm grows and matures, colonisation extends into the deeper regions of the gingival sulcus, where oxygen tension is lower, and nutrient availability is limited. Typically this process starts between 2 – 7 days (Uzel et al. 2011) This is facilitated by the complex metabolite chains allowing newly acquired species to subsist from the waste products of their biofilm neighbours, combined with the acquisition and propagation of anaerobic late colonisers which require the establishment of suitable hypoxic environments to thrive (Socransky and Haffajee 2005). In response to the detection of microbial antigens in close approximation to the vulnerable junctional

epithelium, plus the direct impact of microbial toxins and metabolites, gingival inflammation ensues. This leads to the classical phenotype of gingivitis: oedema, erythema and occasionally symptoms of discomfort or pain. Such signs and symptoms result from local vasodilation and an increase in both vascular and tissue permeability, facilitating recruitment of neutrophils and other leukocytes to eradicate the microbial insult (Honda et al. 2006).

In a large proportion of the population, a homeostatic balance is subsequently reached, wherein a low level of chronic inflammation is adequate to control, but not necessarily eliminate the microbial population. However, due to a combination of both microbial and host factors (particularly, but not exclusively, a degree of dysfunctionality in the immune response), the immune system is unable to manage the microbial insult, and can indeed contribute damage to host cells by release of non-specific cytotoxic compounds in response to foreign antigens, such as proteases (Cekici et al. 2014). Such prolonged and escalating inflammatory response results in migration of the junctional epithelium apically, away from the source of the insult. This leads to the formation of a deepened sulcus, termed a periodontal pocket (Figure 1.2.1.3B). As the epithelium migrates apically, the supporting alveolar bone is resorbed concomitantly; the clinical hallmark of periodontal disease. Untreated, such processes can progress until the periodontal support for a tooth is completely undermined, resulting in tooth loss or a number of other adverse local sequelae.

Further to the local effects of periodontal disease, increasing evidence supports links between periodontitis and a number of systemic diseases, including pneumonia. Many such diseases result in large part from an increase in circulating pro-inflammatory signals, such as cardiovascular and metabolic diseases (Bullon et al. 2009). However, as will be discussed in a later section, there is now a considerable volume of evidence to support the direct role of dysbiotic shifts in microbial communities in seeding respiratory infection.
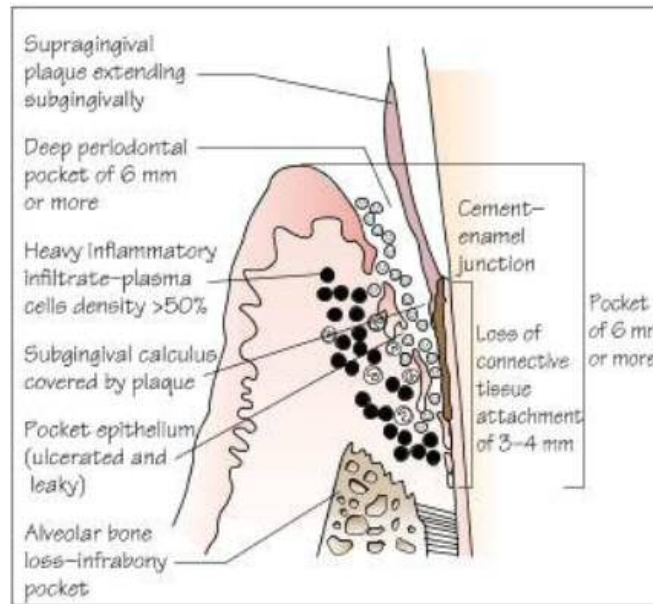
Figure 1.2.1.3B: The periodontal pocket (longitudinal sectional view)

The hallmark feature of the periodontal pocket is the presence of true attachment loss, resulting from migration of the junctional epithelium apically from the cementoenamel junction. There is resorption of alveolar bone in conjunction with apical translocation of the junctional epithelium, which results in loss of hard and soft tissue support to the tooth.

(Image from Clerehugh et al. 2013)

1.2.1.4 Artificial surfaces

Far from a modern phenomenon, the introduction of artificial prosthesis in the oral cavity is known to have been utilised since antiquity (Stern and Sreter 1996). The materials of choice in modern dentistry include composite resins, glass ionomer cements, a variety of ceramics, metal alloys, and polymethyl methacrylate (PMMA). As dental implants continue to increase in popularity, titanium and closely related alloys are used with growing frequency. The potential implications of this change, particularly in later life and if subject to inadequate maintenance and hygiene measures are substantial, but beyond the scope of this review.

An altered microbial community composition has been reported for a range of dental materials. Several studies have examined the microbiota associated with orthodontic appliances (both traditional fixed and removable appliances, and more recent clear retainers). Koopman et al. (2015) found an increase in the relative abundance of periodontal pathogens such as *Selenomonas* and *Porphyromonas* species during treatment with fixed orthodontic appliances, but microbiome composition returned to that of baseline by 12 weeks post-treatment. A further study (Wang et al. 2019) compared the salivary microbiome of patients treated with fixed orthodontic appliances or removable Invisalign© appliances to untreated controls. Similar

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changes in community composition were noted, with an increased relative abundance of phyla and genera associated with periodontal disease. However, oral hygiene was not assessed in this study, and this may have acted as an important confounder. In contrast, biofilms formed on dental composites have been shown to differ only slightly from those present on enamel (Conrads et al. 2019). While there was a significant increase in species richness in biofilms grown on composite materials compared with enamel surfaces, the magnitude of change (an approximately 25% increase) was of questionable clinical significance. Other artificial surfaces, such as drinking bottles, pacifiers and other restorative materials might be expected to impact the oral microbial composition, yet there is a dearth of research into these materials.

Approximately 19% of the UK population wears a full or partial removable denture (Adult Dental Health Survey 2009). PMMA and some metal alloys (typically cobalt-chromium) are typically used in removable prostheses. Unlike the majority of intra-coronal restorations and fixed prostheses, these materials form an interface with the oral mucosa, which potentiates the accumulation of microbes by forming areas of stagnation shielded from cleansing by saliva and masticatory friction. Like the dental hard tissues, such surfaces are non-shedding, and thus without effective cleaning, biofilm formation can proceed uninterrupted. In principle, this poses little problem, as the ability to remove such prostheses should facilitate thorough cleaning by patients. However, individuals in institutionalised care settings, or frail elderly populations who are likely to have multiple comorbidities may lack the motivation, dexterity or mental capacity to effectively clean such prostheses (Coulthwaite and Verran 2007). Oral care provided by staff in institutional care settings can often be suboptimal due to a number of barriers. Care staff often have insufficient training in oral health; many have an inherent declivity to engage in thorough oral hygiene because this is seen as invasive or repellent to some staff; and residents may be highly resistant to oral care, particularly if affected by dementia or other cognitive impairments. Many residents refuse to remove their dental prostheses, or to relinquish control of oral hygiene in an attempt to retain independence, despite mental or physical impairments that may hinder their ability to conduct such care optimally. Finally, oral and denture care may simply be a low priority in comparison to other care needs and be omitted or neglected due to these competing demands and time or resource pressures (Johnson et al. 2017).

A further challenge to effective denture cleansing is the presence of surface imperfections, cracks or degradation with age (Figure 1.2.1.4A & B). While it is typically recommended that dental prostheses be replaced approximately every 5 –

7 years; this is sometimes not pragmatic for a number of reasons. It may be financially non-viable for an individual, or declining motor control may limit a patient's ability to adapt to a new prosthesis, even when techniques are employed to copy the shape of previous dentures. Access to dentistry for frail elderly patients or those in institutional care settings may be limited, and compliance may decline in concordance with progressive cognitive impairment such as dementia. The presence of cracks or surface imperfections provides sheltered niches that can shield microbes, even from diligent hygiene measures. In particular, *C. albicans* hyphae have been shown to infiltrate surface imperfections to anchor mycelial biofilms to acrylic biomaterials (Ramage et al. 2004).

Another frequently encountered clinical problem is atrophy of the alveolar ridges which support dentures. This is particularly problematic in the mandible, where progressive atrophy can lead to translocation of the mental foramen from the lateral surface of the body of the mandible to the edentulous ridge crest. Pressure on the prosthesis during function can then elicit compressive neurogenic pain, which can be sufficiently severe to impede adequate nutrition in some cases. Similarly, pressure exerted on atrophic ridges can elicit pain directly due to inadequate thickness of the alveolar processes. Techniques to avert this issue are limited, and therefore denture soft lining materials, typically polyvinylsiloxane-based, are often employed to cushion compressive forces and distribute pressure over a wider surface area (Ettinger 1993). These lining materials are readily infiltrated by *C. albicans* hyphae, and degenerate at a rapid rate compared to PMMA, to form an irregular, roughened surface that promotes accumulation of microbial biofilm (Bulad et al. 2004). To overcome the issue of enhanced microbial accumulation on denture soft lining materials, a number of researchers have attempted to incorporate antimicrobial agents into the silicone materials used, with variable success (Lefebvre et al. 2001; Chladek et al. 2011; Bueno et al. 2015).

A final feature of removable prostheses that has received surprisingly little consideration in the literature is the potential role of denture biomaterial surfaces as a vector for environmental microbes to enter the oral cavity (Nair et al. 2016). Current advice recommends removal of dentures overnight to reduce the risk of denture stomatitis; while many patients who struggle to tolerate denture-wearing for long periods opt only to wear their prostheses for functional or aesthetic occasions such as meal times or when in public. Storage of removable dentures when not worn is highly variable, ranging from a variety of denture cleanser solutions, a glass of water, or dry storage. It is not uncommon for patients to simply store their prostheses in a pocket or place them loosely upon a convenient surface. Improper storage may

provide an opportunity for atypical, environmental microbes with pathogenic potential to adhere to the acrylic biomaterial surface of the denture, with sufficient time to effectively colonise, propagate and form early biofilm structures prior to reinsertion of the prosthesis. In an immunocompetent individual in a community setting, this transient influx of atypical microbes may pose little threat under normal circumstances, as the combination of host immune response and colonisation resistance of the host microbiome may neutralise the alien colonisers. However, in institutional care settings where endemic outbreaks of highly virulent putative respiratory pathogens such as Methicillin resistant *S. aureus* (MRSA), *Klebsiella pneumoniae, Haemophilus influenza* and *P. aeruginosa* are relatively frequently reported (Cassone and Mody 2015), the potential for the acquisition of a pathogenic microbial reservoir via removable prostheses in susceptible individuals is substantial. Subsequent development of respiratory infection depends on the interplay of multiple stochastic factors to result in dysbiotic modulation of the oral ecosystem that exceeds an individual's immune defence capacity.
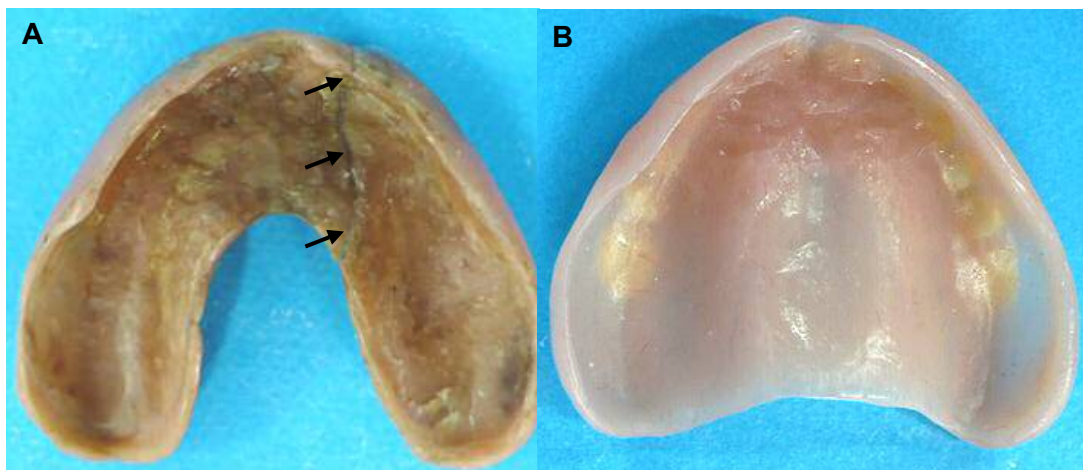
Figure 1.2.1.4: Changes of the denture fit surface with age

A)   An aged denture with poor denture hygiene. Note the acrylic fracture (lower left of image) and accumulation of debris and calculus around the crack (indicated by arrows)
B)   A new denture provided for the same patient. Note the relatively smooth surface and lack of stain, debris and calculus.
(Images from Babiuc et al. 2009)

1.2.2: Modulation of the oral ecosystem

With recognition of the complexity of the oral microbiome and the vast, variable landscapes offered by the oral cavity, the language of microbiology has adopted much of the terminology of ecology. In this paradigm, the influence of one or more factors relating to the host, occurring within microbial populations or occurring as a side-effect of certain dental or medical interventions (*i.e.* iatrogenically) can lead to

dysbiosis: a shift in community composition within the oral microbiome that results in deleterious effects to the host (Azarpazhooh and Leake 2006). An important point to acknowledge in reference to the seeding of infection at distant sites, such as pneumonia, is that here dysbiotic change does not necessarily result in noticeable detriment to the host in every case (at least not in a clinically detectable manner), but instead increases the stochastic risk of seeding and developing the disease. This is a slight, but critical modification to the 'ecological plaque hypothesis' first applied to dental caries and periodontal disease (Marsh 1991, Marsh 1994).

Perturbation of the oral microbiome can occur by two primary mechanisms, summarised in Figure 1.2.2. The influence and interactions of a range of factors can combine to drive dysbiotic community shifts (Figure 1.2.2A). Some factors can be considered 'modifiable' behaviours (*e.g.* smoking, dietary composition, oral hygiene); some are classically considered 'modifiable' (*e.g.* medications affecting salivary flow or immune factors, antimicrobial agents) but from a pragmatic, individual perspective are typically unavoidable without incurring more severe health sequelae; while some are essentially unmodifiable (*e.g.* genetic differences, baseline salivary flow rates and composition). This can either result in the disproportionate growth of one or more species which is either classically pathogenic (Hornef 2015), or a 'pathobiont' – commensal in health, but disease-causing under certain circumstances (Figure 1.2.2B). Such changes occur through both direct effects of the ecological pressure(s), and through other indirect effects of the same pressures on the host. For example, an individual may receive antibiotics to combat a severe urinary tract infection. In addition to the direct effects of oral antibiotics eradicating a large proportion of the resident oral microbiome, providing the opportunity for colonisation by opportunistic pathogens or the promulgation of pathobionts which happen to be resistant to the antibiotic used, the systemic effects of the infection may lead to an increase in pro-inflammatory markers. Simultaneously, appetite and sleep may be reduced, leading to further changes in the microbial composition and increased stress levels. This in turn can result in increased sympathetic nervous tone, which decreases salivary flow. During ill-health, oral hygiene might be expected to decrease in effectiveness, particularly if the individual suffering from a disease is rendered bed-bound. The interplay of these factors will result in a net reduction in the effectiveness of the immune response, further dysregulating the normal control mechanisms of the commensal oral microbiome. Of note, despite close approximation to both the oropharynx and respiratory tissues, the nasal microbiome has been shown to have little impact on the lung microbiome in health (Sakwinska et al. 2014). There is surprisingly scant evidence regarding perturbations in the nasal bacteriome in

pneumonia, but a Swiss cross-sectional study in children found that nasal microbial communities enriched for *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were associated with an increased risk of pneumonia. Furthermore, rhinovirus infection is a known risk factor for subsequent pneumonia development and has been shown to increase the acquisition of *S. pneumoniae* within the nasal microbiome (Karppinen et al. 2017). Overall, there is a surprising lack of evidence regarding compositional changes in the nasal microbiome in pneumonia patients, however.

It is possible that in institutionalised care settings, where opportunistic pathogens are frequently isolated (Kokubu et al. 2008), initial colonisation by such microorganisms could be maintained at a low level. Subsequent change of any ecological factors that govern this relationship could result in overgrowth of this acquired pathogen to drive dysbiotic changes.
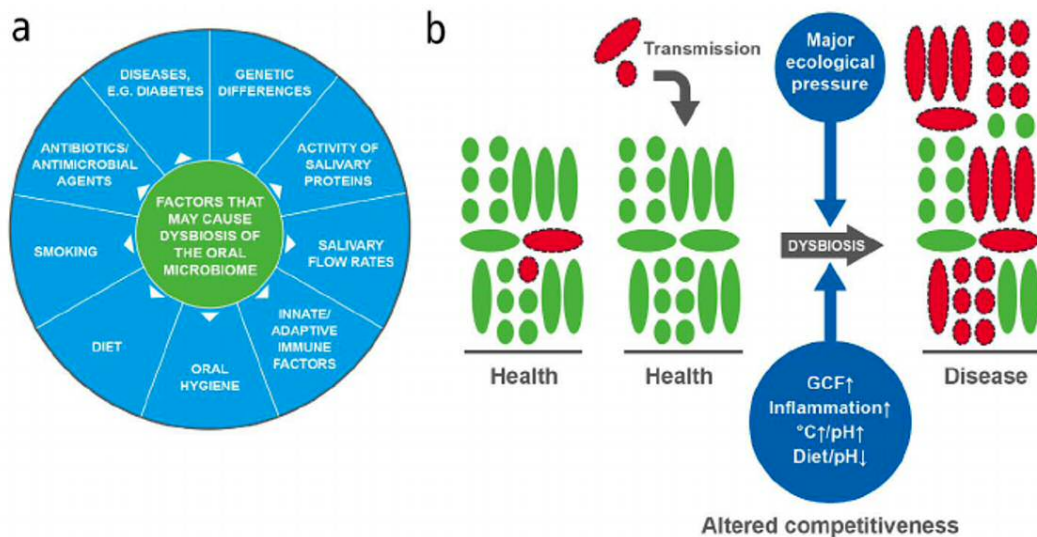
Figure 1.2.2: The ecological selection model of dysbiotic changes in the oral microbiome as a driver of disease

a) A broad range of factors can exert a selective pressure on the oral ecosystem, often with interplay between several factors.

b) This in turn drives either propagation of indigenous pathobionts or permits acquisition of and colonisation by exogenous opportunistic pathogens from the environment. The combination of environmental disturbance and microbial dysbiosis are synergistic. Eventually, this pathogenic microbiome can drive local disease, or seed infection of distant tissues.

(Killian et al. 2016, © Springer Nature, Creative Commons CC BY License.)

Another putative iatrogenic factor which could perturb the normal microbial composition of the oral cavity is the presence of biomaterial surfaces such as removable dentures. The role of such prostheses as vectors for opportunistic pathogens to gain entry to the oral cavity in sufficient numbers for successful

colonisation has been outlined in section 1.2.1.4. However, a further mechanism by which the presence of dental prostheses may promote colonisation by atypical, potentially pathogenic microbes is the very presence of an artificial biomaterial surface.

The 'normal' oral microbiome has evolved in tandem with the host, with a finely orchestrated relationship whereby specific binding sites on host surfaces, or via the glycoproteins of the salivary pellicle, combined with regulation by host immune cells and antimicrobial peptides, results in an overall commensal relationship overall (Sultan et al. 2018). Denture acrylic differs from the natural non-shedding dental enamel surface in hydrophobicity, surface free energy, chemical composition, surface roughness and isoelectric point (Müller et al. 2009). Consequently, the composition of salivary pellicle and microbial adhesive behaviour on acrylic surfaces differs from enamel (Svendsen and Lindh 2009). The evolved biochemical adhesion pathways that regulate 'normal' colonisation of the oral cavity by predominantly commensal microbes (Pedersen and Belstrøm 2019.) may therefore be bypassed, allowing more resilient or rapidly growing environmental pathogens to outcompete typical oral microbes. A key to such atypical microorganisms' survival and formation of robust biofilms recalcitrant to removal is likely influenced by the shelter from saliva provided by dentures.

1.2.3: The multifaceted roles of saliva

Saliva plays an important role in modulating the adhesion of microbes to oral surfaces, including PMMA biomaterials (Kerr and Tribble, 2015). It contains a range of glycoproteins which, as well as lubricating the oral tissues, selectively adsorb to oral surfaces to mediate attachment of commensal organisms such as streptococci and actinomyces. This film of salivary constituents is termed the acquired salivary pellicle (Dawes et al. 2015). The presence of the salivary pellicle on teeth is thought to be one of the major factors influencing the establishment of a 'healthy' microbiome, as oral microbes have evolved specialised adhesion lectins, adhesins, which bind targets within typical pellicle components, outlined in Figure 1.2.3.

Although little evidence exists regarding differences between the salivary pellicle present on teeth compared with artificial surfaces such as dentures and endotracheal tubes, there is a suggestion that pellicle composition differs both in ratio and presence of certain constituents. In particular, pellicles on denture surfaces were found not to contain mucin 7, cystatins or proline-rich-proteins, which could contribute to different patterns of adhesion (Edgerton and Levine, 1992). In addition, the presence of oral inflammation often associated with ill-fitting dentures may result in the incorporation

of serum components into the pellicle, which is thought to contribute to increased *Candida* bioburden and thus is implicated in development of denture stomatitis (Ramage et al. 2004). The implications of these findings regarding colonisation of such surfaces by respiratory pathogens are unknown and more research is needed to elucidate their significance. Despite small differences, the pellicle likely offers similar advantages overall to adherent microorganisms on any non-shedding surface, namely the provision of ligands for binding, and reduction of surface charge, which favours adhesion (Cavalcanti et al. 2016).
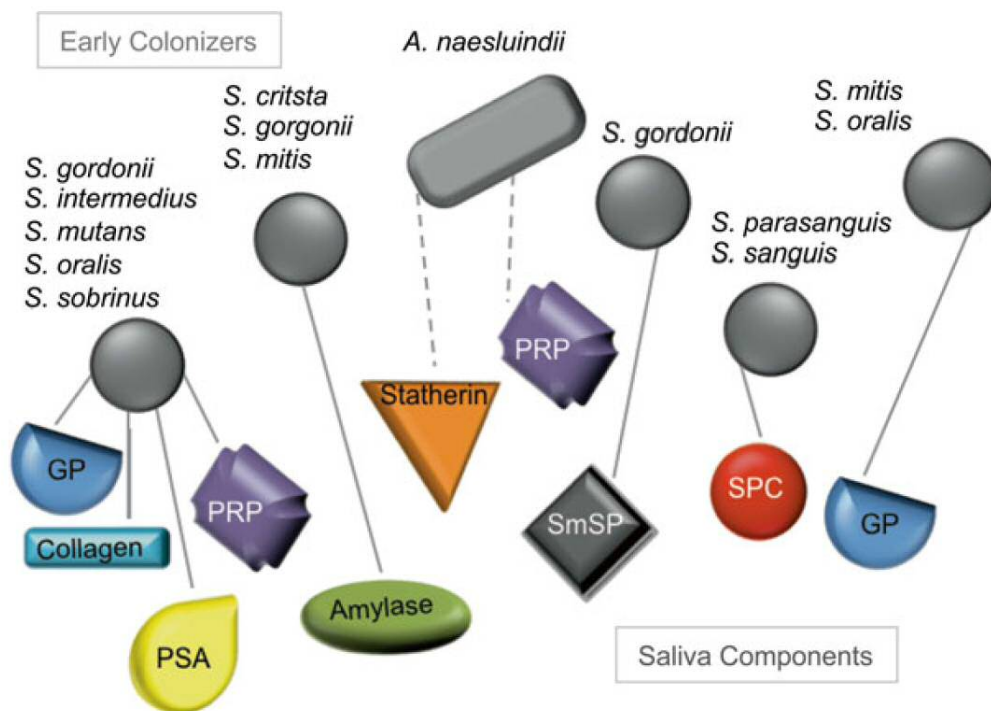
Figure 1.2.3: Saliva receptors for bacterial adhesins

Salivary components offer a variety of potential targets for bacterial adhesion by highly adapted oral bacterial species (early colonizers). Oral prostheses may feature alterations in pellicle composition or reduced pellicle coating and thus alter the patterns of colonisation.
Key: solid lines – adhesins, dashed lines – surface fibrils, GP – glycoprotein, PRP – proline rich proteins, SPC – salivary pellicle component, PSA – parotid salivary agglutinin, SmSP – submandibular salivary protein.
(Image from Kerr and Tribble, 2015)

Saliva has other important functions related to pellicle formation and maintenance of a commensal oral microbiome. Many immunologic components are present within saliva such as histatins, cystatins, IgA, lysozyme and lactoferrin (Humphrey and Williamson, 2001). A putative role of mucins is to localise these antimicrobial substances to the tissues. For instance, by binding to statherin and histatins, mucins 5b and 7 can concentrate them on oral surfaces (Linden et al. 2008). These actions, combined with salivary buffering, clearance and subsequent deglutition, help to

ensure the oral microbiome is regulated. Changes in health, behavioural factors such as smoking, polypharmacy and age-related changes can alter salivary flow, composition and pH (Vissink et al.1996). Such changes may have a significant role in the development of not only oral, but systemic diseases of oral origin. Many such factors are present in individuals in institutionalised care. The majority of residents are frail elderly individuals with multiple comorbidities and a high medication burden (Gordon et al. 2013).

1.2.4: The oral microbiome in health and disease

The constituent, commensal oral microbiome has a role in maintaining both local and systemic health through a number of different mechanisms, summarised in Figure 1.2.3. Some health-associated facets of the microbiome are related to metabolic functions of the microbiome (Huttenhower et al. 2012), and may be more pronounced in the gut proper, such as regulation of the cardiovascular system (Hansen et al. 2015) and antioxidant activities (Menni et al. 2019). However, the possibility for dysbiotic changes within the oral microbiome to result in deleterious effects to the cardiovascular system are clear from the link between metabolic syndrome and periodontal disease, despite the much greater population present within the gut (Koren et al. 2011). Whether periodontal pockets provide a reservoir of pathogenic species which can seed colonisation of the gut has received limited exploration, although early data suggests this may be the case. Lourenço et al. (2018) found that not only did the gut microbiome contain more periodontopathogens in periodontitis patients compared to health controls, but that overall gut microbiome diversity was reduced in such individuals. As with all molecular studies, such research is limited by an inability to distinguish living from dead microbes. Furthermore, the possibility of confounding factors, particularly given the small sample size analysed, cannot be excluded.

Other microbiome features in health, such as resistance to colonisation by exogenous pathogens, play critical roles in the potential association of dentures, colonisation by PRPs and the development of pneumonia.
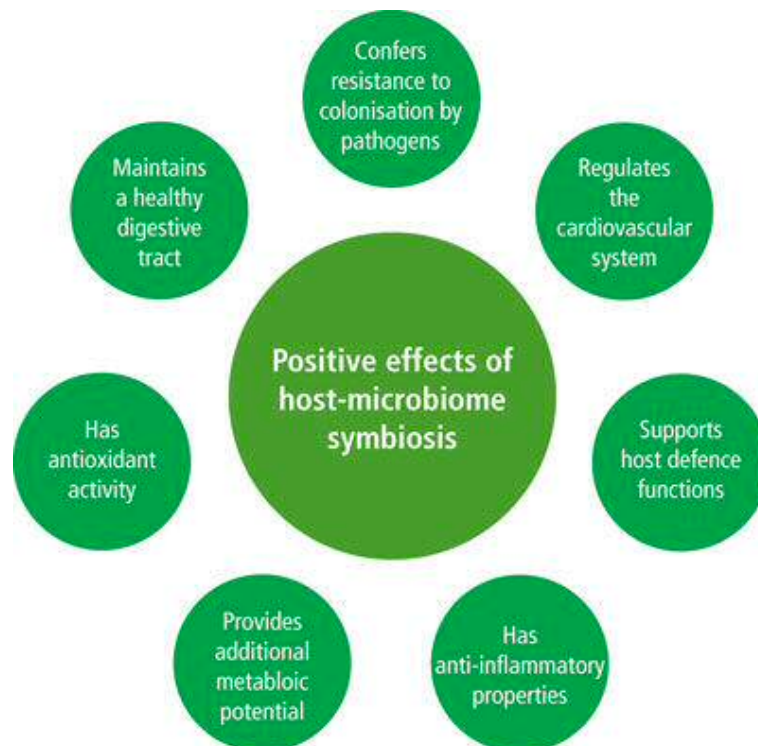
Figure 1.2.4: Beneficial effects of the commensal microbiome to the host

The role of the host microbiome, particularly in the gut, is a growing field of research. Links between microbial nitrite/nitrate metabolic pathways and cardiovascular health have been identified. The role of the gut microbiome in neonates in facilitating normal immune development has been inferred through a combination of *in vitro* studies and cross-sectional observations. Much of the research surrounding pre and pro-biotics has found that modulating the gastro-intestinal microbiome can result in an anti-inflammatory effect. Similarly, disruption of the developing microbiome leads to reduced intestinal antioxidant levels.
(Image from Killian et al. 2016 © Springer Nature, Creative Commons CC BY License.)

1.2.4.1: Symbiotic relationships of host and oral microbes

The role of the gut microbiome in maintaining both local and systemic health is well-documented (Shreiner et al. 2015). However, evidence specific to the role of the oral microbiome is comparatively limited. Much interest has been focussed on the potential pathogenic roles of the oral microbiome in both local and systemic disease, with effects on health framed tangentially as simply the absence of changes associated with such diseases. Some limited cross-sectional data has suggested that the 'core' salivary microbiome can be split into two function groups: a Prevotella or Neisseria predominated group (Takeshita et al. 2015). The former may be associated with poorer periodontal (including plaque and bleeding indices, periodontal pocket depth and tooth loss) and systemic health (such as smoking history, high body mass indices and increasing age), while the latter more health-associated. Clearly, this data is not suggestive of causality, although even in the best-established cases of model disease organisms in the oral cavity, such as *S. mutans* in caries (Simón-Soro and

Mira 2015), or *P. gingivalis* in periodontal disease (Olsen et al. 2017), it is highly challenging to satisfy every condition of causation required to demonstrate a traditional pathogenic role, capable of satisfying Koch's postulates under every circumstance.

One area where the influence of the commensal oral microbiome has been well-documented is the induction of 'immune tolerance'. Many oral streptococci are able to suppress local immune responses, which not only ensures their continued survival, but also leads to the maintenance of normal, healthy oral tissues, despite frequent contact with foreign antigens in the form of the oral microbiome, food and other substances (Devine et al. 2015). This action does not appear to reduce immune response to pathogens, as can be inferred from the increase in pathogenic microbiota in chronically immunosuppressed individuals (Diaz et al. 2013).

The oral microbiome also contributes to metabolism of dietary nitrates, which can enhance cardiovascular health (Blekkenhorst et al. 2018). Dietary nitrates are reduced to nitrites by resident microbes such as Rothia and Neisseria species (Vanhatalo et al. 2018). When swallowed, nitrites can be converted to nitric oxide, which reduces blood pressure through peripheral vasodilatory activity (Tousoulis et al. 2012) and may reduce endothelial pathoses and platelet aggregation (Riddell and Owen 1997). Cumulatively these effects could reduce atherogenic change and risk of adverse cardiovascular events, although evidence to date has focussed only on vascular function in healthy populations. Research exploring the potential for modulation of the oral microbiome to reduce adverse cardiovascular outcomes in at-risk populations is much needed.

Besides the above examples of microbial function either directly or indirectly shaping health, the oral microbiome can reduce colonisation of oral surfaces by pathogenic species through conferring resistance through both passive and active mechanisms.

1.2.4.2: Microbiome diversity and resistance

The concept of colonisation resistance was first derived from observations of increased susceptibility of individuals to intestinal Salmonella infection following antibiotic treatment (Bohnhoff et al. 1954). Subsequent experiments in mice demonstrated that microbes from healthy individuals' faeces could reduce the risk of colonisation by *Salmonella enteritidis* (Bohnhoff et al. 1964). The same concept is today applied through faecal transplant therapy to eradicate recalcitrant *Clostridium difficile* infection in patients, which is a potential sequela of broad-spectrum antibiotic treatment (Kassam et al. 2013).

More recently, an appreciation for the role of the resident commensal oral microbiome in providing colonisation resistance has developed. The mechanisms of colonisation resistance can be broadly classified as passive or active.

Passive colonisation resistance occurs through normal microbial activity, either due to establishment of a large population of commensal species prior to colonisation by potential pathogens, or due to the evolved advantage of the resident oral microbiome in the oral cavity. As has previously been discussed, oral and salivary binding sites have likely co-evolved with commensal oral microbes to maximise the ability of such microorganisms to occupy available oral niches. Additionally, such microbes are highly adapted to metabolise the available nutrients and induce immune tolerance to permit their continued presence and survival. It is not necessary for oral microbes to outcompete alien opportunists for all available nutrients – only a single limiting substrate is enough to prevent effective colonisation by exogenous microbes (Litvak and Bäumler 2019). Oral microbial communities exist largely as biofilms, with complex, dynamic interactions including co-dependent metabolite pathways and synergistic relationships (Huang et al. 2011). This means that would-be colonisers must not only overcome adaptations that confer a selective advantage for the environment, but also co-evolved inter-microbial adaptations that further shape the environment and communities within.

Active resistance to colonisation relates to direct antagonistic activity of microbes to exogenous species (Sorbara and Pamer 2019). While it may be argued that such activity is primarily used by microbes to compete with other endogenous species, and even within the same species for individual competition, the effects on exogenous species are likely far more pronounced due to the co-evolution of endogenous microbial communities under such selective pressures (Hibbing et al. 2010). Many microbes produce antimicrobial substances, such as surfactants, antibiotics, antimicrobial peptides and a variety of substances secreted through Type VI secretion systems (Chassaing and Cascales 2018). While much of our knowledge of such substances is derived from studies of the gut and vaginal microbiome, there is increasing interest in application of pre, pro and synbiotics to the oral cavity (Devine and Marsh 2009). Avenues explored to date include prevention of caries, periodontal disease and oral candidiasis. However, modulation of the oral microbiome through such approaches as a means of reducing systemic disease is an area that remains to be explored.

1.2.4.3: The oral microbiome as a pathogenic reservoir

That the oral microbiome can harbour microbes with pathogenic potential is evident from the presence of oral diseases, such as dental caries (Burne et al. 2012), periodontal disease (Liu et al. 2012) and oral candidiasis (Morse et al. 2019), where a role for oral microbial aetiology is convincingly established. To demonstrate contribution by the oral microbiome to systemic diseases is far more complex, however. Microbes must be shown to be present in the oral microbiome prior to the development of the disease in question, rather than simply to be able to colonise the oral cavity as a consequence of changes associated with the disease. They then must either exert effects to contribute to the pathogenesis of the disease through direct translocation to the disease site, or by inducing a host response with deleterious consequences at the disease site (Hill 1965). While the oral microbiome comprises fungi, bacteria, viruses, archaea and protozoa; the majority of research to date has focussed on the bacterial microbiome. Some researchers are beginning to unravel the importance of fungal and viral microbiota more recently. For brevity and relevance to the study methods reported in this thesis, only the bacterial microbiome will be considered in the following sections.

In the case of the association of periodontal disease with cardiovascular disease, there is evidence to support both dissemination of oral bacteria into the bloodstream (Haraszthy et al. 2000), and adverse proinflammatory mediators in response to the microbial insult to the periodontium. In the case of respiratory infection, the challenge is not in identifying a pathway for microbes to the lungs, which is obvious by their anatomical contiguity. Instead, the requirement to demonstrate causation beyond reasonable doubt is to demonstrate that colonisation of the oral microbiome by the relevant putative respiratory pathogenic species occurs prior to the development of respiratory infection, rather than simultaneously or subsequently (Rogers et al. 2013).

1.2.4.4: Pathogens and virulence - a matter of context

The terminology of microbial infection is sometimes ill-defined, with terms frequently being used interchangeably and without thought to the meaning implied through specific definitions. An example of this is in the use of the term 'virulence factor', often used to describe any microbial function that is directly deleterious to the host (Casadevall and Pirofski 2009). Many authors also describe surface adhesins, factors relating to immune evasion or survival within the host environment as virulence genes when present in a microbial species considered pathogenic. When similar, sometimes identical factors are present in typically commensal species, however, these same virulence factors could now be considered beneficial, and even described

as part of the mutualistic relationship between host and microbes. This is inaccurate and confusing. Instead, the classification of Wassenaar and Gaastra (2001) below is helpful for clarifying the attribution of virulence in pathogenic microbes.
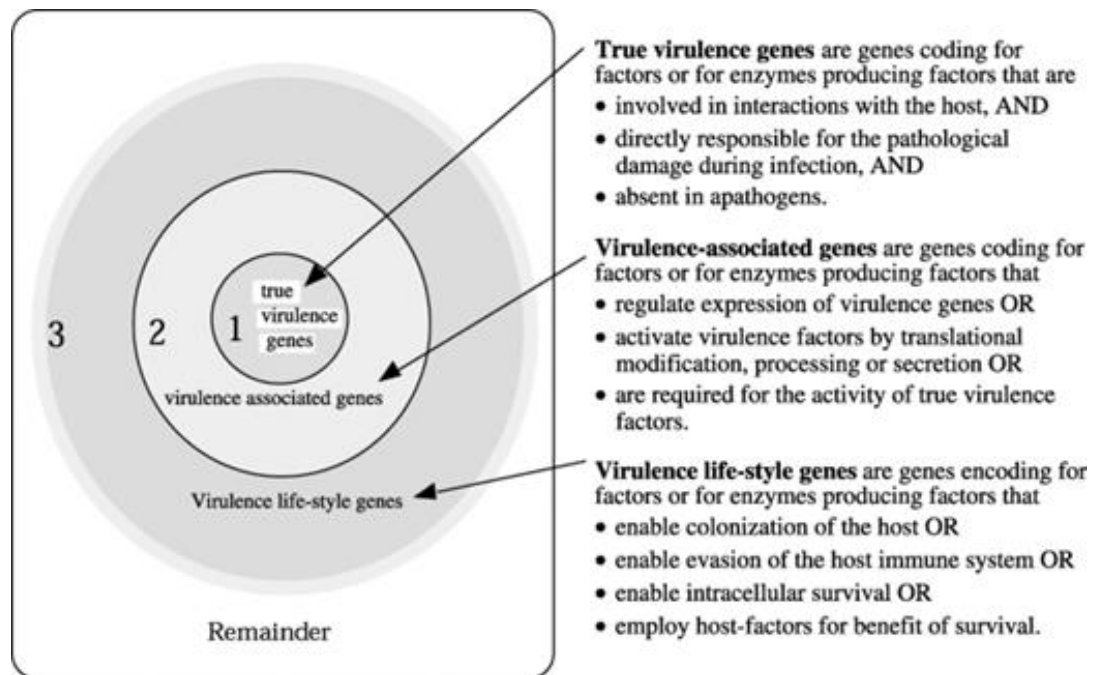
Figure 1.2.4.4: Differentiation of true virulence from virulence-associated or virulence life-style genes

(Figure from Wassenaar and Gaastra 2001)

Of note, this classification refers specifically to genetic regulation of virulence, but can easily be adapted to virulence factors themselves.

Adoption of this model can help contextualise terms such as commensals, opportunistic pathogens (pathobionts) and true pathogens. Under these definitions, only microorganisms capable of producing true virulence genes can be considered exclusive pathogens, while both commensal microbes or pathobionts may possess virulence-associated or virulence life-style genes. Interestingly, horizontal gene transfer between species means that an analysis of species composition alone within microbial communities is not necessarily predictive of pathogen presence (Martínez et al. 2007). Instead, analysis of microbial function through transcriptomics, proteomics or metabolomics is needed (Vayssier-Taussat et al. 2015).

1.2.4.5: Pathogenic potential of the oral microbiome

In light of the above definitions, can the archetypal pathogenic oral microbe associated with oral caries; and can *S. mutans* be considered a true pathogen? A

brief exploration of the often-touted virulence traits of *S. mutans* (Banas 2004) casts some doubt:

- Enhanced adhesion (both sucrose-independent and sucrose-mediated)
- Enhanced biofilm formation
- Lactate metabolic pathway-mediated acidogenesis
- Enhanced aciduricity

None of these traits are unique to pathogens, are shared by most streptococci (Cotter and Hill 2003), although acidogenesis and aciduricity are enhanced in *S. mutans.* Furthermore, caries can occur in the absence of *S. mutans*. As our understanding of the caries microbiome evolves, the role of community interactions and functional redundancy has shown that the role of *S. mutans* is likely overstated, despite this microorganism still being a key player in the development of caries for most individuals (Banas and Drake 2018). It seems likely therefore, that this species is typically an opportunistic pathogen, with no true virulence factors.

The role of the indigenous oral microbiome in the pathogenesis of systemic disease has also been explored. Infective endocarditis can arise in susceptible individuals; especially those with prosthetic heart valves, as a result of bacteraemia during invasive dental procedures (Habib et al. 2009). Streptococci of oral origin have been identified in blood cultures from patients with infective endocarditis, although it should be noted that prior invasive dental treatment, oral health and other key aetiological factors were unknown for this cohort (Douglas et al. 1993). Outside of this acute and somewhat predictable aetiology, the role of the oral microbiome in atherosclerotic changes and ischaemic heart disease (IHD) is more complex. Associations between severe periodontal disease and IHD have been found which cannot be explained by the interaction of confounders such as diabetes, smoking and BMI alone (Humphrey et al. 2008). The triad of microorganisms most frequently associated with severe periodontal disease: *P. gingivalis, Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* have all been found to be capable of directly invading the periodontal tissues to gain entry into the bloodstream (Reyes et al. 2013). These microorganisms have been isolated from atherosclerotic plaque and are able to mediate local inflammation and coagulation to contribute directly to disease (Haraszthy et al. 2000). Furthermore, circulating pro-inflammatory mediators are increased in response to the chronic inflammatory environment of the periodontal pocket, which may also contribute to disease pathogenesis (Loos et al. 2000). This mechanism of disseminated inflammation is likely the primary means by which

periodontal disease negatively impacts glycaemic control and insulin sensitivity in diabetics, as no convincing direct role for the oral microbiome has been discovered to date (Preshaw et al. 2012).

A growing area of research recently has been the putative role of the periodontopathogens *P. gingivalis, Prevotella intermedia* and *Tannerella forsythia* in the development of rheumatoid arthritis (Martu et al. 2017). *Porphyromonas gingivalis* is of particular interest in the pathogenesis of this disease due to its ability to citrullinate proteins, which provides a possible mechanistic pathway for the development of the autoantibodies responsible for rheumatoid arthritis (Cheng et al. 2017). To date, there have been no interventional studies exploring the impact of periodontal treatment on individuals at risk of severe rheumatoid arthritis, likely due to the challenges of identifying a suitably large, homogeneous group of such individuals (van der Pouw Kraan et al. 2003).

There is limited evidence to support a role for the oral microbiome in the pathogenesis of both oral and gastrointestinal cancers (Ahn et al. 2012), and a number of inflammatory gastrointestinal diseases (Lucas López et al. 2017). However, the intimate relationship of microbial community composition with key risk factors such as smoking, alcohol and diet make it highly challenging to unravel specific mechanistic pathways. Furthermore, the role of the immune system and interactions between host immunity and the microbiome add another layer of complexity which may mask the underlying associations. However, the possibility of the oral microbiome influencing gut health is intriguing for three main reasons:

1)   The resident gut microbiome might be expected to override the impact of the smaller oral microbial population

2)   The oral microbiome is more accessible for analysis and direct interventional studies (dietary interventions have a number of non-specific actions that may confound effects)

3)   The common mechanism of the oral microbiome seeding infective diseases or shaping the pathogenesis of disease at distant but anatomically contiguous sites parallels the putative role of the oral microbiome in the pathogenesis of respiratory infections

There is a range of evidence to support the role of both the indigenous oral microbiome in pneumonia, as well as the potential for the oral microbiome to harbour exogenous PRPs following colonisation in susceptible individuals. This comes from three primary areas – ventilator associated pneumonia in intubated intensive care patients (O'Grady et al. 2012); aspiration pneumonia in individuals with reduced neuromotor function (Paju and Scannapieco 2007), such as stroke patients; indirect

evidence linking pneumonia to colonisation of the oral cavity and oropharynx in susceptible individuals without a known aspiration event , using oral health as a proxy marker (Awano et al. 2008). These mechanisms and their inherent commonalities will be explored later to derive a unified model of the oral microbiome in pneumonia.

1.2.4.6: Dysbiosis and ecological shifts

During health, the oral microbiome is largely stable, with established microbial populations resisting colonisation by atypical species through a variety of means (Human Microbiome Project Consortium, 2012). However, a number of exogenous factors may 'destabilise' the environment and alter the composition and behaviour of the microbiome, leading to potentially harmful outcomes to the host (Marsh PD, 2003). This alteration of the oral ecosystem is considered more complex than simply an influx of pathogenic organisms, as typically commensal populations have been found to demonstrate increased virulence-associated or virulent lifestyle traits during such changes (Yost et al. 2015). This change in the microbiome is termed dysbiosis. Of note, such alterations usually result in a decrease in species diversity, although whether this is a cause or symptom of the predominance by more virulent species is unclear (Cho and Blaser, 2012). A notable exception to this trend is the periodontal pocket, where typically increasing diversity is associated with increased disease severity (Abusleme et al. 2013), perhaps due to the generation of a new anaerobic niche in deeper periodontal pockets, and the availability of new substrates and binding sites generated from the host inflammatory response.

1.2.4.6.1: Local sequelae of dysbiosis

The link between pathogenic alterations in the oral microbiome and local disease is clear in plaque-associated diseases such as periodontitis and dental caries. Additionally, the potential for prosthesis-related microbes to cause disease by similar mechanisms is typified by chronic erythematous candidiasis – an inflammatory condition which affects the palatal mucosa of denture wearers.

A significant sequela of inflammatory changes within the oral cavity is the alteration of the environment due to exudate of serum, immunological changes and in more severe cases breakdown of the superficial mucosal layers. These changes have been postulated to favour colonisation of mucosal surfaces by atypical organisms (Scannapieco FA, 1999). For instance, hydrolytic salivary enzymes associated with periodontal disease may contribute to degradation of fibronectin in the oral epithelium, increasing the binding affinity of microbes such as *P. aeruginosa* (Woods et al. 1981). Periodontal microbes themselves, particularly *P. gingivalis* can directly

degrade mucosal surfaces through release of proteolytic enzymes to expose subepithelial adhesive targets for atypical microbes including respiratory pathogens (Gibbons et al. 1990). Release of pro-inflammatory cytokines such as IL1, IL6, IL8 and TNF by oral mucosa and in saliva in response to microbial antigens may impact interactions between respiratory pathogens and these may impact both local host-microbe interactions and for oral microbes and secreted cytokines to enter the airways by microaspiration, impacting their behaviour. The release of proinflammatory cytokines has chemotactic functions. An example is neutrophil recruitment following IL8 release. Neutrophils release proteolytic enzymes and reactive oxygen species in response to contact with foreign antigens, further damaging the epithelial surfaces (Uriarte et al. 2016). Alternatively it may be that oral microbes suppress such immunogenic cascades to permit unchallenged colonisation by atypical pathogens. *Porphyromonas gingivalis* has been found to inhibit IL8 secretion from oral epithelial cells, which aids invasion of the cells (Darveau et al. 1998). The implications of such behaviour in respiratory tissues remains unclear.

1.2.4.6.2: Systemic sequelae of dysbiosis

The potential for systemic disease to be influenced by oral health is well established. Several conditions linked to oral disease, including bacterial endocarditis (Han and Wang, 2013), cardiovascular disease (Tonetti and Dyke, 2013) and diabetes mellitus (Preshaw et al. 2012) have been extensively described in the literature. Given this precedent, it is not unreasonable to expect the oral cavity to exert influence of development of lower respiratory tract infections.

A key consideration in the pathogenesis of respiratory infections is the inherently stochastic nature of the processes involved. In the case of aspiration pneumonia, the disease process mirrors infective endocarditis: a well-defined event leads to a bolus inoculum of microbes reaching the infection site (Marik 2010). A greater oral bioburden, with higher virulence potential is more likely to result in successful infection of the site. Likewise, a greater chance of inoculation occurs in the presence of higher aspiration frequency (van der Maarel-Wierink et al. 2011). In the case of pneumonia that occurs without a known aspiration event however, the exact mechanism is unclear. It could be that 'silent' aspirations, which have been extensively documented, lead to inoculation of infective microorganisms in the lungs (Zaloga 2002). Alternatively, sequential colonisation of the oropharynx, upper respiratory tissues and finally, infection of the lungs may occur as microbes propagate directly (Palmer et al. 2001; Ewan et al. 2009). This possibility seems particularly plausible in the case of highly motile microbes such as *P. aeruginosa*, which can be

observed to coordinate dispersion from an established biofilm and is capable of swarming across a surface to seed a new colony at a distant site (Hall-Stoodley and Stoodley 2005). Finally, the possibility of inhalation of lower numbers of microbes which escape trapping by respiratory epithelial cilia during inhalation, and generate a chronic, low level bioaerosol to the deeper respiratory tissues conveyed by moist air cannot be entirely excluded. It should be noted that some pneumonias are best explained by direct inhalation of aetiological microbes, such as fungal spores (Murray et al. 2019).

1.3: Pneumonia

The 2010 World Health Organisation (WHO) report "Global Burden of Diseases" (Lim et al. 2013) ranks lower respiratory tract infections as the fourth leading cause of death overall, and the most common cause of death due to infection. While in developing countries pneumonia accounts for a high proportion of childhood deaths, in more economically developed Western countries, such as the UK, much of Europe and the USA, pneumonia affects a disproportionate number of elderly patients, with a tenfold increase in European cases noted when patients are older than 65 years of age (Welte et al. 2012). Mortality due to pneumonia follows a similar trend, with 85% of deaths occurring in individuals over the age of 60 (Myles et al. 2009).

In reality the term pneumonia describes a clinical phenotype of acute inflammation in the lower respiratory tract (McLuckie 2009), rather than a specific infective process. Indeed, not all pneumonias are infective in aetiology. Pneumonias can be caused by any of bacteria, fungi, viruses or protozoa; and polymicrobial infections containing multiple species and kingdoms may occur (Cillóniz et al. 2011; Torres et al. 2014). In the UK, the majority of pneumonias are bacterial in aetiology (Welte et al. 2012). Signs and symptoms of pneumonia vary with severity, but include cyanosis, tachycardia and tachypnoea, characteristic chest sounds, cough, dyspnoea, fever, malaise, myalgia and pleurisy (McLuckie 2009). In severe cases requiring hospitalisation, a chest x-ray may be taken, with the finding of radiopaque consolidation in the lung space considered highly suggestive of pneumonia (Mittl Jr et al. 1994) in conjunction with supportive clinical findings (Figure 1.3). Definitive diagnosis can often be challenging due to the lack of specific symptoms associated with pneumonia (Dicpinigaitis et al. 2009).
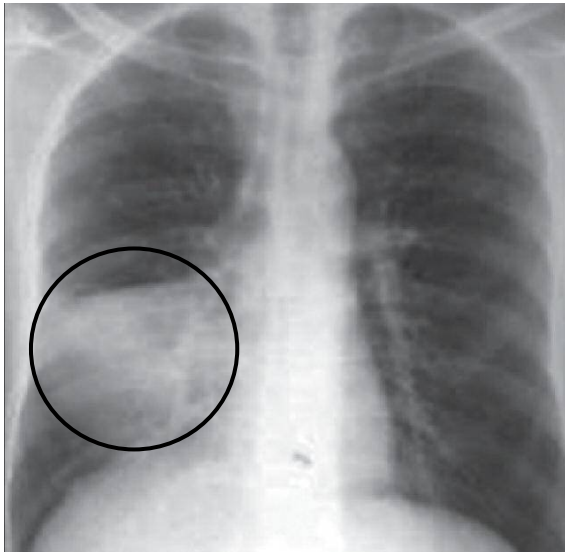
Figure 1.3: Chest radiograph of a patient with consolidation in the middle lobe of the right lung (circled) secondary to pneumonia. Often, radiographic signs are less apparent and can be highly subjective.
(Image from Khadanga et al. 2015 © Nepal Journals Online CC-BY-NC license).

1.3.1: The lungs and respiratory tissues in health

The respiratory system is responsible for supplying atmospheric oxygen to the blood, in exchange for carbon dioxide and other metabolic waste products. The respiratory tract begins with the nares and mouth, and includes the oral cavity and nasal cavity, which then merge in the pharynx. Subsequently, air passes through the larynx into the trachea. From this passage, there are approximately 23 iteratively narrower branching divisions which terminate in the alveolar air sacs (Lumb 2016). These sacs provide a large total surface area which is intimately associated with a dense vascular supply, to allow for the exchange of oxygen and carbon dioxide vital to respiration by diffusion (Weibel 1973).

Inspiration of air occurs through autonomically driven contraction of the diaphragm and intercostal muscles, which expand the chest cavity (Plum 1970). The lung tissues have inherent elasticity, and so the generation of negative pleural pressure leads to expansion of the lungs. In turn, this pressure vector leads to conductance of air from the mouth and nose, through the respiratory tracts to the alveoli. Subsequently, respiratory muscle relaxation results in a diminished chest cavity volume, which, aided by the elastic potential of the lungs, drives air outwards (Faffe and Zin 2009). The distal airways are lined by a surfactant-rich fluid, which reduces friction and helps to prevent collapse of the tissues (Goerke 1998). Typical respiratory rates at rest are highly variable, but in adults usually range from 12 – 20 breaths per minute (RCUK 2015). The neurological networks which interact to regulate breathing are highly complex. A number of psychological, physiological or pathological states can affect respiratory drive. In addition, it is possible to exert conscious control over breathing for a period of time.

An important feature of respiratory function is reflexive responses to foreign bodies or irritants in the respiratory tract: sneezing, coughing, laryngeal closure and bronchoconstriction (Shannon et al. 2004). A reflex similar to coughing, the expiration reflex, is particularly important in protecting the lungs from aspiration injury. In coughing, an inspiratory phase first occurs, followed by a complex expulsion mechanism involving coordinated closure then opening of the glottis to generate a high intrathoracic pressure and high-velocity expiratory airflow to ensure increased shear forces, which increase the change of dislodging and expelling foreign bodies (Chang 2006). The expiration reflex involves the same expulsive phases, without preceding inspiration, as this would only serve to draw in aspirated matter further into the lungs (Tatar et al. 2008). In addition to neuromuscular protection against aspiration, the respiratory epithelium is able to contribute to clearance of foreign material directly.

1.3.1.1: Key features of the respiratory epithelium

The respiratory tissues are lined by mucosa containing ciliated epithelial cells and mucus-producing goblet cells. The cilia represent the first immunological agent of the respiratory tissues, removing foreign particles and microbes indiscriminately. Goblet cells constitutively secrete mucin glycoproteins, which combine with water to form a viscoelastic gel: mucus. There is a continuous layer of mucus covering the respiratory epithelium of the larger airways, which serves to entrap inhaled particulate matter and microbes. Ciliated cells are specialised epithelial cells have hair-like protrusions which beat at a rate of approximately 12-14 beats per second. Ciliary movement towards the head occurs by protuberance of the cilia during the effective stroke, followed by deformation of the cilia during the recovery stroke. The net effect of this hysteresis movement pattern is to propel the mucus layer away from the distal airways, carrying any entrapped microbes or inhaled particles with it (Lumb 2016). Inhaled microbes may also be chemically inactivated in the lungs. The distal airways secrete surfactant proteins and lysozyme in their respiratory lining fluid, which can opsonise foreign antigens, agglutinate microbes, or can even have direct bactericidal activity by increasing membrane permeability (Wu et al. 2003). The airway lining fluid also contains immunoglobulins, primarily IgA in the upper respiratory tracts, while IgG predominates in the distal airways (Lumb 2016). Upon detection of foreign antigens, epithelial cells release a number of pro-inflammatory cytokines and chemokines to attract phagocytes, primarily macrophages, into the respiratory tissues (Boyton and Openshaw 2002). While respiratory cells produce anti-proteases to limit inadvertent

damage to the lungs by leukocyte proteolytic enzymes, in severe infections such enzymes may contribute to tissue damage (Garcia-Verdugo et al. 2010).

Changes in ciliary function, mucus production and composition, and immune function can occur with specific disease conditions, lifestyle factors, and with age. These can affect lung function and predispose individuals to increased risk of respiratory infection.

1.3.1.1.1: Immunology of the respiratory tissues

The components of the immune system can be subdivided into cellular and humoral elements, and may contribut to innate or adaptive immunity, with substantial cross talk between these 4 domains (Chaplin 2010). Following initial bacterial antigen presentation in the lungs, ciliary clearance constitutively removes the majority of microbes. However, if the quantity or virulence of the infective microbes is sufficient to prevent adequate removal, antigenic load from membrane-bound molecules, matrix components, metabolites and virulence factors will increase. This must pass through the mucus layer to reach the epithelium. Dendritic cells present within the respiratory epithelium (termed sentinel cells) and extend cellular processes through the epithelium to 'sense' the lung environment (Burleson et al. 2015). These immune cells, respiratory epithelial cells and fibroblasts all possess membrane bound pattern recognition receptors, capable of recognising conserved microbial components such as LPS, flagellin and lipotechoic acids. Some receptors can also detect damage to tissues, eliciting an immune response in both mechanical and infective trauma. Macrophages are also constitutively present within the alveoli and are capable of recognising foreign antigens through the same mechanisms, leading to phagocytosis and killing of microbes (Kugathasan et al. 2008).

The most well-known pattern recognition receptor is the Toll-like receptor family. These tranmembrane proteins form dimers upon contact with either pattern or damage associated molecular patterns (PAMPs or DAMPs respectively). Dimer formation triggers an intracellular cascade that regulates cytokine production, typically upregulating production of pro-inflammatory cytokines (Kawasaki and Kawai 2014). Additionally, both macrophages and dendritic cells phagocytose antigenic matter, digesting it and subsequently migrating to lymph tissue to present microbial material to T-cells, thus activating the adaptive immune system. The ability of macrophages to act as antigen presenting cells is contentious, with mixed evidence available, but it appears that alveolar macrophages at least possess this ability. They likely play a secondary role to dendritic cells, however (Kugathasan et al. 2008).

Activation of pro-inflammatory cytokines within macrophages and epithelial cells initiates a number of events. Firstly, further recruitment of circulating macrophages and neutrophils is coordinated with local vasodilation and increased vascular permeability to enable migration from the vessels into damaged tissues. Neutrophils are the primary cell of the innate immune system during acute inflammation and possess an arsenal of enzymes and other antimicrobial strategies such as reactive oxygen species and neutrophil extracellular traps (discussed further in Section 1.3.2). Neutrophils are also capable of directly phagocytosing microbes. In response to IL-17 secretion by dendritic cells, epithelial cells upregulate production of antimicrobial peptides (Eddens and Kolls 2012).

Additionally, both epithelial cells and alveolar macrophages produce a range of complement proteins. These proteins contribute to both innate and adaptive immunity by several mechanisms. Activation of the C3 either by the classical pathway in response to the F_c domain of antigen—bound immunoglobulins, or upon binding to microbial surface lectins results in opsonisation by the lectin pathway, clumping microbes and facilitating phagocytosis by macrophages and dentritic cells. C3 can also coordinate with C5 to promote pro-inflammatory signals and upregulate migration and activation of monocytes, as well as providing a substrate for further opsonisation. Any of the three complement pathways can result in formation of the Membrane Attack Complex by C5-9, which directly induces pore formation in target cells resulting in lysis and death. Complement also serves as a regulatory mechanism for antigen presenting cells – presence of activated C3 or C5 promotes T-cell activation by dentritic cells (Pandya and Wilkes 2014).

Natural killer cells are constitutively present within the lungs in health, forming up to 20% of the lymphocyte population. There is some evidence that these cells can be induced by bacteria to release lytic enzymes following activation of Toll-like receptors, in a manner analogous to neutrophils. However, their role in early bacterial infection may be minor, with stronger evidence for a prominent contribution to antiviral defences (Cong and Wei 2019).

These early activities of the innate immune system occur within the first few hours of infection. After approximately 12 hours, T-cell activation and clonal expansion, coupled with antibody production by B lymphocyte-derived plasma cells begins to play a more significant role in the immune response, allowing a more targeted reaction to specific antigens (Burleson et al. 2015). Antigen presentation by dentritic cells (and possibly alveolar macrophages in the lungs) to T-helper (CD4+) and cytotoxic (CD8+) T cells in lymph tissue results in maturation and proliferation of these cells, which are primed to respond to the specific antigen presented. T-helper

cells subsequently activate B-cells (Parker 1993). A subset of both T and B cells form memory cells, which remain dormant for long periods of time, but can rapidly reactivate following contact with the 'memorised' antigen. In the lungs, a subset of T-helper cells, Th1 cells are primarily responsible for enhancing phagocytosis of microbes and directing the activity of cytotoxic T cells. These cells directly kill microbes through release of intracellular granules containing hydrolytic enzymes and other cytotoxic contents. They are also responsible for inducing apoptosis or lysis of infected host cells, particularly in viral infections (Burleson et al. 2015).

Activated B cells can form plasma cells, responsible for antibody production. Initially, low affinity IgM is produced, conferring broad activity against microbes. Subsequently, class switching occurs to IgA, IgE or IgG, with much higher specificity to target antigens (Manis et al. 2002). Antibodies, like complement, can opsonize antigens, enhancing phagocytosis. They may also neutralise the activity of certain microbial toxins. In addition to cytokine production, antibodies activate the complement cascade to upregulate lysis by the membrane attack complex (Bebbington and Yarranton 2008).

The entire immunological process is coordinated within the lung by the zymogen cascades of complement, pro-inflammatory cytokine release and antibody production, to ensure a rapid immune reponse is mounted against the infectious threat. A side effect of such processes can be substantial tissue damage mediated by non-specific immune activity. This can increase the morbidity of pneumonia despite resolution of the initial infective process (Bordon et al. 2013).

1.3.1.2: Important lung changes with age

The respiratory tissues can undergo changes over the course of an individual's life, both as a consequence of local, neuromuscular age-related changes and immune senescence. Additional physiological impairment can occur as the cumulative result of lifestyle factors, particularly smoking, and the effect of comorbidities which can impact on respiratory, neuromuscular and immune function (Sharma and Goodwin 2006).

Age-related kyphosis of the thoracic spine, degeneration of the thoracic vertebrae due to osteoporosis and calcification of the rib cage can lead to decreased chest compliance (the ability of the chest to expand during inhalation) independent of lung compliance. In addition, diaphragmatic contractile strength decreases by up to 25% in individuals over the age of 65, leading to less effective ventilation with each breath (Tolep et al. 1995).

Within the lungs themselves, there is a gradual loss of normal alveolar anatomy and parenchymal tissue which can lead to collapse of the distal airways; effectively reducing functional lung volume (Miller 2010). Damage to the respiratory tissues can occur due to environmental factors, illness or behavioural factors such as smoking or malnutrition. These factors are cumulative, such that even relatively minor effects can amount to a significant decrease in lung function over many years. Longitudinal observational studies have found that decline in lung function can occur from the age of 35 onwards, and the rate of decline may double after an individual reaches 70 (Xu et al. 1995). This may relate to both a reduction in functional alveolar surface area and thickening of the alveolar-capillary membrane, where gaseous diffusion takes place. The epithelial cilia also reduce in beat rate, reducing the speed of clearance for mucus and foreign material that entered the lungs (Lowery et al. 2013).

In addition to the well-documented general immune senescence that occurs in old age, there are specific changes that occur within the lungs (Pawalec and Larbi 2008). A shift towards a more pro-inflammatory state occurs, with increased IgA and IgM, and increased CD4+:CD8+ T-cell ratio and increase in neutrophil number detected in respiratory fluid samples (Meyer 2001; Lowery et al. 2013). Alveolar macrophage behaviour also changes, with a greater proclivity for production of toxic oxygen radicals in response to foreign antigens (Meyer et al. 1996). In concordance, the respiratory lining fluid contains a lower quantity of anti-proteases and antioxidants (Moliva et al. 2014). These changes can compound to result in a low-grade, chronic inflammatory environment in the respiratory tissues.

Beyond changes that directly affect the respiratory tissues and lung function, a number of factors combine to increase the risk of translocation of antigenic material, including microbes, to the distal airways. Aspiration events account for approximately 5-15% of all pneumonias acquired in the community in the elderly and is an independent predictor of mortality (Kikawada et al. 2005). Additionally, it has been found that asymptomatic 'silent' aspirations can occur in up to 71% of pneumonia patients, suggestive that this may be a primary factor in the development of pneumonia and highlighting the potential for the oral microbiome to seed respiratory infections (Kikuchi et al. 1994).

In the elderly, there is a decline in the contractile strength of the masticatory muscles, which can result in difficulty adequately chewing food prior to deglutition. This is especially pronounced in denture-wearing individuals. Motor control by the muscles of the tongue is crucial in initiation of swallowing. Elderly individuals showed atrophy of the tongue muscles and increases in fibrotic tissue, which impacted lingual motor control and could increase aspiration risk (Steele and Cichero 2014). Coupled with

such changes, there is an age-related decrease in saliva production, which may be secondary to comorbidities or medications (Percival et al. 1994). Saliva helps to agglutinate chewed food and provides lubrication to expediate food bolus transit during swallowing (Pedersen et al. 2002).

Once initiated, swallowed food passes into the pharynx, where the peristaltic action of the pharyngeal constrictor muscles ensures transition of the food bolus into the oesophagus (Donner et al. 1985). Videofluoroscopic studies have found that although pharyngeal peristalsis remains unaffected in elderly individuals, there is a delay in the neuromuscular coordination of pharyngeal swallowing, which increases the incidence of coughs after swallowing, and often necessitates multiple swallows to clear food from the pharynx (Bours et al. 2009; Humbert et al. 2009).

Swallowed food from the pharynx passes into the oesophagus through the upper oesophageal sphincter. This sphincter accumulates connective tissue with age, leading to constriction, which can further delay passage of food from the pharynx (Shaw et al. 1995). Oesophageal peristaltic ability declines with age, leading to increased transit time of food in the oesophagus (Gregersen et al. 2008). This can, in turn, lead to further increases in the delay to food leaving the pharynx.

These factors contribute in varying amounts to dysphagia – difficulty swallowing. A background of comorbidities including cerebrovascular accident (stroke), gastro-oesophageal reflux disease or chronic obstructive pulmonary disease have been found to significantly increase the risk of dysphagia symptoms in elderly individuals (Roy et al. 2007). Studies on individuals with, or at risk of, dysphagia are limited, but one group (Pinto et al. 1994) compared the swallowing reflex of elderly individuals with cerebrovascular disease with health age-matched controls. A delay in pharyngeal swallowing was found, which suggested an increase in aspiration risk. Importantly, this could include silent aspirations which are highly challenging to identify due to the absence of overt symptoms (Smith et al. 1999).

The combination of changes associated with aging in the respiratory tissues proper, the oral cavity, immune system and the additional burden of comorbid conditions, environmental exposures and medication-related changes cumulatively contribute to increase the pathogenic potential of the oral microbiome, risk of aspiration of microbes into the distal airways, and likelihood of any aspirated microbes overcoming the immune system to instigate infection. Until recently, it was thought that the healthy lung was a sterile environment. However, as our understanding of the microbiome in health and disease has developed alongside highly sensitive and culture-independent techniques to analyse microbial communities, it has become apparent

that like most areas originally considered to be sacrosanct from the microbial world, the lungs are actually a complex and dynamic ecosystem.

1.3.1.3: An ecological model of the respiratory tissues

Early culture-dependent studies led to the belief that the lungs were a sterile site in health, with the introduction of microbes only in infection (Dickson et al. 2013). However, contemporary molecular methods that do not rely on isolation and culture of microorganisms have demonstrated that the lungs are a diverse ecosystem with dynamic microbial communities (Moffatt and Cookson 2017). The implications of this finding could be transformative: pneumonia could, like many diseases of the gut and oral cavity, be developed through ecological catastrophes which perturb the resident microbiota, rather than simply the result of an inoculum of infective microbes. The majority of research today has focussed on the respiratory bacteriome. However, as growing attention is provided to the roles of fungi and viruses in the 'omics' fields, it is likely that the role of such microorganisms will soon be explored in greater detail (Norman et al. 2014).

The majority of the studies to date have been cross-sectional in nature, with insufficient sample sizes and diverse methodologies and reporting criteria. In the domain of high-throughput sequencing, where error propagation or methodological biases is an acknowledged problem in data interpretation, even small differences in methods can result in highly different findings (Pollock et al. 2018). A further barrier to making inferences from microbiological samples in the lungs is potential contamination of samples from the pharynx, oral cavity and environment. Investigation of the lung microbiome is inherently invasive, which is a potential barrier to participation in such studies (Moffatt and Cookson 2017).

The majority of research to date suggests that in health the lung microbiome is derived from members of the oral and upper respiratory tract microbiota, rather than the external environment or gastrointestinal tract proper (Dickson et al. 2014; Venkataraman et al. 2015; Faner et al. 2017). Although this may be hampered by the contribution of contaminants as discussed above, there is sufficient evidence that the community composition differs from that of the oral cavity or pharynx to support the existence of a separate, derivative microbiome within the lungs. It should be noted that research regarding the lung microbiome in health is very limited, but has found species from the Pseudomonas, Streptococcus and Staphylococcus genera in the healthy lung (Erb-Downward et al. 2011). There is currently insufficient data to draw inferences about changes in community composition in disease, compounded by the striking biogeographic variations noted at different lung sites.

Dickson et al. (2014) have proposed a three-point model of the respiratory ecosystem. The first concept is the view of the lung biogeographical landscape as an adapted island model, originally used in ecology (MacArthur and Wilson, 1963). Here, the transition from the larger airways to the most distal airways can be seen as a series of islands emanating from the mainland of the oral cavity and pharyngeal spaces. The community diversity depends on the net migration rate of microbial species, which is constrained by a number of factors. The closer the next 'island' of respiratory tissue is, the greater the chances of immigration. As each island increases in species richness and diversity, competition for finite resources increases, leading to extinction. Additionally, coughing, ciliary clearance and the immune system further limit the maximum sustainable climax community at a given site. Additionally, an increase in the microbial bioburden of the oral cavity and pharynx lead to a greater increase in immigration rates. Any of the above factors may change over time, and in elderly individuals, many of the host factors that limit the size of microbial populations in the airways are impaired to some extent. This model links closely to the previously outlined mechanism of perturbation of the oral microbiome, which could introduce novel, atypical exogenous pathogens as well as total bioburden, to lead to both an increase in microbial immigration and community virulence traits to the distal airways.

The second concept proposed by Dickson et al. is that there is a heterogeneous distribution of microorganisms that reflects the environmental conditions of each respiratory tissue 'island', beyond simply decreasing diversity and richness. From the perspective of the oral microbiome, this is hardly a paradigm shifting revelation. The logic of this concept relies on the same factors that likely determine the biogeographical distribution of species in the oral cavity: changes in habitats due to environmental gradients in any factor such as surface binding factors, pH, oxygen tensions or anatomically determined niches can provide a selective advantage for certain species over others (Xu et al. 2015). Current sampling methods typically rely on bronchoalveolar lavage, which collects from a large, non-specific volume of the lung. There is currently no convincing data to support this concept from clinical studies therefore, but findings from the gut (Donaldson et al. 2016), oral cavity (Bik et al. 2010), and a trend towards appreciating the increasing parallels with ecology in the microbial world (Robinson et al. 2010) all point towards this concept being likely true.

The third concept unifies the prior two conceptual schemata to explain the pathogenesis of pneumonia. As the lung ecosystem is a complex, adaptive system with many interacting and interdependent factors, small changes in a given factor do

not necessarily yield proportionate effects. Instead, the principle of emergent properties is applied to explain the apparently spontaneous development of a seemingly coordinated disease phenotype as the result of a large number of simple interactions within the ecosystem. This is essentially the application of a systems biology approach to host-microbiome interactions. Here, the factors critical for the pathogenesis of pneumonia: low biodiversity, high microbial bioburden and a dysregulated host inflammatory response, can be seen not to necessitate a single major catastrophic event such as gross aspiration, but can be understood in the context of a convoluted series of clinically undetectable events.

This model has been borne out to some extent in subsequent research by a group from the same centre (Venkataraman et al. 2015). Here, it was found that the lung microbiome in healthy individuals was primarily composed of species which had originated in the oral cavity, with increased similarity to the tonsillar microbiome compared to gingival sites. When compared to samples obtained by oral rinse, there was only minimal difference in the relative abundances of lung microbiome samples ($R^2 = 0.86$). However, in respiratory disease, there was a departure from the predicted neutral distribution of species in lung microbial communities, suggesting active selection of certain species. Similarly, it is known that in the lungs of cystic fibrosis patients, infection by *P. aeruginosa* and other typical pathogens is related strongly to changes in the host environment (Folkesson et al. 2012). A number of studies have demonstrated that microbial diversity in the lungs of individuals with stable cystic fibrosis is high (Surette 2014; Carmody et al. 2015; Coburn et al. 2015), suggesting that fundamentally the same ecological principles apply, simply with altered environmental pressures. As previously mentioned, the above principles apply most strongly to bacterial infections, unlike pneumonia due to fungal spores (Murray et al 2019), which may occur by direct inhalation of an infective inoculum. A key component of the environmental pressures which can shape microbial communities in the lung is the role of the host inflammatory response.

1.3.2: Inflammatory responses in the lungs

Inflammation is the result of the immune response to foreign antigens. Inflammatory responses are protective against microbial insult in the majority of instances and neutralise infective threats effectively. However, in some diseases, either a dysregulated or exaggerated inflammatory response, or even a proportional inflammatory response in some tissues can lead to increased morbidity and mortality due to both local and systemic sequelae (Bordon et al. 2013).

The innate immune system is the first line of defence against microbial infection. In the distal airways, the alveolar epithelial barrier forms the interface between the external environment and the pulmonary blood supply. It is therefore a highly vulnerable site which requires elaborate, highly effective protection. This comes in part from the entrapment and subsequent removal of inhaled matter by the epithelial cilia and mucus layer in the higher airways (Bals and Hiemstra 2004). Alveolar surfactant also has immunological roles in addition to contributing to lung compliance (Goerke 1998). Surfactant contains a number of antimicrobial peptides, proteolytic enzymes and reactive oxygen species, which act in a non-specific manner. Contact with antigenic material results in the production of a number of pro-inflammatory cytokines and chemokines by the respiratory epithelium (Knapp et al. 2005). Two of the four surfactant proteins also act as chemokines and are able to agglutinate and opsonize foreign cells (Waters et al. 2009). The immunoglobulins; primarily IgG, IgA and IgE are also present in the airway lining fluid, which can coordinate vasodilation and diapedesis of leukocytes (Burnett 1986).

The first immune cells to contact microbial antigens in the lungs are dendritic cells, present in the respiratory epithelial layer, and alveolar macrophages in the surfactant and mucus layers. The dendritic cells are responsible for engulfing antigenic material and presentation of antigens to T cells residing in lymph nodes, thus bridging the innate and adaptive immune systems (Serti et al. 1986). The resident alveolar macrophage population is able to phagocytose invading microorganisms (Goldstein et al. 1974). Additionally, both macrophages and epithelial cells secrete pro-inflammatory cytokines and chemokines in response to contact with microbial antigens, to recruit neutrophils (Delclaux and Azoulay 2003). Neutrophils, like the alveolar macrophages, are able to travel through the capillary endothelium and epithelial cell layer, now permeabilised primarily due to the action of VEGF and TNF-α released by the airway epithelial cells. Like macrophages, neutrophils are able to phagocytose and digest microbes. However, the reagents used to kill microbes by neutrophils in endosomes, such as reactive oxygen species and proteolytic enzymes, can be released into the surrounding environment, either accidentally or in an attempt to eradicate necrotic or infected host cells. This can lead to further injury to the lungs which may result in worse outcomes (Craig et al. 2009). A recently discovered neutrophil mechanism of extracellular killing; neutrophil extracellular traps (NETs) have received considerable attention due to evidence suggesting that these may contribute to host cell damage for some time after the infective episode has ceased (Papayannopoulos 2018). Kolaczkowska et al. (2015) found that tissue damage was reduced if NET formation by neutrophils was blocked. Subsequently, CD4+ and

CD8+ T lymphocytes, with B lymphocytes provide the next line of defence in the lungs. B lymphocytes are able to produce pathogen-specific antibodies, while T lymphocytes can differentiate further to kill infected host cells, microbial cells and further mediate inflammatory responses (Moldoveanu et al. 2015).

Much of the host immune response is stimulated and coordinated through the production of pro-inflammatory cytokines and chemokines, produced not only by immune cells but the epithelium. There is growing interest in these markers, as specific patterns of cytokines may help to differentiate microbial infection from other respiratory diseases, thus offering diagnostic potential.

1.3.2.1: Diagnostic potential of inflammatory markers

A number of inflammatory mediators have been evaluated for their potential to act as either a diagnostic aid in pneumonia, or to enhance prognostication of individuals with respiratory infection. These range from relatively non-specific markers of systemic inflammatory burden, such as C-reactive protein (Menéndez et al. 2009), to more specific attempts to determine the likely aetiological microorganism through analysis of the profile of a number of key cytokines (Shankar et al. 2016). Typically, inflammatory markers are researched in the hospitalised population, likely out of the logistic convenience of access to potential participants and increased likelihood of proof-of-concept in more severe disease presentations. As with much of the research relating to pneumonia, targeting populations in the community and hospital settings, with varying risk of developing pneumonia over time, ideally tracking such individuals from health, through a period of respiratory infection would be the most effective way to assess potential biomarkers for both diagnosis and prognostication. This would be highly challenging, prohibitively expensive and ethically questionable, however (Goossens and Little 2006).

The use of systemic, host-derived biomarkers could potential offer an alternative to current methods of microbiological investigation, which is highly unreliable in guiding diagnosis of pneumonia. While rapid methods such as MALDI-TOF and culture-independent techniques offer the potential for rapid identification of microbes directly from obtained samples (Xiao et al. 2012), the sampling process itself can be unreliable and prone to false negative results (Huijts et al. 2013). Similarly, grading disease severity in individuals is highly subjective, and typically involves the use of multifactorial scoring systems, with considerable disagreement among experts regarding decision-making around admission of pneumonia patients (Niederman 2009).

The ideal properties of biomarkers (Christ-Crain et al. 2008) are to allow:

1)   Rapid diagnosis

2)   Prognostication

3)   Guidance for therapy/treatment

Another consideration is the balance between false positive: false negative results (*i.e.* type I: type II error rate). Arguably, false negatives are more problematic in principle, as this could further delay identification of disease and therefore treatment for patients with respiratory infection. However, it should be noted that overtreatment is not without consequence, for both individual patients and the wider population. Antimicrobial resistance is reported to be an impending crisis facing developed nations (Roca et al. 2015), and already outbreaks of microbial infections demonstrating resistance to all available antibiotics have been reported (Kuo et al. 2004; Souli et al. 2008). Pneumonia typically affects frail, elderly individuals with pre-existing morbidity. In this group in particular, injudicious use of antibiotics could lead to further systemic sequelae that reduce survival, or quality-of-life in the event of survival (Pea 2015). When combined with the non-specific nature of many of the signs and symptoms of pneumonia, it is therefore critical to explore potential biomarkers in this population.

1.3.2.2: Potential biomarkers for bacterial pneumonia

Biomarkers evaluated for pneumonia are typically combined with clinical signs and symptoms to attempt to corroborate disease outcomes. One study (Müller et al. 2007) analysed the combination of 5 key clinical features with procalcitonin, which is produced by a range of cells in response to microbial infection, and C-reactive protein, which is produced by hepatocytes in response to raised circulating levels of IL-6. They found an increase in diagnostic accuracy of CAP from 0.79 to 0.92 by including these biomarkers with clinical features. This finding adds to a growing evidence base of the role of procalcitonin for identifying a range of microbial infections. Use of this marker in point-of-care testing has grown, with rapid, high sensitivity devices now available (Masiá et al. 2017). Similarly, procalcitonin has been evaluated as a therapeutic guide, with a 50% reduction in antibiotic use demonstrated in one randomised controlled trial (Christ-Crain et al. 2004), and a reduction in duration of antibiotic treatment by 7 days in a separate trial (Christ-Crain et al. 2006). A number of cytokines have also been explored for their potential utility in both diagnosis and prognostication of pneumonia. However, there are a number of challenges in measuring circulating levels of cytokines. The concentration of many molecules, particularly IL-1 and TNF are often below the window of linearity for most testing methods, rendering measurement highly variable and unreliable. Most

circulating cytokines vary in level substantially over time and are short-lived (Christ-Crain and Opal 2010).

Levels of circulating IL-6 and IL-10 have been used in a prospective cohort study in CAP patients to predict the risk of fatal complications 90 days following discharge (Yende et al. 2008). These findings were seen despite no notable differences in disease severity, comorbidities or experience of sepsis during hospitalisation. Additionally, the majority of subjects had normal clinical signs and apparent clinical stability. This corroborates the findings of a previous multicentre cohort study which evaluated plasma IL-6, IL-10 and TNF levels in patients who had developed sepsis secondary to CAP (Kellum et al. 2007). Again, higher levels of both IL-6 and IL-10 were associated with increased mortality at 90 days. While these studies provide prognostication aids which may help guide treatment, they offer diagnostic potential for sepsis secondary to pneumonia, but not pneumonia itself.

One longitudinal study followed a group of healthy elderly individuals (n=3,075) over a 6.5-year period (Yende et al. 2005). Levels of circulating TNF, IL-6, and CRP were measured within 2 weeks of baseline. The primary outcome for the study was development of CAP, using a Time-to-Event (TTE) analysis. A total of 161 participants developed CAP over the course of the study. Elevated levels of both TNF and IL-6 were individually predictive of CAP development, albeit weakly, while elevation of both markers in an individual led to an odds ratio for CAP of 2.8 (95% CI 2.3 - 5.6), regardless of other risk factors. CRP was not predictive of pneumonia risk. The utility of serum TNF-α has also been shown as a biomarker in prognostication of functional impairment following CAP in a single-centre study of 301 hospitalised pneumonia patients (El Solh et al. 2006). Functional impairment, in turn, may predict a higher change of future readmission and death.

Endeman et al. (2011) evaluated a panel of key inflammatory mediators, including IL-1, IL-6, IL-8, IL-10 and IL-12 in patients hospitalised with CAP (n=201). While levels of cytokine expression in the acute phase response were highly variable, with more than a 5-log range of the levels of IL-6, there was a statistically significant association between the levels of IL-6 and IL-1RA with pneumonia caused by *Streptococcus pneumoniae*. More recently, a retrospective analysis of BAL fluid samples from lung transplant patients with pneumonia, tracheobronchitis or colonisation without respiratory infection demonstrated that the levels of a panel of cytokines including IL-1β in pneumonia samples were significantly higher than those of colonised samples (Shankar et al. 2016). Unfortunately, the authors did not report any assessment of difference in cytokine profiles with differing infective microorganisms, but this may

have been prohibited by the small sample size of this study. This study is important however for showing the potential value of locally expressed cytokine levels, rather than circulating levels in determining infection status.

1.3.3: Aetiologies and epidemiology of pneumonia

Pneumonia occurs due to an inflammatory response in the lungs, causing fluid accumulation and thus respiratory distress. This is normally, but not exclusively, the result of infection. The source of infection may be from multiple putative sources including the oral cavity, and oral prostheses. While certain microorganisms are commonly associated with lower respiratory tract infections (Table 1.3.3), in a large proportion of cases, the causative organism remains undetected (Andruska et al. 2015). To date, no robust microbiologic data exists for patients who are treated on an outpatient basis rather than in the hospital setting, presumably due to the difficulty of recruiting, monitoring and following such patients up over time. Aetiological microorganisms of pneumonia can be viral, bacterial, fungal, or polymicrobial (Cillóniz et al. 2016). In the UK and much of Europe, however, bacterial pneumonia is the most frequent presentation (Torres et al. 2014).

A wide range of possible risk factors exist for pneumonia. Of note amongst these is oral hygiene, which has been implicated both directly and indirectly as an independent risk factor (Almirall et al. 2008, Torres et al. 2013).

Pneumonia can occur due to a wide number of insults to the respiratory system, and thus multiple different classification systems have been proposed. However, the most widely accepted classification divides the disease according to the setting in which it was acquired, either community or hospital, with a further group of patients demarcated by immunosuppression (Ewig et al. 2010). Notably, pneumonia in immunosuppressed patients is frequently due to atypical microorganisms, with the fungus *Pneumocystis jirovecii* a common pathogen (Kazanjian 2020). This triad of pneumonia can be further subdivided, for instance, patients who suffer with CAP can be separated into those who are treated successfully in an outpatient setting, and those who require hospitalisation, suggesting more severe disease. Likewise, hospital acquired pneumonia (HAP) can be grouped by those patients who contracted pneumonia 48-72 h after onset of mechanical ventilation (ventilator associated pneumonia, VAP) and those who did not.

These groupings are important because they are purported to be associated with different microbial aetiological profiles and thus may inform treatment decisions. For instance, VAP patients hold the greatest risk of infection by multidrug resistant (Porzecanski and Bowton, 2006) pathogens such as *Pseudomonas aeruginosa* and

extended spectrum β-lactamase (ESBL) producing Enterobacteriaceae, while CAP patients treated on an outpatient basis are more likely to be infected with *Streptococcus pneumoniae,* or a viral source (Welte et al. 2012).

More recently the American Thoracic Society (ATS) has proposed a fourth addition to the pneumonia classification system (Hospital-Acquired Pneumonia Guideline Committee of the American Thoracic Society & Infectious Diseases Society of America, 2005), termed healthcare-associated pneumonia (HCAP). The introduction of HCAP as a category has been controversial. Proponents suggest that patients who are in frequent contact with hospital and healthcare settings, such as nursing home residents, healthcare workers and 'at-home' dialysis users can present with pneumonia that would be traditionally classified as CAP yet features microbial profiles and outcomes more closely in alignment with HAP. There is therefore a call for more aggressive, broad-spectrum treatment in such cases due to an increased risk of MDR organisms. However, this argument is obfuscated by the large number of overlapping risk factors present in this cohort of patients. While some large studies in the USA and Europe have supported this classification (Kollef et al. 2005, Carratalà et al. 2007), work by Chalmers et al. (2011) suggests that, at least in the UK, the different profiles are more related to an increased number of comorbidities, treatment restrictions (such as do not resuscitate orders), functional impairments, and poorer immune status. Indeed, a systematic review examined the predictive power of the HCAP classification to determine risk of infection by MDR organisms. It was found that this classification discriminates the risk of MDR infections poorly and leads to considerable overtreatment of patients when comorbidities and age were accounted for (Chalmers et al. 2014). In response to this finding, scoring systems which evaluate risk of MDR pathogens on an individual patient basis have been developed, with promising results (Webb et al. 2016). The current classification system used in most of Europe and the USA is summarised in Figure 1.3.3.
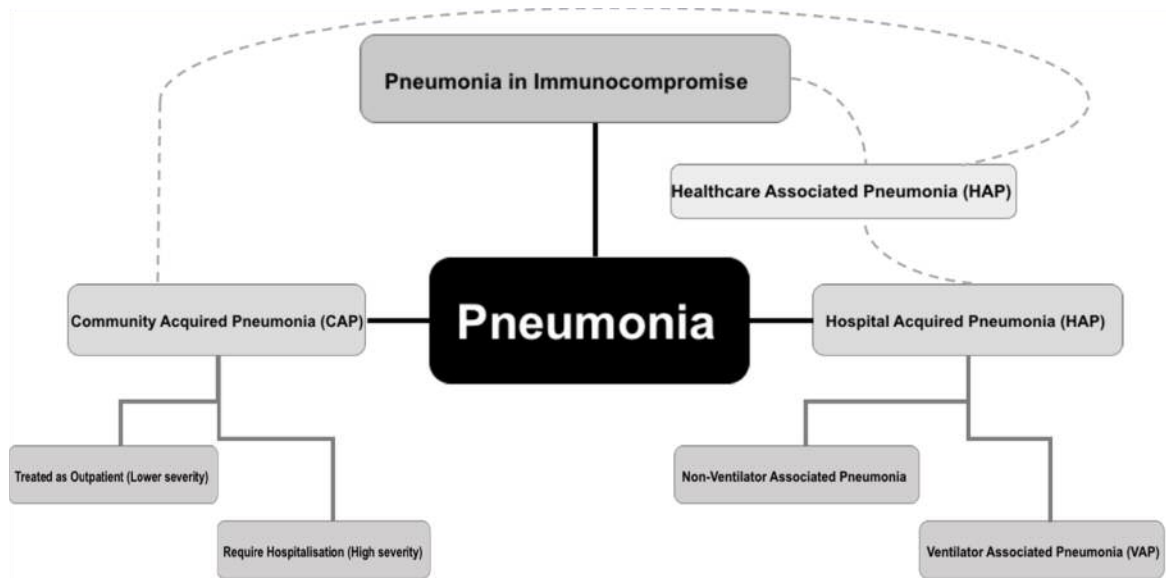
Figure 1.3.3: Schematic of pneumonia classification
The triad of pneumonia categories is represented by CAP, HAP and pneumonia in immunocompromised individuals. Both CAP and HAP are further subdivided – these additional categories provide useful predictors of both aetiologic microbes and outcomes. However, the more recent classification of HCAP by the ATS as a form of CAP with aetiology and outcome more closely related to HAP has been contentious. This is primarily due to possible confounding by failing to acknowledge overlap between HCAP and immunocompromised cohorts, amongst other individual factors such as functional status and treatment restrictions (indicated by dashed lines). Adapted from ATS guidelines (2005) and Ewig et al. (2010).

Due to the diverse settings in which pneumonia is found, little meaningful insight is gleaned by considering pneumonia at a global level. Likewise, the division of pneumonias into CAP, HAP and VAP is entrenched in literature, so aggregate data across all forms of pneumonia is lacking. Of relevance to the UK, multiple studies in the USA and various regions of Europe have considered mortality and morbidity related to pneumonias, as well as the economic burden involved.

Overall, CAP mortality in Europe was 27% at 6 months following diagnosis (Walden et al. 2014). Estimated mortality in a UK cohort was found to be highly related to patient age, with patients under the age of 65 demonstrating mortality of 5.6%, while patients over the age of 85 had a 47.2% mortality rate (Welte et al. 2012). This is likely due to the high number of comorbidities in such patients, but may also reflect the summation of risk factors for virulent, or antibiotic-resistant pathogens in this group, such as poor functional status (Murcia et al. 2010), poor oral hygiene and higher risk of previous hospitalisation or nursing home residence (Yoshikawa and Marrie, 2000).

Mortality due to VAP has been a source of controversy, with historical estimates of crude mortality misrepresenting the true attributable mortality due to VAP rather than

comorbidities. A meta-analysis of intervention data by Melsen et al (2013) found that overall, the directly attributable mortality rate in VAP was 13%. In this analysis, trauma and medical patients demonstrated close to zero attributable mortality, presumably due to the high number of competing pathoses likely to result in death in the ICU, not least the aetiologic cause of ICU admission. However, surgical patients who developed VAP had an attributable mortality of 69%.

Table 1.3.3: Common aetiologic bacteria in pneumonia

Species	Type of pneumonia most commonly associated with	References
Streptococcus pneumoniae	CAP	Welte et al. 2012, Spoorenberg et al. 2014, Quartin et al. 2013, Jones RN, 2010
Haemophilus influenzae	CAP/HAP/VAP	Welte et al. 2012, Spoorenberg et al. 2014, Quartin et al. 2013, Jones RN, 2010
Legionella spp.	CAP	Welte et al. 2012, Spoorenberg et al. 2014, Jones RN, 2010
Staphylococcus spp.	CAP/HAP/VAP	Welte et al. 2012, Spoorenberg et al. 2014, Quartin et al. 2013, Jones RN, 2010
Moraxella catarrhalis	CAP	Welte et al. 2012, Jones RN, 2010
Mycoplasma pneumoniae	CAP	Welte et al. 2012, Spoorenberg et al. 2014, Jones RN, 2010
Chlamydophila spp.	CAP	Welte et al. 2012, Spoorenberg et al. 2014, Jones RN, 2010
Coxiella burnetii	CAP	Welte et al. 2012, Spoorenberg et al. 2014
Pseudomonas aeruginosa	HAP/VAP	Quartin et al. 2013, Jones RN, 2010
Acinetobacter spp.	VAP	Quartin et al. 2013, Jones RN, 2010
Klebsiella spp.	HAP/VAP	Quartin et al. 2013, Jones RN, 2010
Escherichia coli	HAP	Quartin et al. 2013, Jones RN, 2010
Enterobacter spp.	HAP/VAP	Quartin et al. 2013, Jones RN, 2010
Proteus mirabilis	HAP/VAP	Quartin et al. 2013, Jones RN, 2010

1.3.4: Diagnostic and treatment challenges in bacterial pneumonia

Diagnosis of pneumonia is challenging (Ottoson and Evans, 2014), and successful initial antimicrobial therapy is a significant predictor of survival for both CAP (Bartlett et al. 2000) and HAP (Lynch JP, 2001) patients. By definition, CAP, HAP and VAP diagnoses are based on the likely setting for acquisition of the pneumonia. Additionally, CAP can be modified based on individual risk factors (Webb et al. 2016) or using the umbrella HCAP grouping (ATS 2005) to stratify risk of MDR pathogen presence. Mild cases of CAP tend to be the result of atypical infectious organisms such as mycoplasma pneumoniae or seasonal viral outbreaks. VAP is particularly

challenging to diagnose, due to the nonspecific nature of clinical signs and absence of reliable diagnostic biomarkers due to competing inflammatory 'noise' from critical illness. It can be further differentiated (Dias et al. 2013) into early onset (within 72-96 h onset of mechanical ventilation), or late onset (after 96 h onset of mechanical ventilation). Late onset VAP patients have significantly poorer outcomes, are more commonly infected with MDR microorganisms such as *P. aeruginosa* and methicillin-resistant *S. aureus* (MRSA).

Diagnosis by microbiological culture is often possible in VAP patients due to relative ease and acceptability of access to the distal airways by either bronchoalveolar lavage or protected specimen brushing. When present, both diagnostic methods offer reasonable specificity, but lack adequate predictive value to singularly support or negate clinical diagnosis (Woske et al. 2001). The high number of cases in which no causative organism is found for pneumonias by culture methods suggests that the clinical utility of these is poor. However, present culture-independent molecular techniques are costly, and have not yet replaced conventional methods in widespread clinical practice within the NHS.

On a population basis, the rapid increase in antibiotic resistance, particularly concerning in light of the dearth of new antibiotic development, presents a growing challenge for clinicians (Laxminarayan et al. 2013). Concerns about driving antibiotic resistance through unnecessary prescription of broad-spectrum antibiotics must be balanced against the need for rapid and aggressive empirical treatment in severe cases of pneumonia. Guidelines from the European Respiratory Society for treatment of CAP emphasise the need to stratify patients by severity of illness, to modify treatment according to results of bacterial cultures where indicated, and to attempt to treat patients on an outpatient basis due to both savings in cost and reduction in mortality and morbidity associated with nosocomial infections (Espana et al. 2003). Likewise, the joint ATS/IDSA guidelines for treatment of VAP emphasise avoidance of broad-spectrum therapy in cases of low severity and with a low index of suspicion for the presence of resistant pathogens.

Often severe cases of pneumonia require empiric treatment which cannot be deferred until microbiologic investigations have been reported to inform therapeutic strategy. The identification of biomarkers for early diagnosis of pneumonias and specific pathogens or resistance profiles has gained interest in light of this, particularly in VAP patients where antibiotic resistance and associated mortality can be high.

1.3.5: The oral ecosystem and pneumonia – an integrated model

The association between the oral microbiome and pneumonia is complicated by the presence of multiple interdependent confounders which can modulate the oral ecosystem through both local and systemic effects. Many of these factors may simultaneously affect the host response systemically and the ecosystem of the lung directly.

Factors can be broadly divided into host, microbial and iatrogenic factors (Figure 1.3.5). Host factors can be locally impactive, such as anatomical niches, or systemic, such as poorly controlled diabetes. A third subdivision includes factors which are primarily behavioural in origin, such as smoking. In reality, no particular host factor fits neatly into one subcategory outside of innate anatomical variance. For instance, reduction in oral hygiene could be primarily a behaviourally driven choice (Lenčová et al. 2006) or localised to specific areas of the dentition. The resultant gingival inflammation could have systemic effects which alter immune function (Jeffcoat et al. 2014; Liljestrand et al. 2018). Alternatively, reduced effectiveness of oral hygiene could be secondary to neuromotor impairment with associated loss of functional independence (Arai et al. 2003). Likewise, poorly controlled diabetes is clearly a systemic condition, but may be driven by behavioural changes in dietary intake (Kavanagh et al. 2010). Altered diet, particularly excessive sugar intake may have local effects, encouraging proliferation of cariogenic microbes in dental plaque (Staat et al. 1975). While smoking is clearly behavioural in that it is an active choice, nicotine addiction is considered to be a medical condition (Benowitz 2010). Smoking has well-documented impact on oral health (Millar and Locker 2007), the systemic immune response (Sopori 2002) and the lungs (Jayes et al. 2016). It is therefore highly challenging to separate the impact of such factors on the oral microbiome from their effects on interdependent systems and tissues.

Microbial factors, while clearly driven by the local oral environment and regulated by the immune system, can be considered as an independent set of risk factors. The 'virulence potential' of the microbiome as a whole can be modified by spontaneous mutation, horizontal gene transfer, and the acquisition of exogenous pathogens from the environment (Jenkinson and Lamont 2005). Pathogenicity must be inferred in the context of the oral ecosystem, which varies between individuals and over time. Interactions between microbes, may both shape the microbial community composition at a given site and enhance host tissue damage, either directly or indirectly. For example, competitive interactions between *P. aeruginosa* and *C. albicans* result in the modulation of virulence factor expression by both

microorganisms, mediated primarily by quorum sensing signalling pathways (Fourie et al. 2016).

A third set of factors which receive less attention in the literature are iatrogenic sequelae of healthcare. The most widely documented example is the impact of a range of medications on the oral ecosystem, due to their immunomodulatory effects, influences on salivary flow and composition and direct impact on the composition of microbial communities (Ciancio 2004). Use of a steroid inhaler is a known risk factor for the development of oral candidiasis, with the Candidal growth correlating with the strength, frequency and site of the steroid inhaler dose. Similarly, treatment with medications that induce xerostomia, or most notably the use of radiotherapy in head and neck oncology patients increases caries risk substantially (Gupta et al. 2015). The use of broad-spectrum antibiotics is known to reduce the diversity of the salivary microbiome, although it appears that unlike the gut microbiome, this recovers relatively rapidly after completion of antibiotic therapy (Zaura et al. 2015).

Contact with institutionalised care settings is a known risk factor for acquisition of highly virulent pathogens associated with a variety of infections recalcitrant to treatment (Friedman et al. 2002). This includes both *P. aeruginosa* and *S. aureus*, which are frequently resistant to first-line antibiotics, compounding medical challenges (Nordmann et al. 2007). Individuals in intensive care are at risk of dental plaque colonisation by these organisms (Zhanel et al. 2008). Similarly, there is an increased rate of colonisation of the oral microbiome in nursing home residents by potential pathogens (Ewan et al. 2009). Such institutions create a focal point where many systemically unwell individuals with variable functional and cognitive status are gathered, often with multiple long-term medications or medical treatments and in frequent contact with the hospital environment. All of these factors increase the risk of acquiring environmental pathogens and a host environment that could lead to dysregulated, disproportionate growth of opportunistic pathogens to drive dysbiosis of the oral microbiome.

The presence of artificial surfaces directly disrupts the oral ecosystem, by creating multiple new niches for colonisation. The acrylic biomaterial surface itself offers new binding sites for microbial attachment and is likely to feature an adsorbed salivary pellicle which differs in composition from elsewhere in the oral cavity (Svendsen and Lindh 2009). Masticatory function is typically reduced in denture wearing individuals, which not only results in changes in nutritional status and diet but can also lead to reduced desquamation and shear stress on microbial biofilms as softer foods are preferred (Emami et al. 2013). The interface between the denture-fit surface and mucosa results in an area of stagnation where any resident microbes are shielded

from saliva and shed squames are retained *in situ*, providing a novel source of nutrients to microbes (Coulthwaite and Verran 2007). Dentures are also removable, and so may directly acquire microbes from the surrounding environment or hands, acting as a vector for translocation of substantial colonies of atypical, potentially pathogenic species to the oral cavity.

It is proposed here that the changes to the oral microbiome known to accompany intubation in mechanically ventilated patients in intensive care units (Sands et al. 2017) parallel the introduction of dentures in perturbing the oral ecosystem. This in turn, coupled with altered host factors can drive dysbiotic changes in the oral microbiome and thus increase pneumonia risk. As has been previously outlined, the oropharynx provides the primary habitat which seeds the respiratory microbiome, and therefore it is reasonable to assume that an increased abundance of putative respiratory pathogens in the oral cavity could result in increased immigration of such species to the respiratory tissues (Dickson et al. 2014). The likelihood of successful colonisation of the lungs shares many common risk factors with denture-wearing, frail elderly populations, such as functional impairment, immune senescence and a range of comorbidities (Felton 2009; van der Maarel-Wierink et al. 2011).

The presence of an endotracheal tube provides a direct conduit to the airway by bypassing the normal protective cough and swallow reflexes. This device also necessitates open-mouth posture, which encourages drying of the oral cavity, and may increase the potential for contact with environmental pathogens (Munro and Grap 2004). While dentures do not share such features, frail elderly populations are at higher risk of dysphagia, have been shown to asymptomatically micro-aspirate oral contents frequently, and may have reduced saliva flow due to age-related changes or secondary to medications or disease (Zaloga 2002; van der Maarel-Wierink et al. 2011). The mechanisms underpinning the acquisition of environmental pathogens or overgrowth of opportunists in the oral cavity, migration of such species to the distal airways, successful colonisation and propagation of pathogens to overwhelm the immune response culminating in the development of bacterial pneumonia are essentially unchanged regarding the influence of the endotracheal tube in VAP and dentures in other forms of pneumonia.
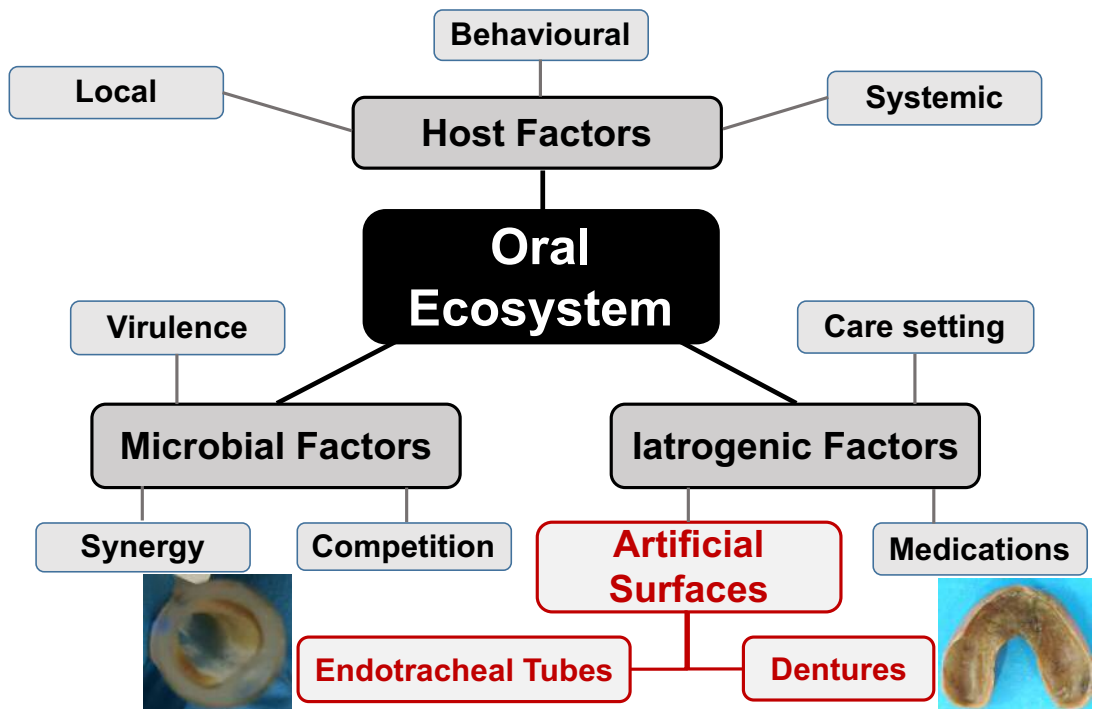
Figure 1.3.5: Factors which can modulate the Oral Ecosystem

A number of interdependent factors govern the oral environments and therefore the composition of microbial communities that reside within each habitat. These can be subdivided further, although such divisions are arbitrary, with considerable overlap and interaction between each. The presence of artificial biomaterial surfaces such as endotracheal tubes or dentures can be considered an iatrogenic factor which may influence microbiome composition. Factors can summate to encourage the acquisition and accumulation of putative respiratory pathogens which can increase the risk of pneumonia in susceptible individuals.

1.4: Aims

The overall aims of this project were:

- Development and characterisation of a denture acrylic biofilm model incorporating respiratory pathogens (WP1)

- Evaluation of microbe-microbe and epithelial-microbe interactions in an oral mucosal tissue model infected with denture acrylic biofilms (WP2)

- Exploration of denture-associated microbiomes and associated salivary cytokines in pneumonia patients, compared with care home residents with no respiratory infection.   (WP3)

- Development of novel antimicrobial silicone denture-liner materials to prevent biofilm formation (WP4)

- Evaluation of a novel microwave delivery system to eradicate denture acrylic biofilms (WP5)

CHAPTER 2: DEVELOPMENT AND CHARACTERISATION OF A MODEL ACRYLIC BIOFILM INCORPORATING ORAL 'COMMENSAL' MICROORGANISMS AND PUTATIVE RESPIRATORY PATHOGENS

2.1: Introduction

It is well-documented that microbial life on surfaces exists predominantly in the form of multi-kingdom and multi-species communities called biofilms, rather than planktonically (Stoodley et al. 2002). This is especially pertinent to the healthcare setting, with estimates that approximately 80% of all healthcare-associated infections (HAIs) are related to microbial biofilms (NIH, 2002). Typically, biofilm formation is conceptualised into discrete stages in a developmental model (Figure 2.1); presented as a highly orchestrated sequence of events that parallels portrayals of multicellular organism development, including the manifestation of emergent properties, cooperative and coordinated growth and maturation (Flemming et al. 2016). However, this interpretation is not substantiated by direct evidence, and much of the observational data purported to support such a schema could be plausibly considered through a number of alternative narratives (Eberl et al. 2001, Ghigo JM 2003, Nadell et al. 2008, Oliveira et al. 2015).
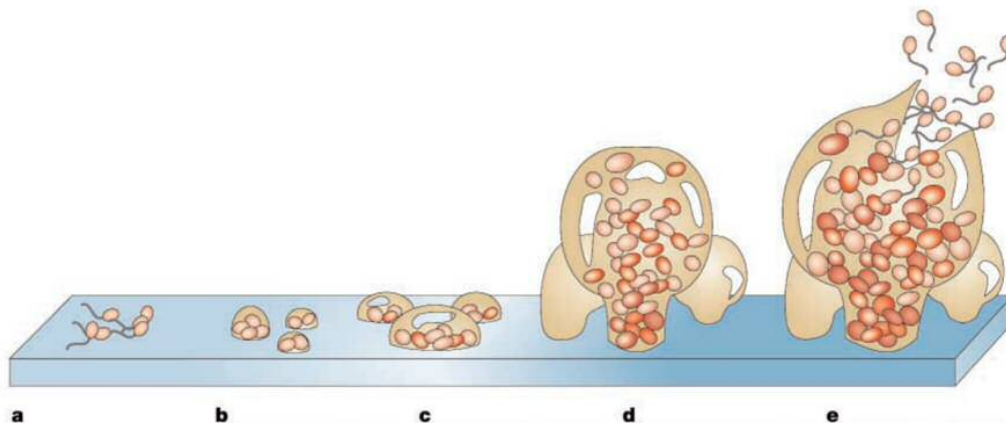
Figure 2.1: The developmental model of biofilm formation

a) Initial (reversible) adhesion of microbes to a surface or substrate
b) Attachment (Irreversible) of microbes with early deposition of Extracellular Polymeric Substrate (EPS)
c) Early biofilm maturation by clonal expansion of colonising microbes and formation of basic biofilm architecture
d) Maturation proper – here continued clonal expansion of colonising microbes is coupled with binding and attachment of further microbes, which may be from different species, to EPS components.
e) Biofilm dispersal – in response to certain molecular signals, population density and environmental changes, microbes may return to planktonic form to seed colonisation of new sites.
(Image from Davies D, 2003)

Much of the applied research that uses *in vitro* biofilm models to test anti-biofilm or antimicrobial products, decontamination or sterilisation methods, or the impact of biomaterial surfaces relies on simple approaches to generating biofilms, with

microbes typically added as a 'bolus' inoculum. Some groups have attempted to mirror the developmental model of biofilm formation by adding representative microbial species in a sequence intended to parallel the order of successional colonisation that would occur *in vivo*, although the validity of such an approach is debateable (Foster and Kolenbrander, 2004; Ammann et al. 2013; Violant et al. 2014). The majority of methods developed for biofilm modelling are focussed primarily on different options for analysis, with an increasing emphasis on monitoring biofilm development over time, or accurately assessing the diffusion and activity of molecules of interest within biofilms (Azeredo et al. 2017).

2.1.1: Approaches to *in vitro* biofilm development

Historically, most clinically directed biofilm models used devices that made analysis over time impossible, requiring samples to be sacrificed at each point of examination. The use of flow chambers has enabled dynamic biofilm development to be examined over time; using microscopy or serial sampling of the biofilm (Tolker-Nielsen and Sternberg, 2011). More recently, microfluidic devices have been employed to examine a wide range of biofilm physicochemical properties and monitor the development of biofilms under a variety of conditions over time (Kim et al. 2012). Such devices also allow a high level of control over the spatial organisation of the biofilm and environmental factors, making them ideal for perfusion models or assessment of the impact of different surface chemistries or nanotopographies.

The simplest methods used to model biofilms involve a batch system (McBain 2009), wherein a microbial inoculum is applied to a surface for a period of time (typically between 30 min – 4 h), followed by rinsing to remove non-adherent microbes and the addition of growth medium. The samples are then incubated for 24 h or longer; a time period which is broadly accepted to mark the point at which biofilm maturation begins for many microorganisms. Many tests including the majority of minimum biofilm eradication concentration (MBEC) protocols use this 24 h interval as an endpoint (Ceri et al. 2001). The ability to perform multiplex assays using microtitre plates or more complex equipment such as a Calgary device (Figure 2.1.1.1), has led to this method being popular for screening antimicrobial or anti-biofilm compounds (Ceri et al. 1999). However, biofilms generated using batch systems typically feature reduced extracellular polymeric substrate and simpler architectural structure, limiting their ecological and clinical validity (Wang et al. 2014, Hödl et al. 2014). Owing to their simplicity and capacity for high throughput assays, a batch biofilm modelling system selected for the work presented in this and subsequent chapters.
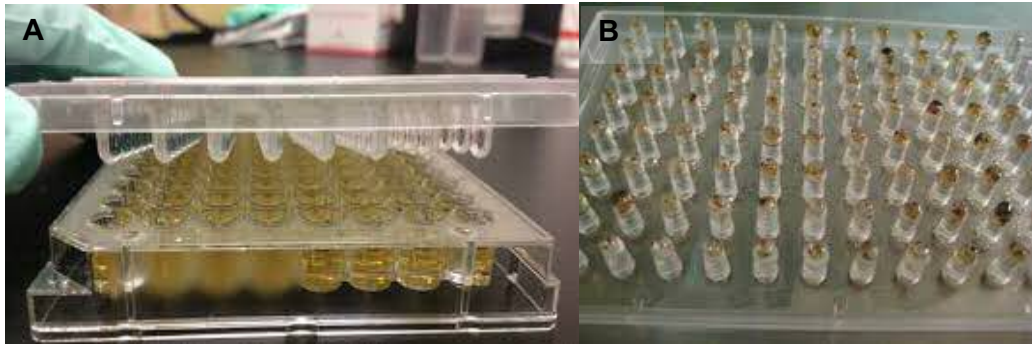
Figure 2.1.1.1: The Calgary biofilm device

A) A modified 96-well microtitre plate with PTFE pegs on the lid. These rest within growth medium or microbial inoculum, to allow microbial colonisation and biofilm formation over time on their surface.
B) Once the experimental endpoint is reach, the pegs can be inverted for further processing and analysis of biofilms.
(Image from PhysOrg 2011 https://phys.org/news/2011-12-method-toxins-oilsands.html)

A number of alternative modelling techniques have been introduced which overcome some of the limitations of the batch system, albeit with reduced throughput; increased technical demand, cost; and in many cases a requirement for specialist equipment. Many of the systems also employ a continuous flow of nutrient rich medium, which substantially increases the risk of contamination through operator error.

Several variations can be found of the rotary biofilm reactor, where samples are grown on coupons attached to rotors in a cylindrical container containing growth medium. After inoculation, the rotors spin to create flow of medium, which generates shear stress on the biofilms; influencing EPS deposition, biofilm structure and composition to more closely mirror those found in many natural environments. This system can be employed in either a batch format, with a standing reservoir of medium, or using a continuous infusion of growth medium and removal of waste medium via an exit port at a set height on the cylindrical chamber (Figure 2.1.1.2). Thus, both rate of nutrient delivery and simulated 'flow' rate can be manipulated (McBain 2009). Such systems offer the ability to control flow, nutrient and waste delivery, but are prone to contamination and allow only a maximum of 24 samples per run, requiring the same experiemental conditions to be met within a single experiment. They are also less suitable for antimicrobial testing where only a limited quantity of antimicrobial is available for testing owing to the large quantities of media employed.
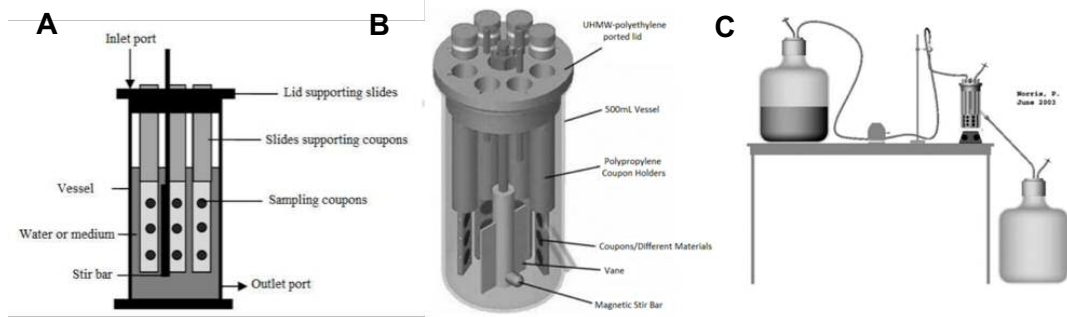
Figure 2.1.1.2: Bioreactor design and experimental setup

A)   Cross sectional diagram of typical bioreactor design (image from Gomes et al. 2014)

B)   The CDC biofilm bioreactor is probably the most well-known and widely used bioreactor (image from Williams and Bloebaum 2010)

C)   Basic experimental setup for bioreactor studies (image from http://www.biocleanh2020.eu/index.php/project/esrsfh/esr3m © BioClean Project)

A similar format is featured in the Constant Depth Film Fermenter (CDFF). However, this device uses coupons placed in wells which are arranged to create a depression in which biofilm formation can occur. Again, culture medium is fed by flow into the chamber, but is directed to each sample through a scraper bar that brushes against the upper surface of the wells (Figure 2.1.1.3). Consequently, once the biofilm has grown to the full height of each well, any additional microbes are removed from the surface. Over time, this generates biofilms with a highly even surface topography, allowing excellent standardisation for perfusion modelling and facilitating easier analysis by 3-D microscopy such as CLSM. It has also been claimed that this method is particularly relevant to oral biofilm modelling, as the scraping action simulates brushing or frictional forces from movement of the tongue, lips and buccal surfaces; while the thin layer of growth medium applied intermittently over time may be representative of pulsatile nutrient spikes in the oral cavity (Pratten 2017). However, the same limitations for antimcirobial assays are true of this method, and the scraping action across a fixed biofilm layer may lead to an artificially regular surface which could impact biofilm behaviour by changing surface:volume ratios and affecting nutrient exchange, chemical penetration, and microtopography.
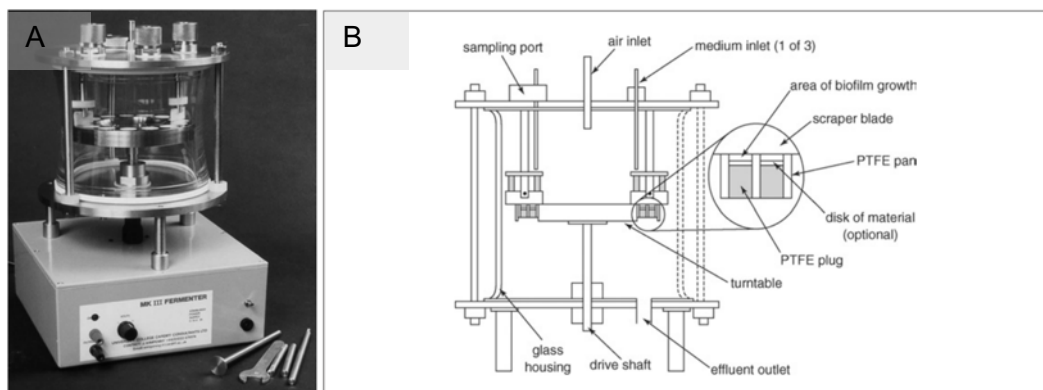
[Overleaf] Figure 2.1.1.3: The constant depth film fermenter (CDFF)
A)   The assembled CDFF (empty)
B)   Schematic of CDFF demonstrating how biofilm thickness is controlled by the scraper blade (images from Pratten 2017)

A number of different devices (Figure 2.1.1.4) involve the transverse flow of growth medium across a surface upon which a biofilm is seeded. The first such device to employ this design was the Robbin's device, which consisted of coupons mounted on a cylindrical pipe so that their surfaces enter the liquid stream. The modified Robbin's device derived from this (McCoy et al. 1981) uses a square-section tube, with sample ports allowing removal of individual samples without disrupting the remaining samples. This permits both high throughput analysis and time-series experiments to be undertaken. Due to their linear design, both methods result in a nutrient gradient forming across the samples as growth medium is used by each biofilm it passes (Hall-Stoodley et al. 1999). Similarly, as a consequence of metabolic activity by the constituent microbes, an inverse toxin gradient may form across the samples. The impact of such changes along the device length have not been characterised, although there is some evidence of differences in microbial adhesion across the sample positions (Linton et al. 1999). These systems are highly applicable to systems with a constant shear flow, such as water pipes. However, their relevance to oral biofilms may be limited, as microniches in the oral cavity are exposed to negligible shear flow for long periods of time followed by sudden, extensive shear and frictional forces. An experimental setup with a timed period of pump activity to mimic this activity could be a useful model for oral biofilms however. While throughput is generally better in these devices, there is still a limited ability to assay a large number of samples with different experimental conditions simultaneously. The ability to apply antimicrobials present in limited quantity also remains problematic.
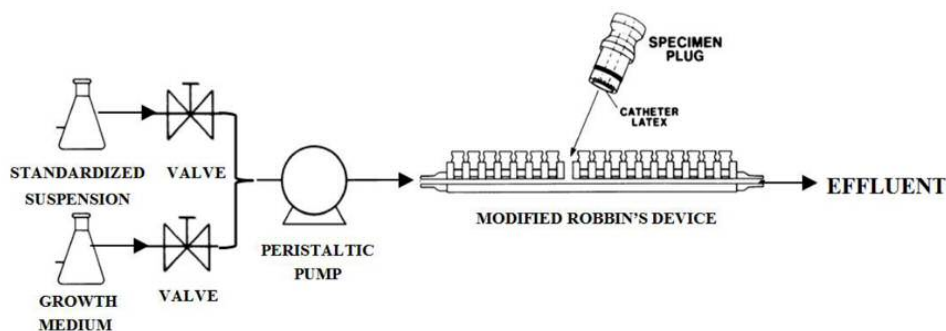
Figure 2.1.1.4: The modified Robbin's device

Note that specimen plugs protrude into the flowing growth medium. This makes the modified Robbin's device similar to a Calgary device in setup, with the added benefit of being able to manipulate shear stress and continuously replace nutrients through flow of medium.
(Image from Gu et al. 2015, © Gu, W 2015, Creative Commons Attribution License).

Similar methods include flow-cell devices, such as modified chamber slides with growth medium drip-fed across the surface or fed by peristaltic pump. These devices offer an advantage over the modified Robbin's device in that samples are held in parallel, so that nutrient availability is equivalent for each biofilm. However, this comes at the cost of the number of biofilms that can be generated per run. A major advantage of many such devices is the capability to image samples in real time, permitting evaluation of biofilm development under different conditions; diffusion rate of substances through the biofilm; or biological effects of compounds, without the need to sacrifice samples for analysis. Microfluidic biofilm modelling devices offer a promising means to combine the benefits of flow-based methods with the higher throughput offered by bioreactors. Presently such devices are not frequently employed in biofilm research, but a number of groups have demonstrated their effectiveness for evaluating biofilm development, anti-biofilm surfaces and antimicrobial compounds (Kim et al. 2010; Skolimowski et al. 2010; Hong et al. 2012).

2.1.2: Influence of adherence patterns on biofilm development

Adhesion of microbes to a surface is considered by most researchers to mark the initiation of biofilm development. Consequently, extensive research to characterise factors affecting adherence and surface retention of microbes has been undertaken (Garrett et al. 2008; Dufrêne 2015; Carniello et al. 2018). In the oral cavity, surfaces are rapidly coated with a glycoprotein salivary pellicle, which could modulate innate surface characteristics (Lendenmann et al. 2000). However, there is some evidence that underlying surface properties are maintained through this layer (Teughels et al. 2006). Alternatively, artificial saliva formulae can be used, which aim to replicate such preconditioned surfaces.

Two key properties of a surface have been found to exert significant influence on levels of microbial colonisation and retention by microorganisms: surface roughness and surface free energy (SFE). A threshold R_a value of 0.2 μm has been widely accepted as the point at which microbial adhesion is enhanced, with a general finding that further increases in R_a lead to concomitant increases in adhesion (Teughels et al. 2006). No maximum threshold for roughness has been defined, although it seems intuitive that beyond a certain level, the only increase in microbial surface coverage will be due to an increased surface area presented by a highly rough material for colonisation. In contrast, a recent systematic review by Dutra et al. (2018) suggested that no specific threshold roughness value exists, and any polishing procedure appears to decrease microbial adherence, regardless of roughness. The underlying mechanisms which may explain this observation have not yet been elucidated.

In general, the more hydrophobic a surface is (*i.e.* lower SFE), the greater the microbial adherence. However, this may be species and even strain specific. Importantly, SFE and surface roughness interact, meaning that an increase in surface roughness of a material tends to increase hydrophobicity of an already hydrophobic (contact angle less than 60°) material, while more hydrophilic materials (contact angle greater than 86°) increase in hydrophilicity with increasing roughness. Materials with a contact angle between 60-86° appear to undergo no change in SFE with changes in surface roughness (Teughels et al. 2006).

The majority of research evaluating impact of surface properties on microbial adhesion is conducted *in vitro*, often using mono-species biofilms, and so the applicability of this evidence to the clinical setting is questionable. It is likely that surface imperfections that shield microbes from mechanical removal and shear forces in the oral cavity will play a significant role in directing biofilm formation, but such conditions are highly difficult to reproduce robustly in the laboratory. Importantly, modifications to SFE may influence surface chemistry. Similarly, polishing procedures may alter the surface chemistry by exposing molecules not normally present in high quantities on a finished material surface. Given the potential for such compositional changes; the influence of microbial species, strain, cell-cycle stage and receptor-mediated interactions to alter initial patterns of adherence, it is difficult to reach any meaningful conclusion with reliability. Further *in vivo* verification of the influence of surface properties on microbial adhesion is needed.

It is also contentious whether initial patterns of adhesion have any meaningful impact on eventual biofilm formation (Miller et al. 2015). Specific interactions between the salivary pellicle with microbes (Scannapieco 1994; Ruhl et al. 2004; Nobbs et al. 2011), and microbes with one-another (Kolenbrander 2000; Jenkinson and Lamont 2005; Peters et al. 2010) are well-documented and could hypothetically exert influence over the composition, structure and resilience of the mature biofilm. However, a number of additional factors may be of greater importance in a clinical setting.

When considering removable prostheses such as dentures, there is considerable tensile and shear force exerted through saliva interposing the mucosa and the denture-fit surface. Such forces will occur maximally during insertion and removal of the prosthesis – indeed, the surface tension of saliva helps retain dentures, particularly in the case of complete maxillary dentures. However, there will also be submaximal, cyclical loading during masticatory function and speech, due to

functional movement of the denture against the underlying mucosa. Such forces may cause disruption of the outer surfaces of the biofilm.

The salivary pellicle may contribute to initial patterns of adhesion, which may influence biofilm composition through species-specific interactions promoting adhesion of certain microbes, or similarly inhibiting colonisation. Saliva is also continuously washed over the surface of the established biofilm, and contains many components with antimicrobial activity, as well as potential nutrients and buffers which can modulate the local microenvironment (de Almeda et al. 2008; van't Hof et al. 2014). However, it has been shown that the biofilm matrix helps to shield the constituent microorganisms from host defences, either by screening antigenic sites or inhibiting the diffusion of antimicrobial agents (Stewart and Costerton 2001; Otto 2006; Limoli et al. 2015). Consequently, once a mature biofilm has been established, there may be little effective antimicrobial activity from host defences. This is particularly true of biofilms colonising the denture fit surface, as the acrylic biomaterial shields the biofilm from the oral cavity, forming an area of stagnation (Chandra et al. 2001).

Another crucial factor to consider is the role of lifestyle factors such as diet, smoking and the effects medications or medical conditions on shaping biofilm development and composition. The pulsatile bursts of substrate availability associated with nutritive intake can vary drastically in frequency, quantity and composition. One of the primary factors which may influence biofilm behaviour is nutrient availability (Jefferson 2004); and, particularly in the case of carbohydrate fermenting microbes including most streptococci, life in the oral cavity likely consists of long periods of starvation between meals. An adaptation to this condition is the sequestration of dietary sugars in the extra-cellular polymeric substrate in the form of dextrans and glucans (Lemos et al. 2005). These biofilm matrix components serve as structural matrix components which enhance both adhesion and resistance to removal for the microbial communities, while simultaneously providing a reserve of nutrients in case of prolonged periods of starvation. Where carbohydrate intake is limited, there is a decrease in the relative abundance of *Streptococcus mutans* with a concomitant increase in *Streptococcus sanguinis* in dental plaque biofilms (De Stoppelaar et al. 1970). Smoking is an important lifestyle factor known to contribute to a wide range of oro-pharyngeal and systemic diseases. Tobacco smoke can drive dysbiotic changes in oral biofilms through a number of mechanisms. Decreases in oxygen tension favours an increase in facultative or strict anaerobic species, including streptococci (Wu et al. 2016). Additionally, tobacco smoke leads to a more acidic salivary pH, which favours acid-

tolerant microbes (Grover et al. 2016). These factors combined may contribute to the increase in dental caries found in smokers (Benedetti et al. 2013).

Cigarette smoke contains an abundance of toxicants which may directly modulate microbial communities while simultaneously encumbering host defence cells and stimulating a pro-inflammatory response. The effects of such compounds have been shown to contribute to biofilm formation by a number of putative respiratory pathogens (PRPs) through a variety of (likely synergistic) mechanisms. In laboratory experiments using *S.* aureus, biofilm formation has been found to be induced by tobacco smoke through stimulation of accessory regulator A (*sarA*) and required for biofilm formation (*rbf*) genes. Concomitantly, fibronectin binding protein A (*fnbA)* gene expression is upregulated, promoting adherence to mucosal surfaces. The available evidence suggests that these effects are mediated through increased oxidative stress, with antioxidant treatments abrogating biofilm formation (Kulkarni et al. 2012). Similarly, experiments with *P. aeruginosa* have found that induction of genes associated with pili and flagellae assembly, with concurrent suppression of key quorum sensing genes promotes biofilm formation in the presence of tobacco smoke components (Antunes et al. 2012). Overall, there are limited mechanistic insights into the direct effects of tobacco smoke on microbial behaviour and virulence.

An important factor that differentiates biofilms colonising removable biomaterial surfaces, such as dentures, is the increased likelihood for the introduction of environmental contaminants. Upon removal and insertion, dentures come into contact with fingers, possibly other inert surfaces, and may be left in vessels for extended periods of time which may be open to the environment. In care facilities and other environments where endemic outbreaks of highly virulent, potentially drug-resistant microorganisms are not infrequent, this may result in a substantial increase in the likelihood of colonisation and infection by PRPs, particularly if denture hygiene is suboptimal. To date, there has been no high-quality research evaluating such routes to infection. Indeed, to reliably trace contaminants and identify the precise point of infection would be highly challenging to assess on even a modest scale.

2.1.3: Synergy and competition in biofilms - impact on virulence

The introduction of atypical microbial species, which may include PRPs, to 'normal' denture-associated oral biofilms may result in unpredictable changes in behaviour of constituent microorganisms, due to the nature of the interactions between such species. While the majority of dental research has understandably focussed on the development of 'normal' oral microbial communities and orofacial diseases, there has been interest in the interactions between key pathogenic microbes frequently

implicated in respiratory infection and nosocomial disease and members of the typically commensal oral microbiota. The most thoroughly studied of such microorganisms are: *Candida albicans*, which is widely considered to be an opportunistic pathogen in immunocompromised patients; *Pseudomonas aeruginosa*, particularly in the context of cystic fibrosis, ventilator-associated pneumonia and chronic wound infections; and *S. aureus*, which is isolated from a wide range of healthcare-associated infections (Percival et al. 2015).

Cystic fibrosis is a genetic disorder that affects multiple organs. In the lungs, deficiency in chloride ion transport across the epithelial membrane leads to reduced mucociliary clearance and increased viscosity of mucous secretions (Flume et al. 2009). Individuals with cystic fibrosis are susceptible to respiratory infection, particularly by *P. aeruginosa*. In the 1980s, research first began to acknowledge the potential for oropharyngeal colonisation by *P. aeruginosa* to be a likely antecedent to colonisation and infection of respiratory tissues. Several studies examined interactions between *P. aeruginosa* and predominant oral microbes such as streptococci and Actinomyces species; focussing on coaggregation behaviour to offer insight into the successful colonisation of the oropharynx by this atypical, Gram-negative aerobe, despite the preestablished mature microbial communities that populated this environment (Komiyama and Gibbons 1984; Komiyama et al. 1985). Further research by Komiyama et al. (1987) found that of 22 strains of *P. aeruginosa* isolated from a total of 17 cystic fibrosis patients, there were between 73 – 96% positive coaggregation reactions with commensal oral microbes. Biochemical inhibition testing suggested that these reactions were protein-mediated and not lectin-dependent, although this characterisation was not exhaustive. More recently, it has been found that periodontal pathogens enhance invasion of respiratory epithelial cells by *P. aeruginosa*, and these species co-localise within cells (Pan et al. 2009). While this study did not examine specific interactions between the two species, the important biological effects on human tissues make this an interesting avenue of research to pursue. Importantly, such research reinforces the paradigm of colonisation of the oral cavity by PRPs to form a nidus which can seed infection of respiratory tissues.

There is a lack of research examining possible interactions between *S. aureus* and the oral microbiome, particularly in the context of respiratory infection. However, interactions of both *P. aeruginosa* and *S. aureus*, with the polymorphic fungus *C. albicans* are perhaps some of the most extensively studied in medical microbiology. Of note, evidence from animal models (Carlson 1987; Roux et al. 2009; Roux et al. 2013), and limited evidence from clinical studies of pneumonia and

chronic wound patients have demonstrated that polymicrobial infections involving combinations of any of this triad of microbes appears to result in increased virulence, and worse outcomes in the infected host, when compared to mono-microbial infections alone (Liou et al. 2001; Schlecht et al. 2015; Serra et al. 2015). This finding is especially interesting as the majority of interactions between *P. aeruginosa* and *C. albicans* are thought to be antagonistic, while those between *S. aureus* and *C. albicans* appear to be primarily mutualistic or synergistic. Complicating matters further, some authors have suggested that the nature of interactions between *P. aeruginosa* and *S. aureus* differ in nature depending on the sequence of events. Each of these relationships will be elaborated in more detail below.

Pseudomonas aeruginosa produces an extensive arsenal of virulence factors (or virulence-associated factors), which may act on host tissues and other microbes. Of particular note, the secreted phenazine pyocyanin, which has pleiotropic effects, appears to be responsible for the killing of *Candida* hyphae by *P. aeruginosa* in co-culture models, in addition to type IV pili and lectin-mediated interactions (Fourie et al. 2016). Regulation of such behaviour occurs through key quorum sensing molecules, which have been characterised extensively in knockout studies. Pyocyanin also inhibits germ tube formation by *C. albicans* to prevent hyphal formation and ensure the fungus remains in yeast form. However, the interactions are not unidirectional. The candidal quorum sensing molecule farnesol, associated with the yeast cell morphology, inhibits phenazine synthesis and thus pyocyanin production in *P. aeruginosa* (Cugini et al. 2007)*.* Another clinically important consequence of inhibition of this pathway is the disruption of rhamnolipid production, which is associated with *P. aeruginosa* swarming motility. This ability has been suggested to contribute to the pathogenesis of ventilator-associated pneumonia by *P. aeruginosa* (Köhler et al. 2010)*.*

Both farnesol and pyocyanin are damaging to host tissues, primarily through the generation of reactive oxygen species (Fourie et al. 2016). Thus, competitive or inhibitory interactions between microbes can lead to increased damage to host cells and initiation of a pro-inflammatory cascade, as host tissues suffer the destructive impact of inter-kingdom warfare. Such changes likely further encourage colonisation and proliferation of both species, as inflammatory changes and increased permeability of the mucosal lining offers new binding sites. In contrast, there is evidence to support a commensal relationship between *S. aureus* and *C. albicans* in mixed infections. Like *P. aeruginosa*, *S. aureus* is frequently implicated in nosocomial, and particularly medical device-associated infection, and endemic outbreaks of methicillin-resistant *S. aureus* (MRSA) can result in high levels of

morbidity and mortality in institutional care settings (Albrich and Harbarth 2008). Isolation of both *C. albicans* and *S. aureus* from bloodstream infections is common, and associated with increased length-of-stay, and poorer outcomes. In murine models of infection, inoculation with both microorganisms results in increased mortality (Peters and Noverr 2013).

The potential for synergism between these two microbes has been explored in laboratory studies. Interestingly, the *S. aureus* strain ATCC 25923, a clinical isolate frequently employed in research due to its ability to form biofilm readily *in vitro*, failed to form meaningful biofilms in serum compared to brain-heart infusion media. However, when co-cultured with *C. albicans*, a dense polymicrobial biofilm was generated; suggesting that *C. albicans* was required to initiate adhesion in the experimental conditions used (Harriett and Noverr 2009). Unlike *P. aeruginosa*, the staphylococcal cells appeared to preferentially associate with candidal hyphae when examined by fluorescence and scanning electron microscopy. It has also been found that vancomycin tolerance is increased in dual species biofilms compared with *S. aureus* monotypic biofilms. However, to date, neither detrimental nor beneficial effects conferred to *C. albicans* when grown with *S. aureus* have been described. There was no difference in microbial biomass, resistance to antifungals such as amphotericin B, nor any difference in invasive potential in *C. albicans* in dual versus mono-species biofilms.

The term 'microbial hitchhiking' refers to the utilisation of one microorganism by another to invade tissues. There is good evidence that *S. aureus* can gain access to tissues and the bloodstream through this mechanism. The hyphal morphology of *C. albicans* is responsible for invasive behaviour, and *S. aureus* has been found to have a strong affinity for the agglutinin-like sequence 3 adhesin (Als3p) – a cell wall protein specific to candidal hyphae. Murine models have demonstrated close coaggregation of *S. aureus* allowing translocation through the mucosal barrier in murine models. Als3p knockout mutants of *C. albicans* were unable to form hyphae or invade tissues, and this was accompanied by the absence of *S. aureus* invasion of tissues and the bloodstream, corroborating this finding (Schlecht et al. 2015).

While interactions between *S. aureus* and *C. albicans* appear to favour the bacteria without conferring any benefit to the yeast, there may be as yet undiscovered advantages to *Candida* in such a relationship. Further research is needed to explore such interactions in the context of the host response, given the importance of both microorganisms in nosocomial infection.

A more complex relationship appears to exist in polymicrobial biofilms comprising both *P. aeruginosa* and *S. aureus*. Here, the nature of interactions between these

two bacteria has been examined primarily in the context of chronic wounds, as colonisation by either species is associated with delayed healing, promulgation of chronic inflammation and increased morbidity. These microorganisms pose the additional challenge of frequent multi-drug resistance which, when combined with the increased antimicrobial tolerance conferred by the biofilm lifestyle and typically poor perfusion of chronic wounds, can lead to highly recalcitrant infections.

Initial adherence to the epithelial cell surface and colonisation of tissues by *S. aureus* is highly efficient. *Staphylococcus aureus* possesses a number of surface adhesins which bind with high affinity to mucosal components, such as the fibronectin-binding proteins FnBPA and FnBPB, the fibrinogen-binding Clumping Factors ClfA and ClfB and the various "sas" surface proteins; among others (Foster et al. 2014). Findings from an *in vitro* 'wound-like model' suggest that when co-inoculated together, *S. aureus* initially inhibits attachment and growth of *P. aeruginosa* (DeLeon et al. 2014). Yet, in culture-media co-inoculation of both species results in the eradication of viable *S. aureus* by *P. aeruginosa.* When *S. aureus* was allowed to colonise the wound model and form a biofilm over a 48 h period, addition of *P. aeruginosa* resulted in successful secondary colonisation by this microorganism. While the mechanisms of this interaction were not evaluated, these findings suggest that either changes in *S. aureus* metabolism and gene expression mean that Pseudomonal attachment is no longer inhibited, or staphylococcal biofilm matrix components provide alternative binding sites for this species.

Additionally, once established biofilms had formed in wound models, antibiotic tolerance increased for both *P. aeruginosa* and *S. aureus* for gentamicin and tetracycline, albeit only crossing the threshold of statistical significance in the case of the latter. Gentamicin is a cationic molecule and is susceptible to interactions with charged components of biofilm matrix. However, tetracycline resistance is primarily conferred by intrinsic efflux activity in Gram negative bacilli such as *P. aeruginosa*, and so the mechanism promoting resistance within *S. aureus* remains unclear.

These findings were corroborated by Alves et al. (2018), who found that despite *S. aureus* predominating in early co-culture biofilms, later attachment and growth of *P. aeruginosa* on pre-established *S. aureus* biofilms was enhanced. This result seemed to be mediated by surface proteins, although the specific mechanisms were not examined. The authors built upon findings of previous research by showing increased adherence to a human keratinocyte monolayer by *P. aeruginosa* in co-culture infections, with simultaneous increased internalisation of *S. aureus* cells. The authors interpreted this as an increase in invasivity of *S. aureus*, although whether this was due to a change in behaviour of this species or secondary to enhanced cell

membrane disruption by *P. aeruginosa* was not explored. Interestingly, both healing time in a scratch wound assay and pro-inflammatory response by keratinocytes to infection was highest in *P. aeruginosa* monotypic infections; lowest in *S. aureus* infections; and demonstrated an intermediate response in co-culture infections.

Naturally, most mechanistic research exploring microbial interactions is conducted in the laboratory setting, using simple, reductionist models to provide a setting in which confounders can be highly controlled. Caution is therefore needed when extrapolating such findings to clinical settings, where subtle nuances combine iteratively to drastically alter the environmental context that microbial actions take place in. This concept intersects with the notion of emergent properties that has been promoted within the field of systems biology. Improvements in microbial sampling from biofilms such as LASER-capture microdissection, which allows isolation of specific microorganisms from a given biofilm environment; combined with high throughput 'omics' methodologies, which allow open and unrestricted exploration of community composition, gene and protein expression, and metabolic profiling may converge to facilitate the detailed characterisation and simultaneous 'antireductionist' experimental design needed to generate clinically driven hypotheses. However, *in vitro* studies still have a crucial role in validating such hypotheses in greater detail and elaborating the mechanisms and pathways involved.

2.2: Aims

The primary aims of this work package were to:

1. Develop a simple *in vitro* biofilm model on denture acrylic incorporating representative oral microbes and putative respiratory pathogens *S. aureus* and *P. aeruginosa*

2. Characterise the model biofilms using a range of quantitative and qualitative techniques

3. Evaluate the impact of microbial interactions on the expression of a range of virulence-associated genes of putative respiratory pathogens *S. aureus* and *P. aeruginosa*

Hypotheses

- Preconditioning of acrylic surfaces by artificial saliva will increase attachment, adherence and biofilm biomass of the oral commensals *S. sanguinis*, *A. viscosus* and *C. albicans*, but not *P. aeruginosa* or *S. aureus*

- Initial attachment and adhesion levels will impact biofilm composition for each species tested

- Biofilm species will occupy distinct biogeographical niches within the biofilm

- Co-habitation in biofilms with oral commensal microbes will modulate virulence gene expression by *P. aeruginosa* and *S. aureus* compared with single species biofilms

2.3: Materials and Methods

2.3.1: Production of polymethyl methacrylate (PMMA) coupons

Oracryl powder and liquid monomer were combined in a 1:1.5 (w/v) ratio and mixed thoroughly until smooth and without air bubbles. The mixture was poured into a Polytetrafluoroethylene (PTFE) mould which was compressed between two glass slides to provide a smooth, polished finish and reduce surface irregularities in the material. The mould produced discs of 3 mm thickness and 10 mm diameter. After allowing the material to cure for at least 1 h at room temperature, samples were removed from the mould, any sharp surfaces or irregularities removed with 120 grain silicone carbide paper, then soaked in water for at least 5 days prior to use, to remove any uncured monomer. Coupons were sterilised by autoclaving at 121 ºC for 15 min prior to use.

Sterile acrylic samples were aseptically transferred into either 10 ml of artificial saliva (AS), or 10 ml of water in sterile universal containers and incubated aerobically at 37 °C for 24 h prior to use. After a single use, coupons were disposed.

2.3.2: Preparation of artificial saliva preconditioning solution

The formulation of AS solution used has previously been published (Russel and Coulter, 1975) and used by our research group in a number of studies. Briefly, 5.0 g/L proteose peptone (Oxoid), 2.5 g/L porcine stomach mucin (Oxoid), 2.0 g/L yeast extract (Oxoid), 1.0 g/L lab lemco powder (Oxoid), 0.35 g/L sodium chloride (Fisher), 0.20 g/L potassium chloride (Fisher), 0.20 g/L calcium chloride dehydrate (Fisher) were combined in 1 L of distilled water. The solution was aliquoted into 100 ml glass bottles and autoclaved at 121°C for 15 min. A 40% (w/v) urea solution was filter sterilised and 125 µl/L added to the AS solution immediately prior to use.

2.3.3: Selection and culture of microbial strains

Three species were selected to represent normal oral commensal microbes, frequently found in high abundance within the oral cavity. Two potentially pathogenic bacteria, both associated with a high severity of pneumonia and frequently isolated from health care associated infections were also used. The strain and rationale for each species is detailed in Table 2.3.3A.

Table 2.3.3A: Reference strains used for *in vitro* biofilm model

Microorganism	Strain	Rationale	Isolated from
Candida albicans	ATCC 90028	Common commensal yeast found on high number of denture surfaces. Strain is effective at forming biofilms.	Blood
Streptococcus sanguinis	NCTC 7863	Representative early coloniser of oral cavity	Bacterial endocarditis
Actinomyces viscosus	ATCC 15987	Representative anaerobic early coloniser of oral cavity	Periodontal disease
Staphylococcus aureus	NCTC 6571	Pathogenic species commonly isolated in respiratory infections and healthcare associated infections	Not specified
Pseudomonas aeruginosa	ATCC 15692	Pathogenic species associated with increased severity of pneumonia and high likelihood of antibiotic resistance	Infected wound

Microbial species were incubated for 24-48 h under appropriate conditions (summarised in Table 2.3.3B). One colony was then aseptically transferred into a suitable liquid growth medium and incubated in the same conditions for 8-12 h (aerobes) or 18-24 h (*A. viscosus)* to approximate mid-log phase of growth.

Table 2.3.3B: Growth conditions for microbial species

Microorganism	Agar	Growth Medium (10ml)	Growth Conditions
Candida albicans	Sabouraud Dextrose Agar (SDA, LabM)	Yeast Nitrogen Base (YNB, BD Difco) supplemented with 100mmol d-glucose (Fisher Scientific) (~1.7 mmol/L NaCl, 100 mmol/L glucose)	Aerobic incubator at 37 °C
Streptococcus sanguinis	Colombia Agar (LabM) supplemented with 5% defibrinated horse blood (TCS Biosciences) plus Colistin and Nalidixic acid	Brain heart Infusion broth (BHI, Lab M) (~11 mmol/L glucose, 100 mmol/L NaCl)	
Pseudomonas aeruginosa	Pseudomonas selective agar (LabM) supplemented with Cetrimide and Nalidixic acid		
Staphylococcus aureus	Mannitol Salt Agar (MSA, Lab M)		
Actinomyces viscosus	Fastidious Anaerobe Agar (FAA, Lab M)	Fastidious Anaerobe Broth (FAB, Lab M) (no glucose, ~50 mmol/L NaCl)	Anaerobic Incubator at 37 °C

2.3.4: Inoculation of microbial species for adherence assays and biofilm generation

Bacteria were diluted to approximately $1\text{-}1.5 \times 10^8$ CFU/mL (0.5 McFarland standard) by optical density (OD) at 600 nm, using a spectrophotometer. *Candida* was diluted to approximately 1×10^7 CFU/mL (1.0 McFarland standard). Inocula were prepared either as single species suspensions or combined in equal volumes to produce a mixed inoculum.

2.3.5: Attachment assays

In a 6-well plate, triplicate acrylic samples preconditioned by 24 h submersion in either water or AS solution were aseptically placed. A 50 µL inoculum of the test

microbial suspension (single species or mixed) was added to the surface of each disc. PMMA coupons were then rinsed in PBS immediately to remove any non-adherent microorganisms then placed into new 6-well plates. Negative controls consisted of acrylic coupons processed as above and incubated without an inoculum present.

2.3.6: Adherence assays

Adherence assays were prepared in an identical manner to that described in section 2.3.5. However, after inoculation of the test microbial suspension, PMMA coupons were incubated aerobically at 37°C for 90 min prior to rinsing in PBS. Negative controls consisted of acrylic coupons processed as above and incubated without an inoculum present.

2.3.7: Biofilm generation and maturation

PMMA coupons were inoculated as described above, with either a single species or mixed microbial suspension. Following inoculation, test coupons were incubated aerobically at 37°C for 90 min and rinsed in PBS to remove non-adherent microorganisms. Coupons were then transferred to a new 6-well plate, containing 3 mL FAB, so that the PMMA samples were submerged in culture medium, before incubation for 72 h. Every 24 h, spent culture medium was removed by pipetting, and new, fresh media added. PMMA coupons were not rinsed at 72 h prior to analysis. Negative controls consisted of acrylic coupons processed as above and incubated without an inoculum present.

2.3.8: Characterisation of biofilms

As the model biofilms were intended for subsequent use in evaluation of antimicrobial treatments, quantitative cell-viability was an important outcome measure. Two methods to measure viability were assessed: culture of recovered viable organisms, and confocal laser scanning microscopy of microorganisms on acrylic coupons, after staining with the BacLight Live/Dead kit (Thermofisher Scientific, Loughborough, UK). A graphical summary of biofilm characterisation experiments is provided in figure 2.3.8.

Figure 2.3.8: Flow diagram of experimental input and analyses
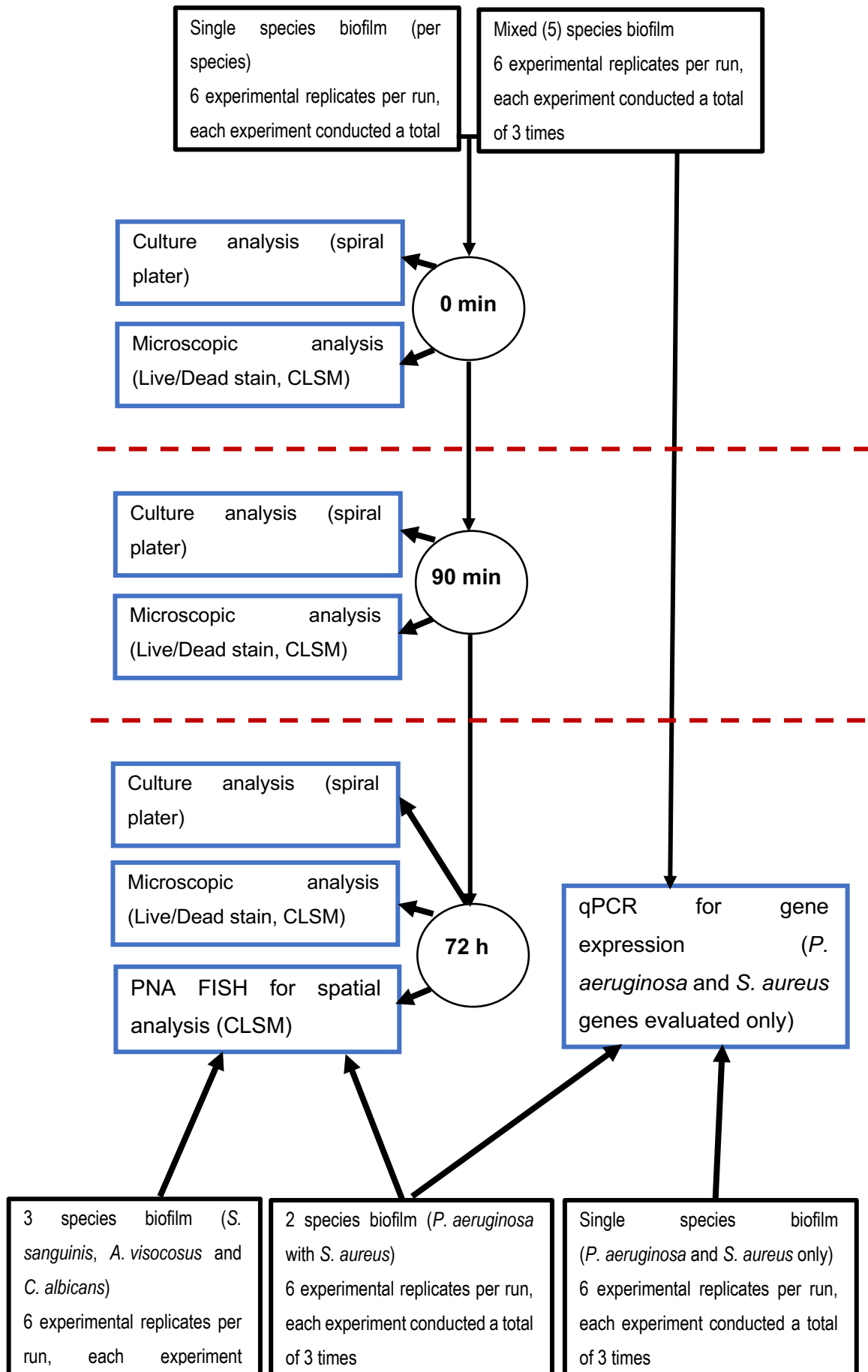
2.3.8.1: Recovery and culture of viable microorganisms

Following rinsing of PMMA coupons in attachment and adherence assays, or 72 h growth in biofilm assays, coupons were immediately transferred to a 10 mL glass bijou containing 1 mL PBS. Samples were vortex mixed for 1 min at maximum speed to remove adherent cells, and the resultant cell suspension transferred by pipetting to a sterile 1.5 mL microcentrifuge tube. Following this, 1:10 serial dilutions were performed to appropriate concentrations, and 50 mL of these final dilutions was applied to an appropriate selective agar plate (Table 2.3.3B) using a spiral plater (WASP – Don Whitley, Sheffield, UK). The plates were then incubated aerobically at 37°C for 24-48 h. Microbial colonies were counted for each species and colony forming units per ml (CFU/ml) calculated. A validation of the effectiveness of vortexing to remove adherent cells was undertaken by staining the coupons with Live/Dead stain and imaging them after this procedure had been undertaken.

2.3.8.2: Confocal laser-scanning microscopy (CLSM) of biofilms using Live/Dead stain

One μl each of SYTO-9 and propidium iodide (PI) from the BacLight Live/Dead assay kit were aseptically added to 998 μl of distilled water, mixed by inversion, and protected from light. Ten μl of this working solution was added to PMMA coupons which had been treated as above for attachment, adherence or biofilm assays. The coupons were incubated with the stain at room temperature for 30 min and protected from light. Negative controls were processed identically and stained to provide a standard for filtering out autofluorescence on images (acrylic autofluoresces strongly in both imaging channels used).

Following incubation, a coupon was inverted onto a glass (0.17 mm) coverslip and viewed at x400 (for *Candida* or mixed inocula) or x630 magnification using a Leica SP5 confocal laser-scanning microscope. The excitation wavelengths used were 488 nm (green; live cells) and 561 nm (red; dead cells); while the emission filters 500-550 nm (green) and 570-620 nm (red) were applied. Images were recorded from 5 randomly selected areas on each coupon. For attachment and adherence assays, single-plane images were recorded. For biofilm assays, a 3-dimensional "Z-stack" was obtained by recording successive images from the acrylic surface to the outer extent of the biofilm (assessed manually) at 1 μm intervals.

2.3.8.2.1: Quantitative analysis of CLSM images using COMSTAT software

Images and Z-stacks were analysed using the COMSTAT 2.1 plug-in in ImageJ. Image files were converted to the OME-TIFF format for analysis. Subsequently files were opened using the COMSTAT plugin and threshold values calibrated to reduce background noise for images. For each image-set (n=45), 3 images were randomly selected for thresholding. Both LIVE and DEAD channels were viewed independently for the purpose of thresholding. A threshold value that offered maximum noise reduction with minimum loss of obviously in-focus cells was manually selected and applied globally to all images. COMSTAT analysis to provide surface area coverage (μm^2) in attachment and adherence assays was undertaken. For biofilm assays, biovolume (μm^3) was calculated.

2.3.8.2.2: Preparation of biofilms for Peptide Nucleic Acid-Fluorescence *in situ* Hybridisation analysis (PNA-FISH)

The methodology for PNA-FISH conjugation to biofilms were adapted from approaches previously employed within our group (Sands et al. 2017, Cavalcanti et al. 2015).

PNA probes were obtained according to the sequences described in Table 2.3.8.2.2; specific for *P. aeruginosa* and *S. aureus*, in addition to a universal bacterial probe. Lyophilised stocks were reconstituted aseptically in sterile, nuclease-free water and mixed by gentle pipetting. From this, 100x master stock aliquots were created for each probe, protected from light and frozen for future use at -20°C. From each stock, the respective working concentration for each probe was prepared in FISH hybridization solution prepared according to the method outlined in Perry-O'Keefe et al. (2001).

Table 2.3.8.2.2: PNA probe sequences

Target species	Working Concentration	Probe Sequence	Reference
Universal Bacterial	300 nM	CTGCCTCCCGTAGGA	Perry-O'Keefe et al. 2001
P. aeruginosa	300 nM	AACTTGCTGAACCAC	Coull & HyldigNielsen (2003)
S. aureus	450 nM	GCTTCTCGTCCGTTC	Perry-O'Keefe et al. 2001

Candida albicans was labelled using a ready-made probe solution available as part of the AdvanceDx Yeast Traffic Light kit (AdvanDX, Vedbaek, Denmark). All probes were protected from light during the procedure wherever possible, and kept at 4 °C. Biofilms containing either mixed or single species inocula (for *P.* aeruginosa and *S. aureus)* were prepared as detailed in section 3.3.7, then fixed by immersion in neutral buffered saline solution for at least 1 h. Similarly, positive controls for the representative PRP species were prepared by heat-fixing a smear of each microorganism onto a glass slide. Following fixation, samples were gently washed in 100 μl PBS x3. This washing procedure was repeated between every subsequent step described. To each biofilm, 50 μl of lysozyme (Sigma Aldrich, Gillingham, UK) solution at a concentration of 10 mg/ml was added; after which samples were incubated at 37°C for 30 min. Subsequently, samples were incubated with 50 μl of proteinase K (Sigma Aldrich, Gillingham, UK) solution at a concentration of 0.1 mg/ml at 37°C for 30 min. Finally, biofilms containing *S. aureus* were treated with lysostaphin (Sigma Aldrich, Gillingham, UK) solution at a concentration of 0.1 mg/ml and incubated at 37°C for 1 h.

To each single species biofilm, and positive control for *S. aureus* and *P. aeruginosa* 50 μl of the respective PNA probe solution was added. The remaining probes were homogeneously combined and 50 μl added to each mixed biofilm sample. Samples were then incubated in a humidified box, protected from light, at 55°C for 2 h to enable hybridisation of the probes. Following this, samples were flooded with 200 μl pre-warmed FISH washing solution prepared as per Perry-O'Keefe (2001) and incubated for a further 15 min at 55°C. This step was repeated twice, after which excess washing solution was removed by carefully blotting the edge of each sample with absorbent paper. Samples were mounted on glass slides with 25 μl of Vectashield™ mounting medium (Vectorlabs, Orton Southgate, UK) and enclosed with 12 mm circular 0.17 mm glass coverslips, sealed with a commercially available nail varnish.

2.3.8.2.3: CLSM of PNA-FISH labelled biofilms

PNA treated samples prepared as described in section 2.3.8.2.2 were imaged using a Leica SP2 confocal-scanning laser microscope. The excitation spectra and emission filters used are outlined in Table 2.3.8.2.3.

3-Dimensional Z-stacks were recorded at 0.1 μm intervals, using a scanning resolution of 1024-1024 pixels and x4 line-averaging. From these multilayer Z-stacks, 3-Dimensional projections were generated using the Leica proprietary image processing software.

Table 2.3.8.2.3: Excitation and Emission Spectra for PNA probes

Target species	Fluorophore	Excitation Wavelength (nm)	Emission Frequency (nm)
Universal Bacterial	CY3	561	550–610
P. aeruginosa	FITC	488	498–540
S. aureus	Cy5	633	635–700

2.3.8.3: Preparation of biofilms for scanning electron microscopy (SEM)

Biofilms were prepared on acrylic coupons as described in section 2.3.7. At the 72 h timepoint, each acrylic coupon was submerged in 2 ml of gluteraldehyde solution (3% v/v) overnight to fix biofilm samples. After gentle washing with PBS, samples were sequentially placed in 50, 70, 90 then 100% ethanol for 30 min each to dehydrate biofilms. Samples were then transferred directly into a 1:2 solution (v/v) of hexamethyldisilazane (HMDS; Sigma Aldrich, Gillingham, UK):ethanol, followed by a 2:1 solution of HMDS:ethanol for 30 min each. Finally, samples were immersed in 100% HMDS solution and left loosely covered in a fume cabinet overnight, until all HMDS had evaporated. Samples were subsequently sputter-coated with gold in a vacuum chamber, under an Argon atmosphere. Coated samples were then carefully mounted onto SEM stubs, using carbon adhesive discs.

2.3.8.3.1: SEM of biofilms

Biofilms were viewed using a Tescan VAGA SEM and imaged at between 5 – 20 kV to assess both surface coverage and more detailed morphological and ultrastructural features of biofilms. Due to samples rapidly becoming highly charged, even at moderate magnification, focus was obtained, then the field of view moved immediately prior to image capture.

2.3.8.4: Generation of biofilms for gene expression analysis

Biofilms were prepared for gene expression analysis using the methods described in section 3.3.7. However, only *S. aureus* and *P. aeruginosa* single-species biofilms were prepared; a combined *S. aureus* and *P. aeruginosa* mixed biofilm, a mixed 'commensal' biofilm and a mixed biofilm containing all 5 species as used in previous sections.

2.3.8.5: Extraction of bacterial RNA from model biofilms

A number of methods were evaluated for extraction of bacterial RNA from the model biofilms on acrylic coupons. Initially, the Qiagen RNeasy proprietary method; a modification of this method employing phenol-chloroform-isoamyl alcohol from earlier work within the group (Cavalcanti et al. 2015); the FastPro Blue RNA extraction proprietary method, which has previously been reported to result in good RNA yields from microbial biofilms (França et al. 2012) were trialled. Finally, a hybrid method was found to provide the best yield and quality of RNA from biofilms. This was performed using a combination of the FastPro Blue kit (MP Biomedicals, Santa Ana, CA, USA) and Qiagen RNeasy clean-up kit (Qiagen, Manchester, UK) and is described below. After 72 h aerobic incubation at 37°C, inoculated acrylic coupons were pooled in triplicate and placed in glass bijoux containing 1 ml of lysis buffer and 500 μg glass beads (500 – 750 μm size). These were vortex mixed at maximum speed for 1 min to remove biofilm cells from the acrylic surface. The resultant cell suspension was transferred by pipette into 1.5 ml screw cap tubes containing 500 μg glass beads (500 – 750 μm size) and kept on ice. These samples were homogenised using a rotary bead-beater (BeadBlaster 24, BenchMark Scientific, Edison, NJ, USA) at a speed of 7 m/s for 30 s, followed by steeping samples in ice for 1 min, repeated a total of 3 times. Tubes were then centrifuged at 13,300 *g* for 3 min to pellet cell debris, and the supernatant transferred by pipette into a 1.5 ml microcentrifuge tube, being careful not to perturb the intermediate layer. The screw top tubes containing cell debris were discarded.

To each microcentrifuge tube (containing approximately 500 μl of supernatant) 700 μl 100% ethanol was added and mixed by pipetting. This liquid was transferred to a silica spin column from the Qiagen RNeasy clean-up kit, and centrifuged at 10,000 *g* for 30 s. The flow-through was discarded, and the process repeated for any remaining lysate supernatant. Then, 350 μl RW1 buffer was added to each column, and again centrifuged at 10,000 *g* for 30s, with the flow-through discarded. Next, 80 μl of a DNAse I – RDD buffer (comprising 70 μl RDD buffer and 10 μl DNAse I solution) was carefully pipetted onto the membrane of each column and incubated at room temperature for 30 min. Following incubation, a further 350 μl RW1 buffer was added to each column and columns were centrifuged at 10,000 *g* for 30 s, with the flow-through discarded. To each column, 500 μl RPE buffer was added. Columns were centrifuged as in prior steps and the flow-through discarded. This was repeated with a final centrifugation for 2 min to dry the spin columns.

To elute bacterial RNA, 10 μl RNase free water was carefully added to the spin column membrane, followed by centrifugation at 10,000 g for 1 min. This process was repeated for a total of 20 μl eluate. RNA eluate was transferred to a 200 μl microcentrifuge tube and kept on ice. Each sample was checked for yield and purity by photospectrometric absorbance at 260 and 280 nm using a NanoVue. Samples below a minimum threshold of 55 ng/μl (needed to generate a target RNA quantity of 500 ng in reverse transcription reactions) were discarded, as were samples failing to give a 260:280 nm absorbance ratio of at least 1.8, indicating high levels of chemical or protein contaminants.

2.3.8.6: cDNA synthesis by Reverse Transcription (RT)

RNA samples were diluted in sterile, nuclease free water to achieve a quantity of approximately 500 ng RNA in 9 μl total volume. Reverse transcription was undertaken using a 2-step protocol, with the Precision nanoScript™ 2 kit (Primerdesign, Camberley, UK). To each of the above 9 μl RNA suspensions was added 1 μl random nonamer primers. Samples were briefly mixed by vortex, then annealed at 65°C for 5 min in a thermocycler (G-storm, Gene-technologies, Braintree, UK), followed by immediate transfer to ice (before cooling of the thermocycler occurred).

A reverse-transcriptase mastermix was then prepared, by combining 5 μl 4X buffer, 1 μl dNTP mix, 3 μl RNase/DNase free water and 1 μl nanoScript 2 enzyme for each reaction. A 10 μl volume of the above mastermix was added to each sample and briefly vortex mixed at medium speed. The thermocycling parameters used for the second stage reverse transcription reaction were 25°C for 5 min followed by 42°C for 20 min. The reaction was terminated by heat inactivation of the RT enzyme at 75°C for 10 min. Following completion of reverse transcription, cDNA was stored at -20°C for subsequent use in qPCR experiments.

2.3.8.7: Quantitative PCR (qPCR) of targeted *P. aeruginosa* and *S. aureus* genes from model biofilms

A range of *P. aeruginosa* and *S. aureus* genes were selected from extensive searching of the literature. Genes involved in adherence, biofilm formation, inter-microbial interactions and associated with virulence in human infection were selected (Table 2.3.8.7A). Initially, primers were selected from the literature, but in a number of cases were found to provide poor amplification under the experimental conditions used. Consequently, a number of primers were redesigned using the PrimerQuest and OligoAnalyzer platforms from the Integrated DNA Technologies website

(https://www.idtdna.com/Primerquest/Home/Index and https://www.idtdna.com/calc/analyzer, respectively). Secondary primer structure reaction enthalpies were screened using the online mfold application (http://unafold.rna.albany.edu/?q=mfold), ensuring that the melting temperature (Tm) of any secondary products was at least 30°C below the Tm to be used for qPCR reactions. Target bacterial gene sequences were obtained via the KEGG genome database (https://www.genome.jp/kegg/genome.html), NCBI microbial genome browser (https://www.ncbi.nlm.nih.gov/genome/browse#!/prokaryotes/); and for *P. aeruginosa* genes, the pseudomonas genome database (https://www.pseudomonas.com/). Where possible, gene sequences were cross referenced using more than one database and were obtained for the specific strains used in qPCR experiments. Where gene sequences for the specific experimental strains used were not possible to obtain, confirmation that the genes existed in multiple strains was sought, with the assumption that such genes would therefore likely be present ubiquitously across the species. The revised primer sequences are detailed in Table 2.3.8.7B.

Table 2.3.8.7A: Target genes and primer sequences initially trialled for qPCR experiments

S. aureus			
Target gene	**Primer Pair**	**Gene Product and Function(s)**	**Reference**
icaA*	5-GAGGTAAAGCCAACGCACTC-3	Polysaccharide intercellular adhesin. Roles in biofilm formation, colonisation of epithelial cells.	Atshan et al. 2013
	5-CCTGTAACCGCACCAAGTTT-3		
icaB*	5-ATACCGGCGACTGGGTTTAT-3		
	5-TTGCAAATCGTGGGTATGTGT-3		
icaC*	5-CTTGGGTATTTGCACGCATT-3		
	5-GCAATATCATGCCGACACCT-3		
icaD*	5-ACCCAACGCTAAAATCATCG-3		
	5-GCGAAAATGCCCATAGTTTC-3		
fnbA*	5-AAATTGGGAGCAGCATCAGT-3	Fibronectin binding proteins A/B. Adhesion to epithelial cells.	
	5-GCAGCTGAATTCCCATTTTC-3		
fnbB*	5-ACGCTCAAGGCGACGGCAAAG-3		
	5-ACCTTCTGCATGACCTTCTGCACCT-3		
clfA*	5-ACCCAGGTTCAGATTCTGGCAGCG-3	Clumping factors A/B. Coaggregation and adhesion to plastic biomaterials.	
	5-TCGCTGAGTCGGAATCGCTTGCT-3		
clfB*	5-AACTCCAGGGCCGCCGGTTG-3		
	5-CCTGAGTCGCTGTCTGAGCCTGAG-3		
cna*	5-AATAGAGGCGCCACGACCGT-3	Collagen binding protein. Adhesion to epithelial cells.	
	5-GTGCCTTCCCAAACCTTTTGAGCA-3		
hla*	5-GCAAATGTTTCGATTGGTCA-3	Alpha haemolysin. Leucotoxin – allows escape after phagocytosis; entry to blood stream.	Burnside et al. 2010
	5-CCATATACCGGGTTCCAAGA-3		
hld	5-TAATTAAGGAAGGAGTGATTTCAATG-3	Delta haemolysin. Leucotoxin – allows escape after phagocytosis; entry to blood stream.	
	5-TTTTTAGTGAATTTGTTCACTGTGTC-3		
spa*	5-CAAACGGCACTACTGCTGAC-3	Protein A. Blocks phagocytosis, immune suppression and evasion.	
	5-CATGGTTTGCTGGTTGCTTC-3		

P. aeruginosa			
Target gene	Primer Pair	Gene Function(s)	Reference
*exoS**	5-CGTCGTGTTCAAGCAGATGGTGCTG-3 5-CCGAACCGCTTCACCAGGC-3	Exotoxins S/T. Disrupt	Fazeli and Momtaz 2014
*exoT**	5-CAATCATCTCAGCAGAACCC-3 5-TGTCGTAGAGGATCTCCTG-3	intracellular actin filaments and inhibits phagocytosis.	
exoU	5-GATTCCATCACAGGCTCG-3 5-CTAGCAATGGCACTAATCG-3	Exotoxin U. Phospholipase activity, promotes neutrophil migration.	
*exoY**	5-TATCGACGGTCATCGTCAGGT-3 5-TTGATGCACTCGACCAGCAAG-3	Exotoxin Y. Mechanism of action unclear but associated with increased virulence.	
*aprA**	5-TGTCCAGCAATTCTCTTGC-3 5-CGTTTTCCACGGTGACC-3	Alkaline protease.Blocks complement opsonization, contributes to immune evasion.	
toxA	5-GGT AAC CAG CTC AGC CAC AT-3 5-TGA TGT CCA GGT CAT GCT TC-3	Exotoxin A. Inhibits eukaryotic protein synthesis causing cell death.	
lasB	5-GGA ATG AAC GAA GCG TTC TC-3 5-GGT CCA GTA GTA GCG GTT GG-3	Pseudolysin. Downregulates IL-6 to impair chemotaxis.	
algE	5-ATGTGTTCGGCGACATTT-3 5-TAGGTCTTGTCGAGGTTGT-3	Alginate biosynthesis.	This study
algG	5-ACAAGCAGGTCAAGGAAC-3 5-CAGGAGATCAGGAACGGA-3	Adherence, biofilm formation, EPS constituent.	
phzA	5-AACGGTTACAGCGGCACAGCCTGTTC-3 5-CTCGACCCAGAAGTGGTTCGGATCCTC-3	Phenazine biosynthetic	Ha et al. 2011
*phzG**	5-TTTCCGAGTCCCTCACCGGGACCATC-3 5-CGCGCTCGCCGAGTTCGGC-3	pathway; Pyocyanin production. Biofilm formation, cell signalling, quorum sensing.	
*pslA**	5-AAGATCAAGAAACGCGTGGAAT-3 5-CACCGTACGGAACAGGATGTAG-3	Psl exopolysaccharide	Crabbé et al. 2008
*pslD**	5-AACGTCGCCACCATCTATGAA-3 5-ATGCGACCGATGAACGGATA-3	biosynthesis. Colonisation of surfaces, biofilm formation.	
pelA	5-CCTTCAGCCATCCGTTCTTC-3		

pelD	5-TAGCCATACTGGGCCTCGAA-3 5-GATGCCGTTCTTCGTCTTCAA-3 5-CGGTCGCTCTGCAGCAA-3	Pel exopolysaccharide biosynthesis. Colonisation of surfaces, biofilm formation.	
lasI*	5-CGTGCTCAAGTGTTCAAGG-3 5-TACAGTCGGAAAAGCCCAG-3	N-(3-oxo-dodecanoyl)-homoserine lactone biosynthesis and signalling. Autoinducer, quorum-sensing.	Sabharwal et al. 2014
lasR	5-AAGTGGAAAATTGGAGTGGAG-3 5-GTAGTTGCCGACGACGATGAAG-3		
rhlI	5-TTCATCCTCCTTTAGTCTTCCC-3 5-TTCATCCTCCTTTAGTCTTCCC-3	N-(butanoyl)-homoserine lactone biosynthesis and signalling. Autoinducer, quorum-sensing.	
rhlR	5-TGCATTTTATCGATCAGGGC-3 5-CACTTCCTTTTCCAGGACG-3		
lecA	5-CACCATTGTGTTTCCTGGCGTTCA-3 5-AGAAGGCAACGTCGACTCGTTGAT-3	Lectins. Adhesion to epithelial cells.	Balasubramanian D 2013
lecB	5-AGACAGCGTAACAATCGAACGAGC-3 5-AGGACGCATCGTTCAGCCAATCTA-3		

*Primers which showed poor amplification and were consequently redesigned (see Table 2.3.8.7B).

Table 2.3.8.7B: Revised primer designs for target genes used in qPCR experiments

	S. aureus	
Target gene	**Primer Pair**	
icaA	5-TATTTACTGGATTGTCGGTTCA-3	
	5-CGTATCTTCAATCGTTTCACTTT-3	
icaB	5-GCAGATGACGATTCACCTAAA-3	
	5-TGAGATTCAAATTGTGATTGACTAAC-3	
icaC	5-AGGTCAATGGTATGGCTATTT-3	
	5-GCTGTGTTGTTCGTAAAGTAAT-3	
icaD	5-ACTTATCGCTATATCTTGTGTCTT-3	
	5-TCTCTTCCTCTCTGCCATT-3	
fnbAB[1]	5-CACGCTGATGTTGTTGAATATG-3	
	5-CTGTTGTATGATCGCTCACTG-3	
clfA	5-AGTAGAGTTTAATACGCCTGATG-3	
	5-TGACATAGAGCGCCAAATTAT-3	
clfB	5-AATCATCACCACAAACGATTTC-3	
	5-TCACTTGCCGTAACTTTATCA-3	
cna	5-ACTGTATCACCGTCTAAGATAG-3	
	5-CCCACCTGTTCACCTTTA-3	
hla	5-CACATATAGTCAGCTCAGTAACA-3	
	5-TATCAGAATCTGCGGCATTAG-3	
spa	5-ACACGATGAAGCTCAACAA-3	
	5-ACTTTGGCTTGGATCATCTT-3	
	P. aeruginosa	
Target gene	**Primer Pair**	
exoS	5-GCGTTTGGGACAGATTGA-3	
	5-ACCATCTGCTTGAACACG-3	
exoT	5-ATGCGGTAATGGACAAGG-3	
	5-GATTCAGGTGCTGGTACTC-3	
exoY	5-GATGACCGCCGATTATGAC-3	
	5-CATATCCTCCCTGCCATAGA-3	
aprA	5-GACCTTCGGCAACTTCAG-3	
	5-TGTTCTGCTCTTCCCAGTA-3	
phzG	5-GAAACCCTCGCCGACATC-3	
	5-AGAACTCCACCGACTCCA-3	
pslA	5-GGGCTGGATTCATCGAGTA-3	
	5-GGAACATAATCACCGTGAGC-3	
pslD	5-CAAACGCACCCTCCTCAT-3	
	5-GTAGCCTGGCGAGTTCTT-3	
lasI	5-TAGGCGTGGAGAAGATGAT-3	
	5-GGGTCTTGGCATTGAGTT-3	

[1]It was not possible to design an acceptable primer pair for fnbA or fnbB individually due to the short span of these genes, thus a primer pair which incorporated the combined sequences for these adjacent genes was used.

2.3.8.8.1: Screening of potential reference genes

Five candidate reference genes, and their corresponding primer-pairs were selected from the literature for *P. aeruginosa* and *S. aureus* (Table 2.3.8.8.1). Primer efficiency was calculated using the LinRegPCR open source software (Ruijter et al. 2009). The stability of each candidate reference gene was assessed using the NormFinder plugin in Microsoft Excel.

Primers were selected based on the consistency of most efficient amplification and stability of each candidate across all experimental conditions. Pairs of primers were also considered during the NormFinder analysis for improved stability.

Table 2.3.8.8.1: Reference genes and primer sequences screened for qPCR experiments

Target gene	Primer Pair	Gene Function(s)	Reference
	S. aureus		
16S	5-GGGACCCGCACAAGCGGTGG-3 5-GGGTTGCGCTCGTTGCGGGA-3	16S ribosomal RNA. Translation – structural component of 30s ribosomal subunit	Atshan et al. 2013
pyk	5-ACTGCTGCTGGTTTATATCC-3 5-TGCAACCGAAATACCGATAG-3	Pyruvate kinase. Glycolytic enzyme.	This study
rho	5-CAGATTTAGAACGCTCAGTAGAA-3 5-CACCTGATAATGTACGACCAC-3	Transcription termination factor Rho. Regulates transcription.	This study
gyrB	5-CCAGGTAAATTAGCCGATTGC-3 5-AAATCGCCTGCGTTCTAGAG-3	DNA gyrase subunit B. Induce or relax DNA negative supercoiling, affects transcription and DNA replication	Rudkin et al. 2012
rpoB	5-GCGAACATGCAACGTCAAG-3 5-GACCTCTGTGCTTAGCTGTAATAGC-3	DNA-dependent RNA polymerase subunit B. Transcription.	Duquenne et al. 2010
	P. aeruginosa		
Target gene	Primer Pair	Gene Function(s)	Reference
proC	5-CAGGCCGGGCAGTTGCTGTC-3 5-GGTCAGGCGCGAGGCTGTCT-3	Pyrroline-5-carboxylate reductase. Amino acid biosynthesis.	Savli et al. 2003
rpoD	5-GGGCGAAGAAGGAAATGGTC-3 5-CAGGTGGCGTAGGTGGAGAA-3	RNA polymerase sigma factor D. Promotes transcription,	

		particularly for cell growth.
fabD	5-GCATCCCTCGCATTCGTCT-3 5-GGCGCTCTTCAGGACCATT-3	Malonyl CoA-acyl carrier protein transacylase. Fatty acid biosynthesis.
ampC	5-AGATTCCCCTGCCTGTGC-3 5-GGCGGTGAAGGTCTTGCT-3	Beta-lactamase precursor. Hydrolyses a broad range of Beta-lactam antibiotics.
pbp2	5-CCGCCACTACCCGCTGAAG-3 5-TGCCGTGCAACTCGCTCTC-3	Penicillin binding protein 2. Peptidoglycan cell wall synthesis.

2.3.8.8.2: qPCR conditions and data analysis

All qPCRs were undertaken using the Precision®FAST qPCR Mastermix with LOW ROX (Primerdesign, Camberley, UK). Bacterial cDNA samples prepared as described in section 2.3.8.7 were thawed on ice, then diluted 1:5 in nuclease free water. Each 20 µl reaction volume consisted of: 10 µl Precision®FAST qPCR Mastermix solution, 8 µl of RNase/DNase free water, 1 µl of the relevant primer mix (0.5 µl of each forward and reverse primer) and 1 µl of template cDNA suspension.

All reactions were aliquoted in duplicate in BrightWhite PCR 96 well-plates sealed with Optical adhesive seals (PrimerDesign, Camberley, UK). Negative controls were included by adding an additional 1 µl of nuclease-free water to reaction wells rather than RNA.

The QuantStudio 6 Flex Real Time PCR system (Applied Biosystems™, California, USA) was used for thermocycling and fluorescence analysis for the reactions. Thermocycling parameters used were: an initial Hot Start Enzyme activation step of 95 °C for 2 min, followed by 40 cycles of denaturation (95°C for 5 s) and annealing (60°C for 20 s). As a final step, upon completion of the 40 cycles, melt curve analysis was undertaken automatically for each reaction. Thresholds were applied automatically by the QuantStudio 6 software across all reactions, indicating a point at which all reactions were undergoing a logarithmic increase in fluorescence intensity. Cycle threshold values were obtained for each reaction, defined as the cycle number at which each reaction crossed the threshold line defined above.

Changes in gene expression were calculated using the Pfaffl method (Pfaffl 2001) by imputing efficiencies for each primer calculated using the program LinRegPCR. The equation used was:

$$R = \frac{(E_{target})^{\Delta CP_{target}\,(control\,-\,sample)}}{(E_{ref})^{\Delta CP_{ref}\,(control\,-\,sample)}}$$

where:

- R = ratio of gene expression
- E = primer efficiency
- ΔCP = change in crossing point (or cycle threshold) value
- Target = gene of interest
- Ref = reference (housekeeping) gene

2.4: Results

2.4.1: Attachment of microbes to acrylic coupons - influence of preconditioning by AS and single or mixed inocula

There was a difference in the impact of preconditioning with artificial saliva in single species and mixed species inocula (Figure 2.4.1A and B). In single species inocula, artificial saliva led to increased recovery of *S. sanguinis* from acrylic biomaterial surfaces compared to water alone. There was a trend for this effect on *A. viscosus* also, although this did not reach the threshold of statistical significance. Overall, *P. aeruginosa* was recovered in substantially greater number compared to the other microorganisms evaluated, with an approximate 1 log greater recovery than any other microorganism.

However, in the case of mixed inocula, there was a reduction in the number of pseudomonas colony forming units (CFU) recovered from acrylic coupons, with a concomitant increase in the number of *S. sanguinis* and *A. viscosus.* The impact of preconditioning by AS was enhanced for these microorganisms also, with both species demonstrating highly statistically significant increases in CFU recovery from acrylic surfaces preconditioned with AS. It was not possible to identify *C. albicans* from samples, likely because *P. aeruginosa* grew heavily on the Sabouraud Dextrose agar used for Candida recovery.

The impact of preconditioning with AS appeared to differ to culture analysis when microbial surface area coverage of acrylic biomaterials was analysed from CLSM images however (Figure 2.4.1C). Here the trend for *S. sanguinis* for higher area coverage by total cells and live cells on acrylic coupons preconditioned in water. This pattern was similar for *C. albicans, S. aureus* and *P. aeruginosa*. Only *A. viscosus* demonstrated greater surface area coverage on acrylic biomaterial surfaces preconditioned with AS, although this did not reach the threshold of statistical significance. There was a high degree of variability noted in surface area coverage for all samples, which is reflected in the large error bars. When evaluating mixed inocula, there was an increase in both viability of microbes and surface area coverage of acrylic coupons after preconditioning with AS. However, as quantification of such images does not discriminate between species, it is not possible to ascertain if this increased surface area coverage is due to greater attachment of *S. sanguinis* and *A. viscosus*, as was seen in culture experiments. The overall area covered by mixed inocula was also substantially higher than the addition of each single species inocula (up to 10-fold greater). Representative CLSM photomicrographic images for each species and condition are displayed in Figure 2.4.1D-O.
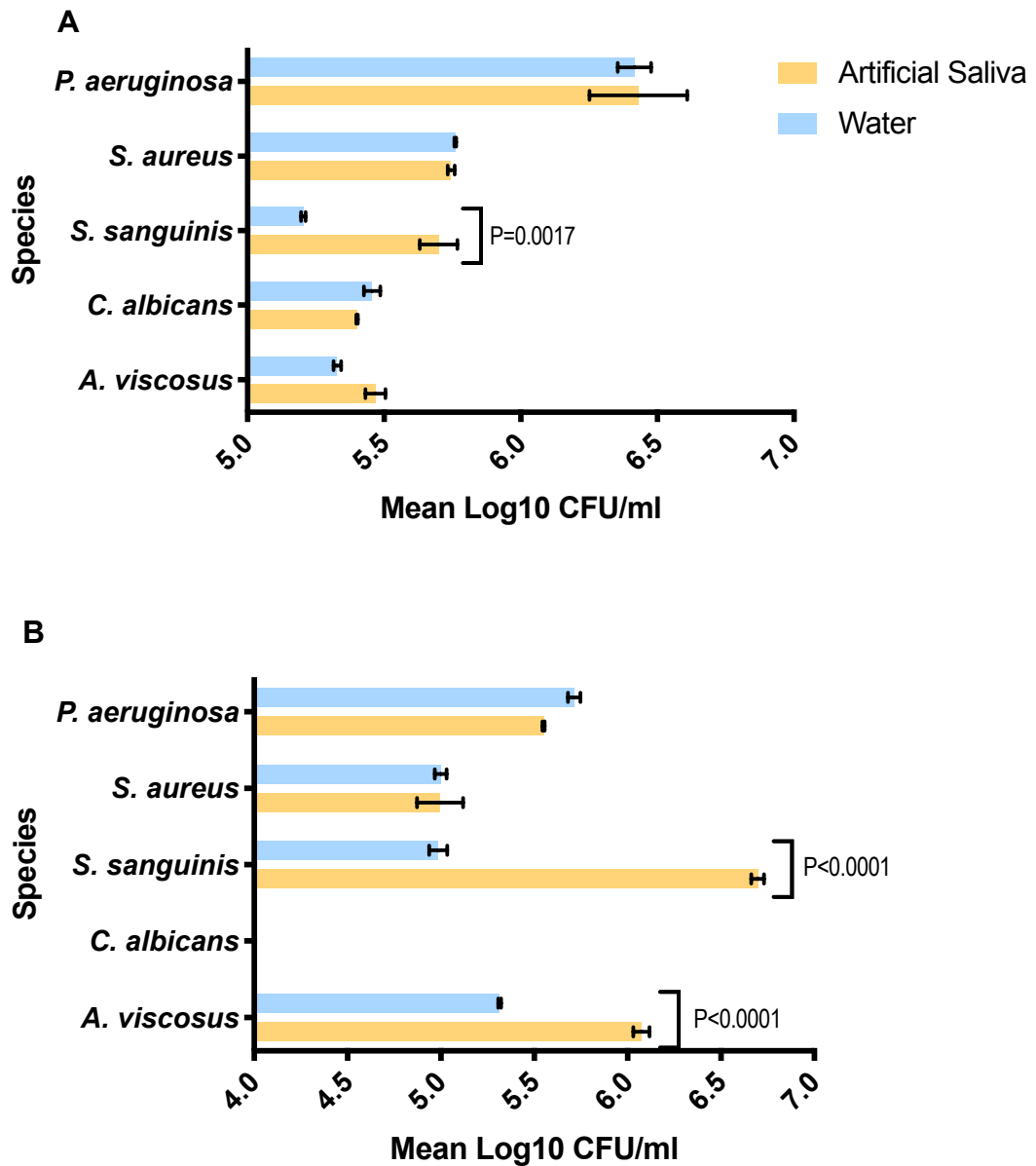
Figure 2.4.1: Mean colony forming units recovered from acrylic surfaces rinsed immediately after inoculation

A)   Single species inocula
B)   Mixed species inocula
AV – *A. viscosus*
CA – *C. albicans*
SS – *S. sanguinis*
SA – *S. aureus*
PA – *P. aeruginosa*
Note the logarithmic scale (log10 axis).

All samples were analysed in triplicate and experiments repeated twice.

C
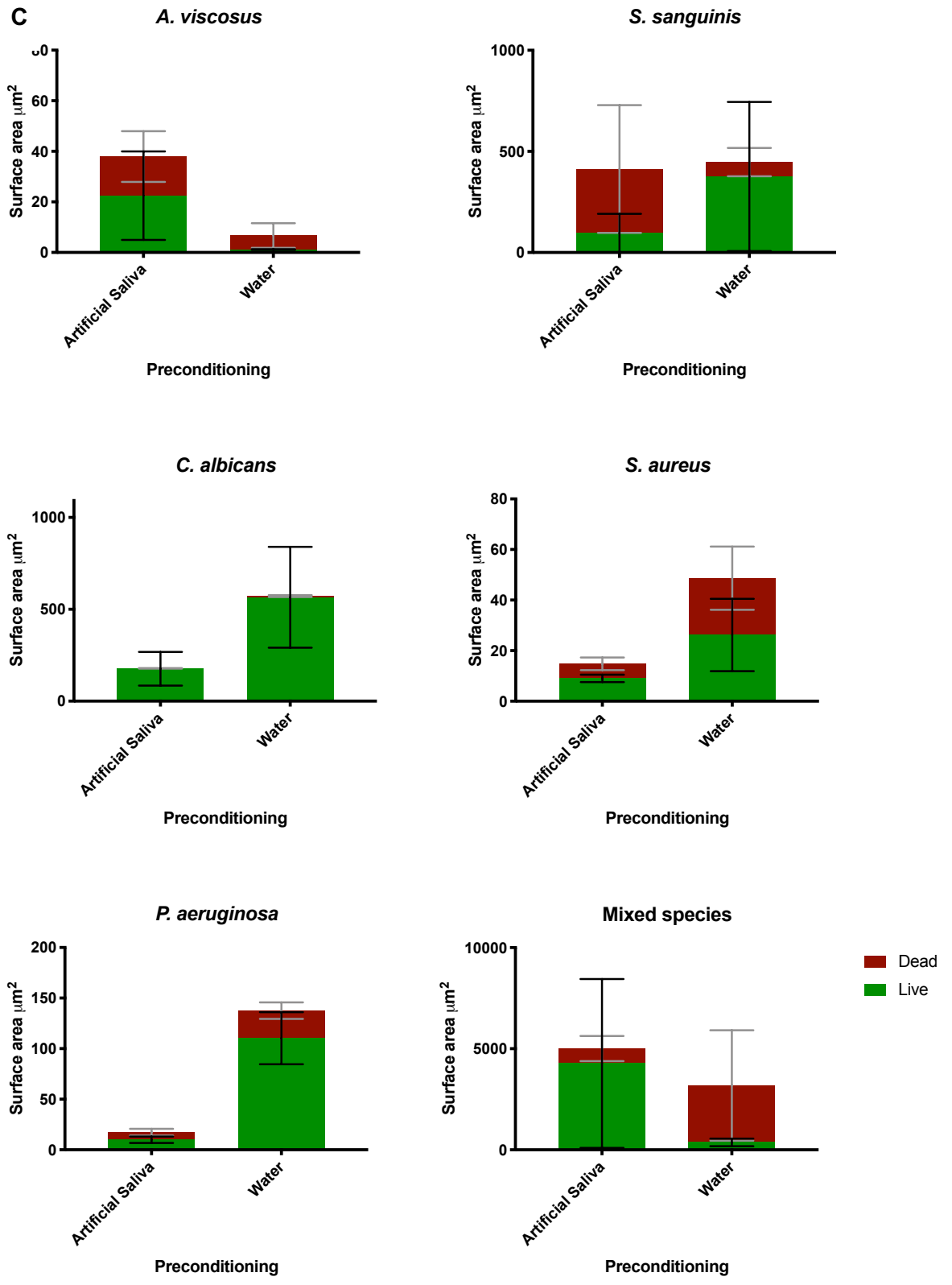
Figure 2.4.1C: Median surface area (μ m²) occupied by microbes on acrylic surfaces rinsed immediately after inoculation, detected by CLSM using Live/Dead stain.

Error bars represent standard deviation. All acrylic samples were imaged at five random locations, in triplicate with experiments repeated 2 times. Note that scales differ for each inoculum.
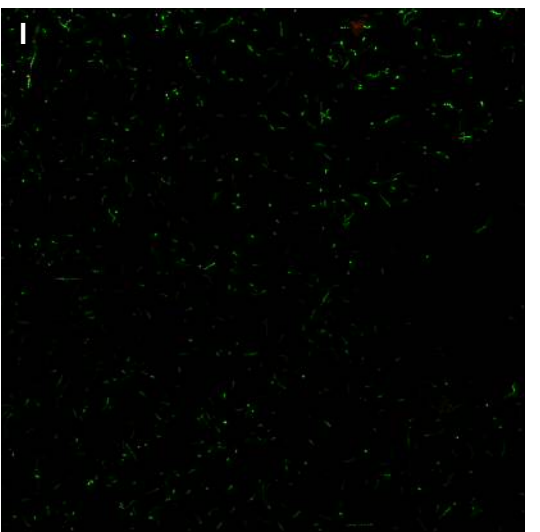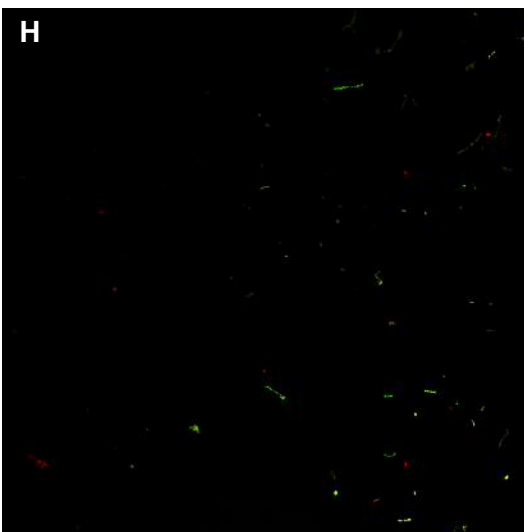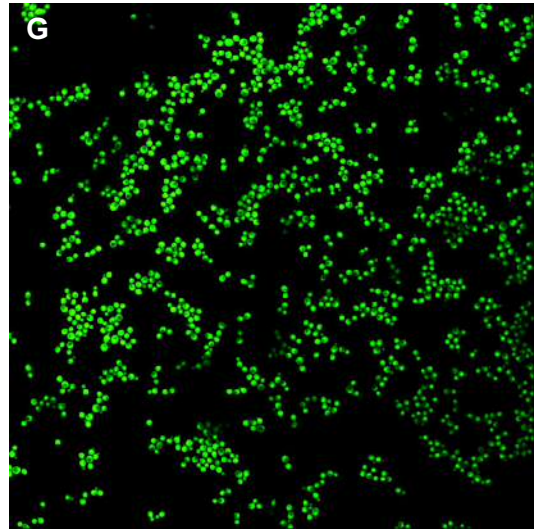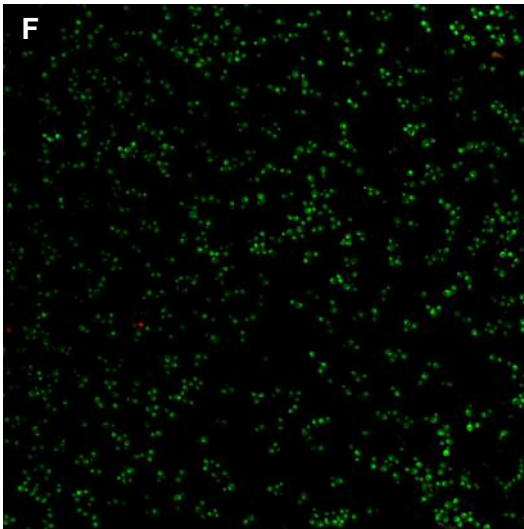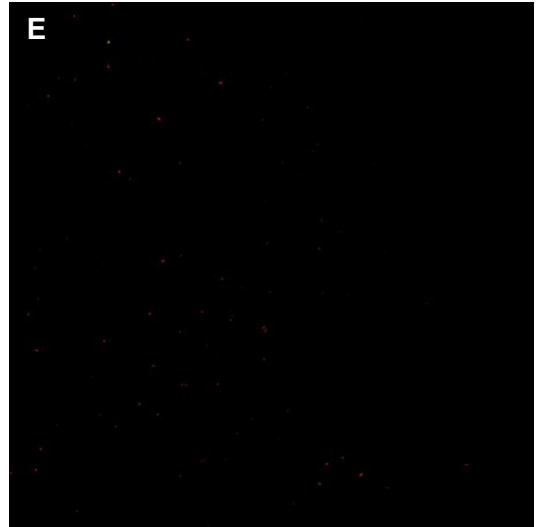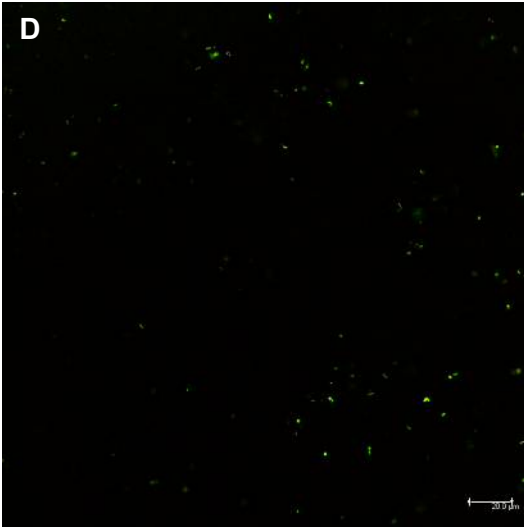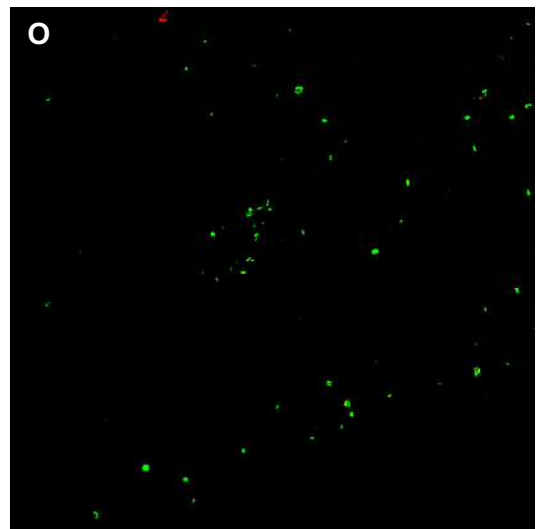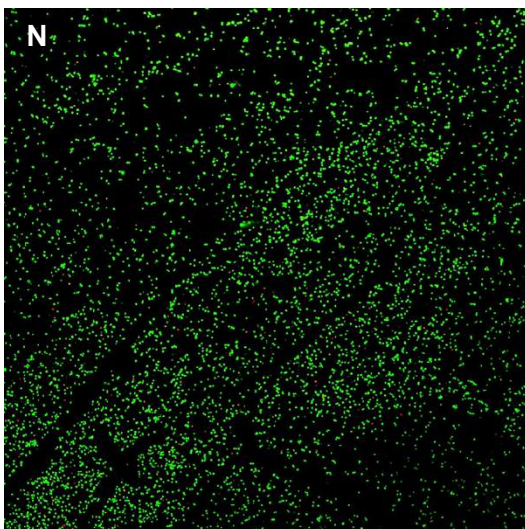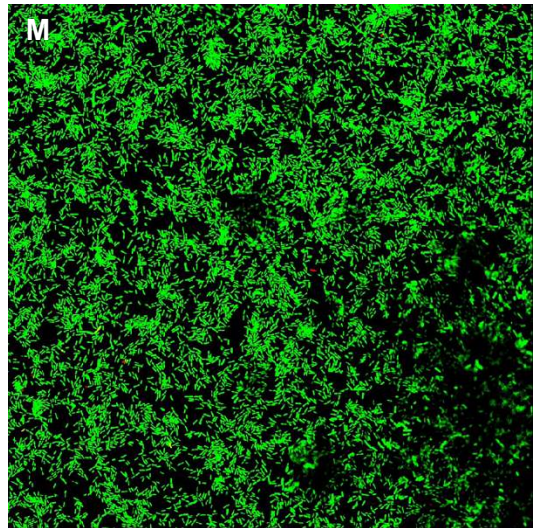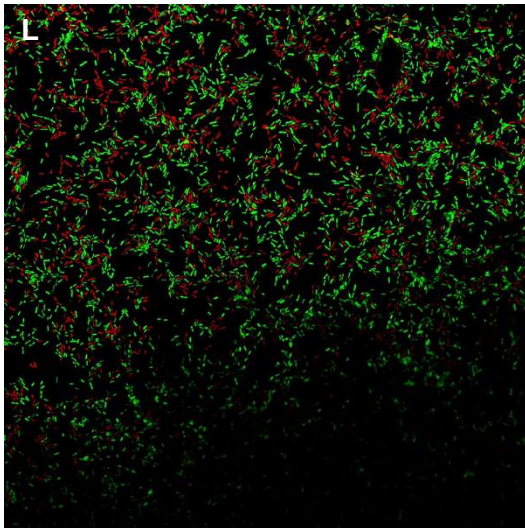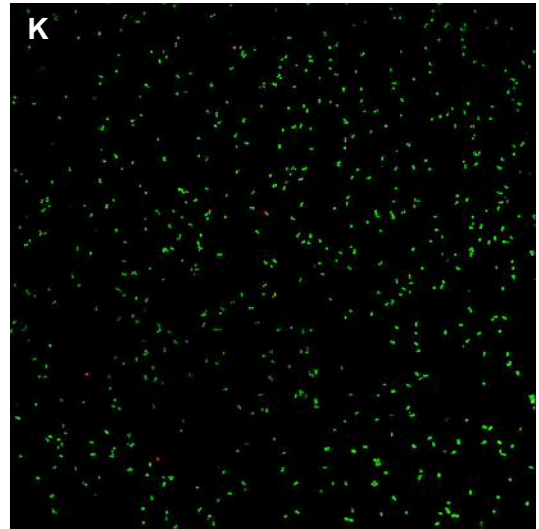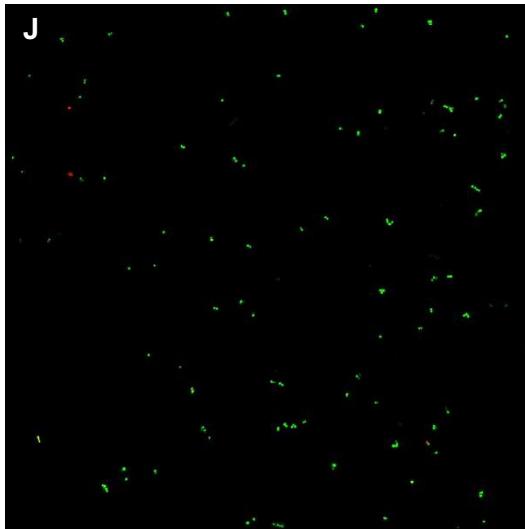
Figure 2.4.1: CLSM photomicrographs of microbial adherence assays on acrylic surfaces
D, E – *A. viscosus* **F, G –** *C. albicans* **H, I –** *S. sanguinis* **J, K –** *S. aureus*
L, M – *P. aeruginosa* **N, O –** Mixed species inoculum
D, F, H, J, L, N show microbes attached to acrylic surfaces preconditioned with artificial saliva
E, G, I, K, M, O show microbes attached to acrylic surfaces preconditioned with water

2.4.2: Adherence of microbes to acrylic coupons - influence of preconditioning by AS and single or mixed inocula

After 90 mins adherence time, there was a change in the proportions of microbes recovered from acrylic coupon surfaces, as well as a change in the impact of preconditioning with AS of single species inocula (Figure 2.4.2A). Both *C. albicans* and *S. aureus* were recovered in lower quantities from AS preconditioned coupons compared to water preconditioning. In contrast, *P. aeruginosa* was recovered in significantly greater quantities from AS preconditioned acrylic surfaces. Both *S. sanguinis* and *A. viscosus* were recovered in approximately equal quantities from acrylic biomaterial surfaces preconditioned with either water or AS, in contrast to initial attachment patterns.

In mixed inocula however (Figure 2.4.2B), *S. sanguinis* and *A. viscosus* retained the statistically significant increase in CFUs recovered from AS preconditioned acrylic. Again, it was not possible to identify *C. albicans* from mixed inocula.

Findings from CLSM image analysis (Figure 2.4.2C) demonstrated an increase in surface area coverage from both viable and total *S. sanguinis* and *A. viscosus* single species inocula on AS preconditioned acrylic surfaces; corroborating the findings of culture studies. There was also a clear decrease in surface area coverage of *P. aeruginosa* and *C. albicans.* None of these findings met the threshold of statistical significance, due to the high variability between images. Interestingly in mixed species inocula, both the total surface area coverage and proportion of viable cells was decreased on acrylic biomaterial surfaces preconditioned with AS. Comparison between culture and CLSM studies for mixed inocula is tenuous at best, as no *C. albicans* grew in the presence of *P. aeruginosa* on SDA, while quantitative CLSM image analysis does not account for the contributions of different microbial species to surface area coverage. Representative CLSM photomicrographs are displayed in Figure 2.4.2D.

A
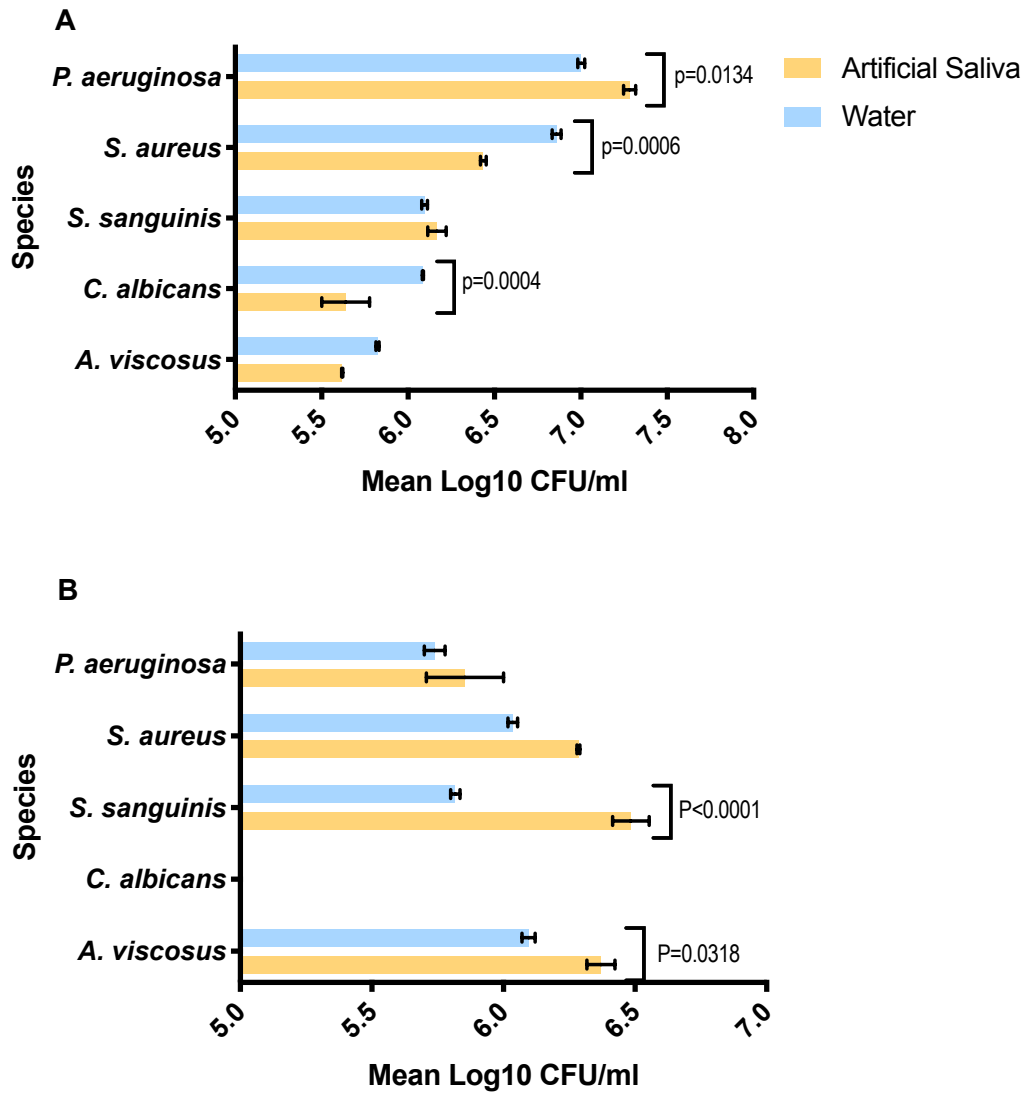
Figure 2.4.2: Mean colony forming units recovered from acrylic surfaces rinsed 90 min following inoculation

A)   Single species inocula
B)   Mixed species inocula
AV – *A. viscosus*
CA – *C. albicans*
SS – *S. sanguinis*
SA – *S. aureus*
PA – *P. aeruginosa*
Note the logarithmic scale (log10 axis).

All samples were analysed in triplicate and experiments repeated twice.

C
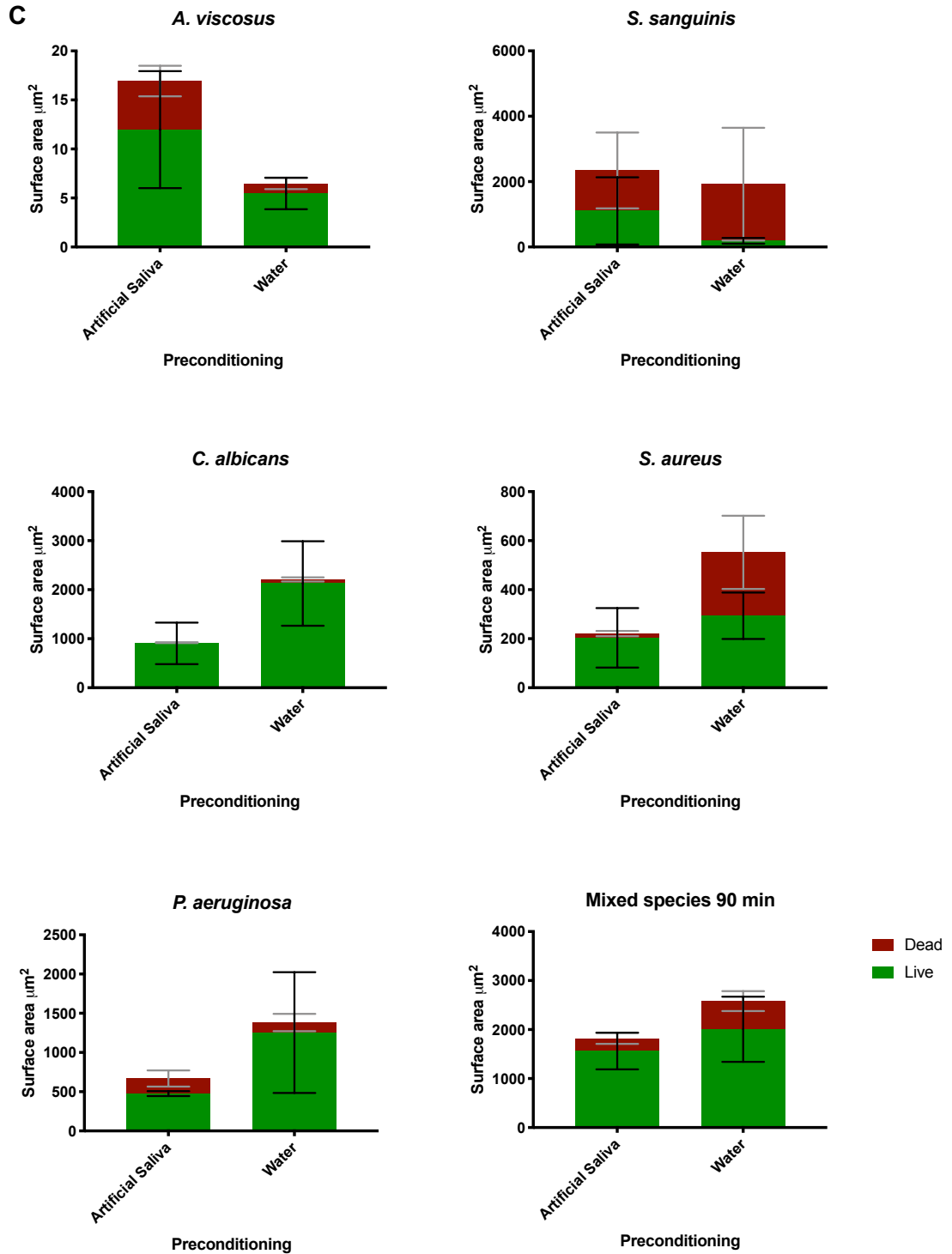
Figure 2.4.2C: Median surface area (μ m²) occupied by microbes on acrylic surfaces rinsed 90 min after inoculation, detected by CLSM using Live/Dead stain.

Error bars represent standard deviation. All acrylic samples were imaged at five random locations, in triplicate with experiments repeated 2 times. Note that scales differ for each inoculum.
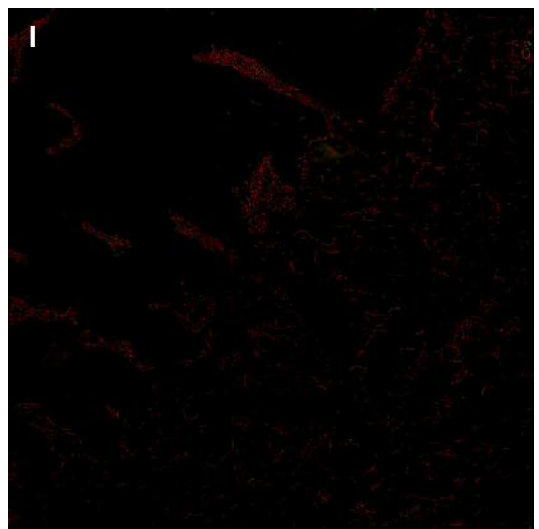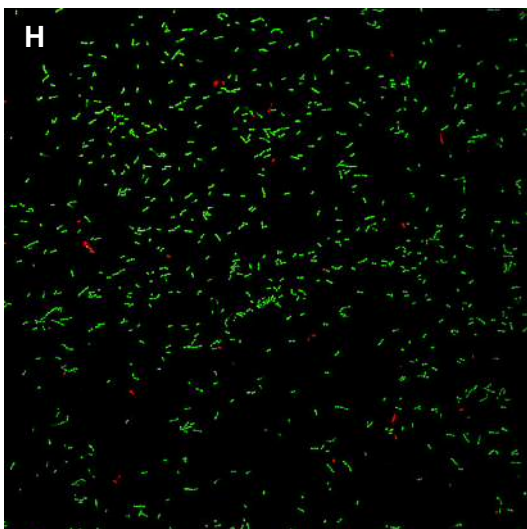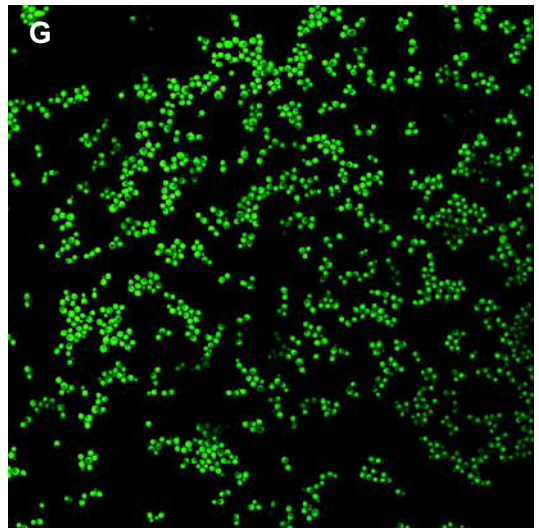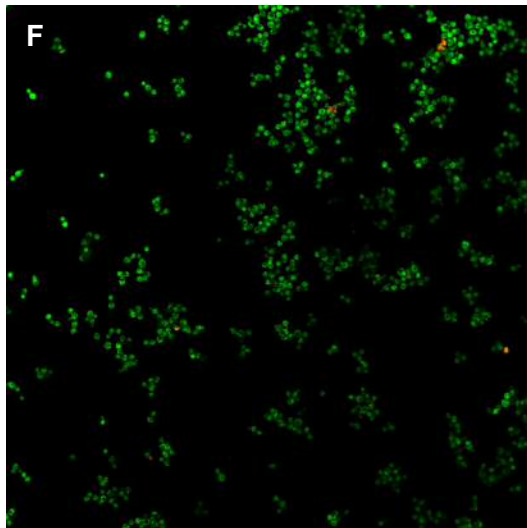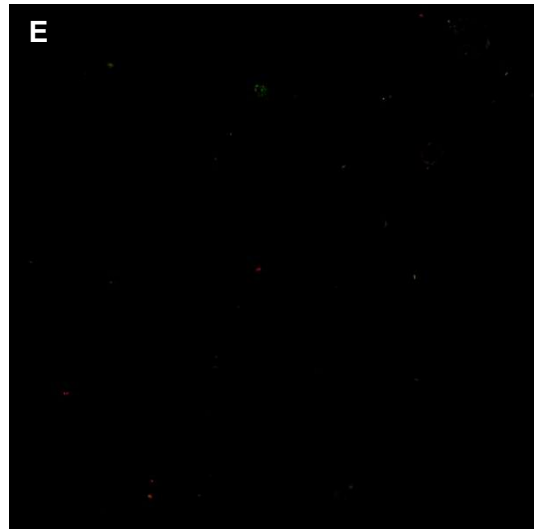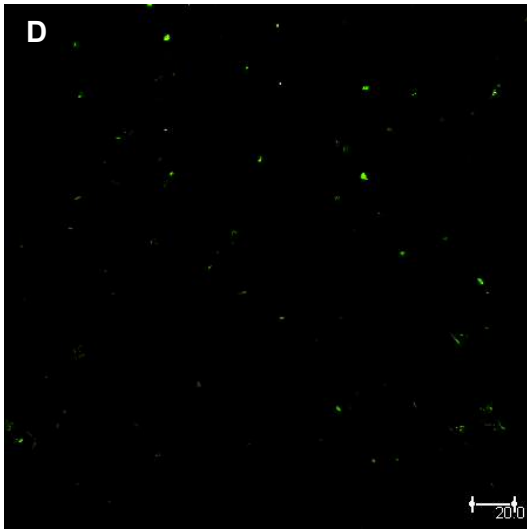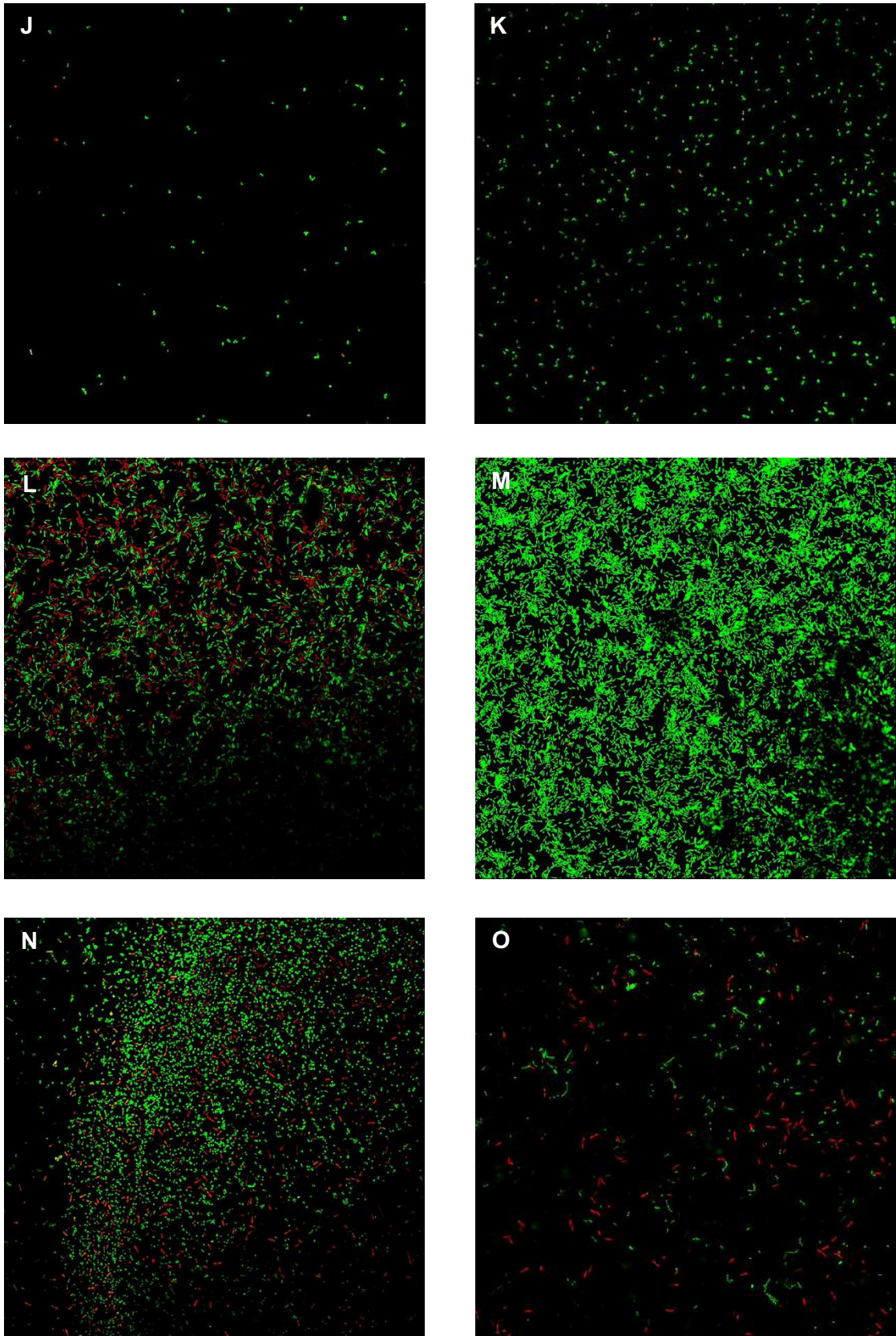
Figure 2.4.2: CLSM photomicrographs of microbial adherence assays on acrylic surfaces
D, E – *A. viscosus*　　**F, G –** *C. albicans*　　**H, I –** *S. sanguinis*　　**J, K –** *S. aureus*
L, M – *P. aeruginosa*　　**N, O –** Mixed species inoculum
D, F, H, J, L, N show microbes attached to acrylic surfaces preconditioned with artificial saliva
E, G, I, K, M, O show microbes attached to acrylic surfaces preconditioned with water

2.4.3: Characterisation of acrylic biofilms

2.4.3.1: Recovery of viable microorganisms by culture

Analysis of biofilms at 72 h revealed that the impact of preconditioning of acrylic surfaces on initial attachment and adherence patterns did not appear to affect subsequent biofilm formation. For both single species (Figure 2.4.3.1A) and mixed species (Figure 2.4.3.1B) inocula, there was no difference in recovery rate from acrylic biomaterial surfaces preconditioned with AS or water for *A. viscosus, C. albicans, S. sanguinis* or *S. aureus.*

In contrast to findings after 90 min of adherence, *P. aeruginosa* biofilms yielded a lower CFU recovery from AS preconditioned acrylic surfaces, compared to water, although the effect size was small. However, in mixed species biofilms, this trend was reversed, with increased recovery from AS preconditioned acrylic. Again, the effect size was relatively small, although consistent across samples. As was previously noted, it was not possible to identify viable *C. albicans* from mixed species biofilms on SDA, due to overgrowth of *P. aeruginosa* on these agar plates.
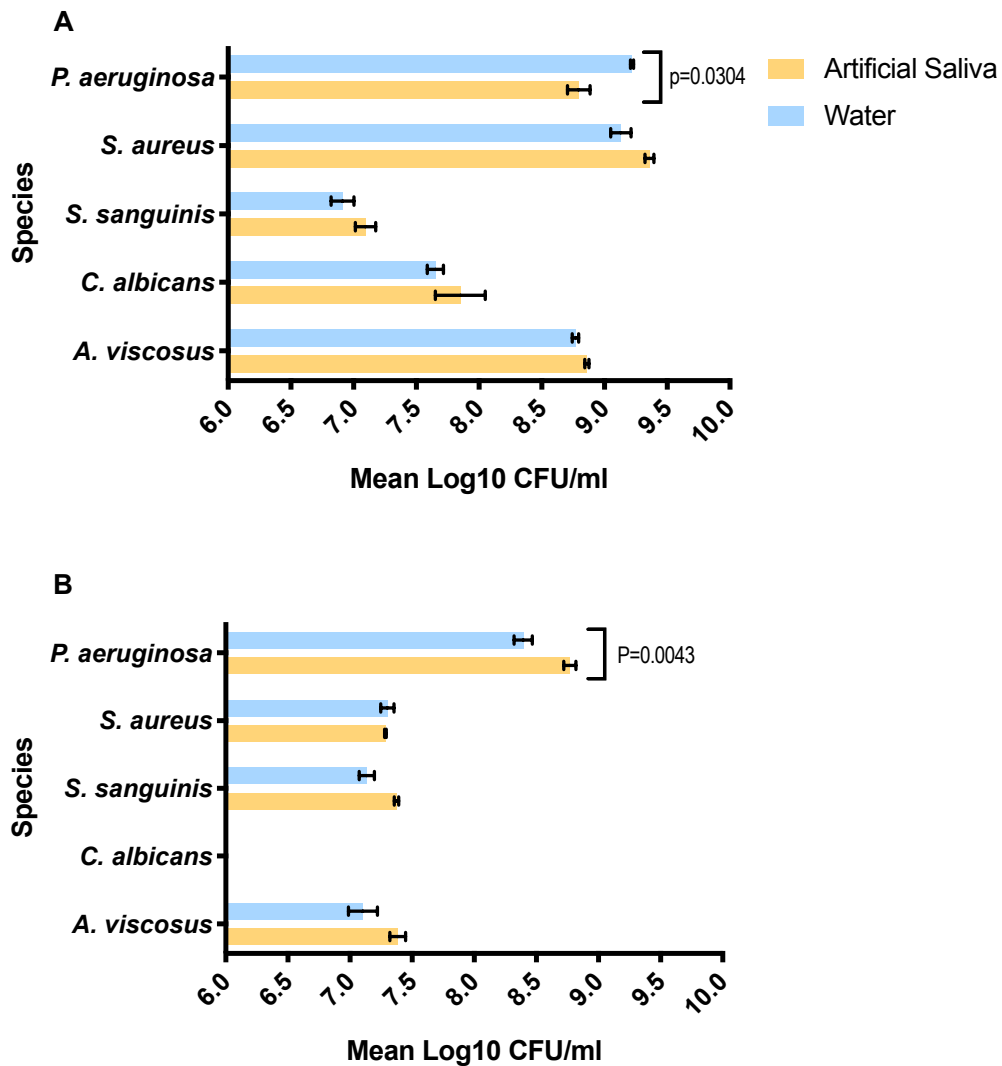
Figure 2.4.3.1: Mean colony forming units recovered from 72 h biofilms grown on acrylic surfaces

A)   Single species inocula
B)   Mixed species inocula
AV – *A. viscosus*
CA – *C. albicans*
SS – *S. sanguinis*
SA – *S. aureus*
PA – *P. aeruginosa*
Note the logarithmic scale (log10 axis).

All samples were analysed in triplicate and experiments repeated twice.

2.4.3.2: Quantification of Live/Dead-stained biomass viewed by CLSM with COMSTAT 2.0

As was found in earlier sections, quantitative analysis of CLSM images appear to corroborate culture studies. There was no clear difference in biomass within biofilms

grown on acrylic biomaterial surfaces preconditioned with either water or AS (Figure 2.4.3.2A). Approximately 50% of microbes were viable in image analysis in the case of *A. viscosus, C. albicans* and mixed inocula. Both *P. aeruginosa* and *S. aureus* showed a smaller proportion of viable biomass (approximately 30%), while *S. sanguinis* biomass was comprised of around 60% viable cells. Mixed species biofilms had reduced biomass compared to single species biofilms in the case of *A. viscosus, P. aeruginosa* and *S. aureus*, and were approximately equivalent in biomass to single species biofilms of *C. albicans* and *S. sanguinis.* Example 3D projections of z-stacks recorded of mixed species biofilms are displayed in Figures 2.4.3.2B and C.
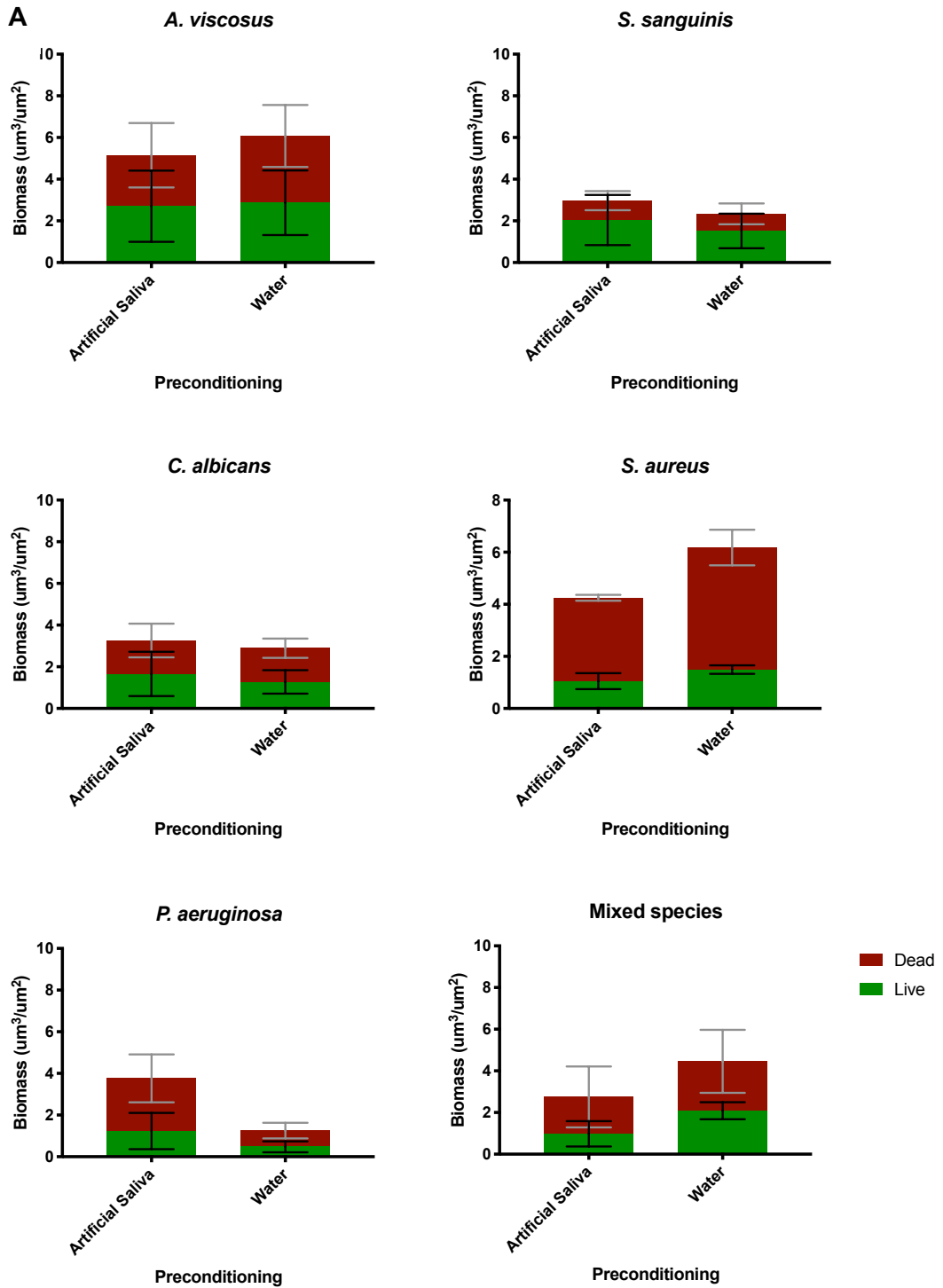
Figure 2.4.3.2A: Median biomass (μ m^3/μ m^2) within 72 h biofilms grown on acrylic surfaces, detected by CLSM using Live/Dead stain.

Error bars represent standard deviation. All acrylic samples were imaged at five random locations, in triplicate. With experiments repeated 2 times.
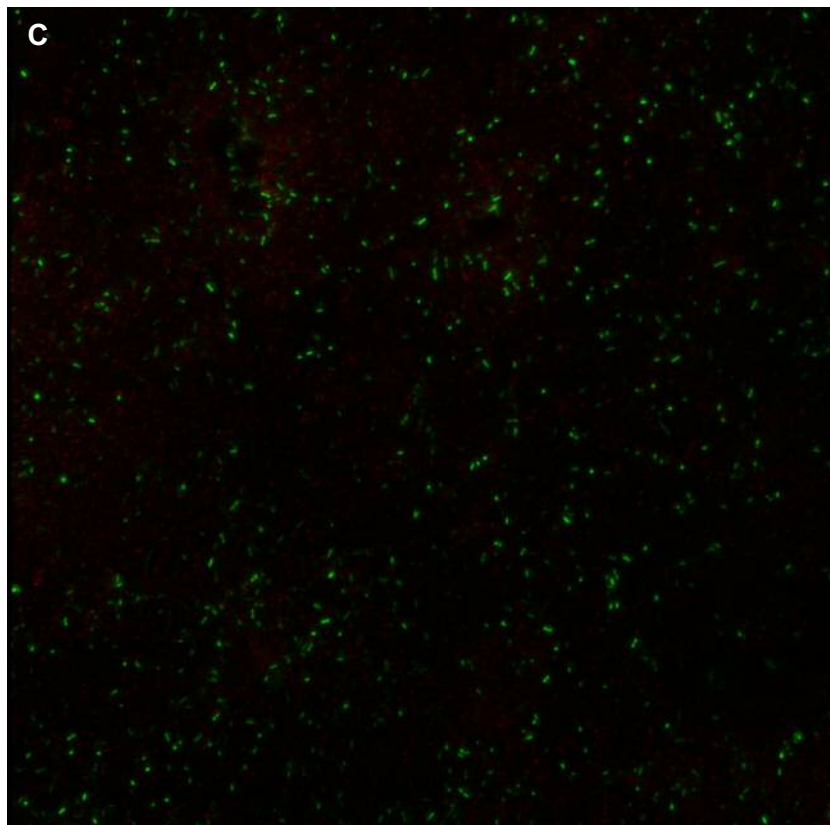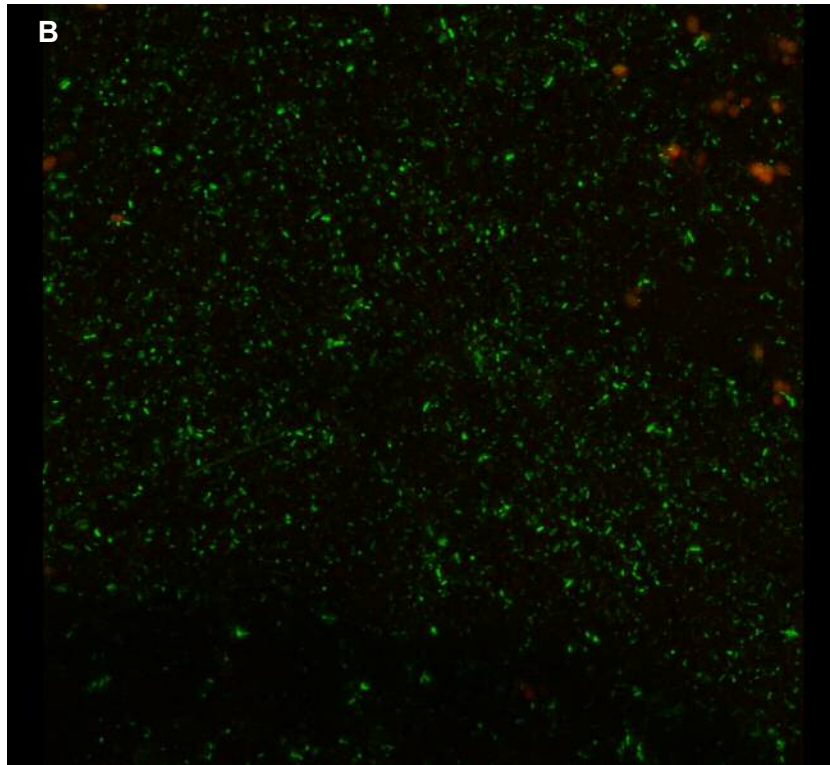
Figure 2.4.3.2B and C: (filtered) 3D projections of mixed species biofilms captured by CLSM

B) Mixed species 72 h biofilm grown on acrylic biomaterial surface preconditioned with artificial saliva

C) Mixed species 72 h biofilm grown on acrylic biomaterial surface preconditioned with water

2.4.3.3: Validation of culture recovery of viable organisms

To establish the validity of vortex recovery of viable microbes, mixed species biofilms were grown and treated using the protocol in section 2.3.8.1. Subsequently, remaining biofilm cells were stained with Live/Dead stain and analysed as per section 2.3.8.2. This demonstrated that the majority of biofilm cells were removed during vortex treatment and showed no preferential retention of viable cells from either AS or water preconditioned acrylic biomaterial surfaces (Figure 2.4.3.3).
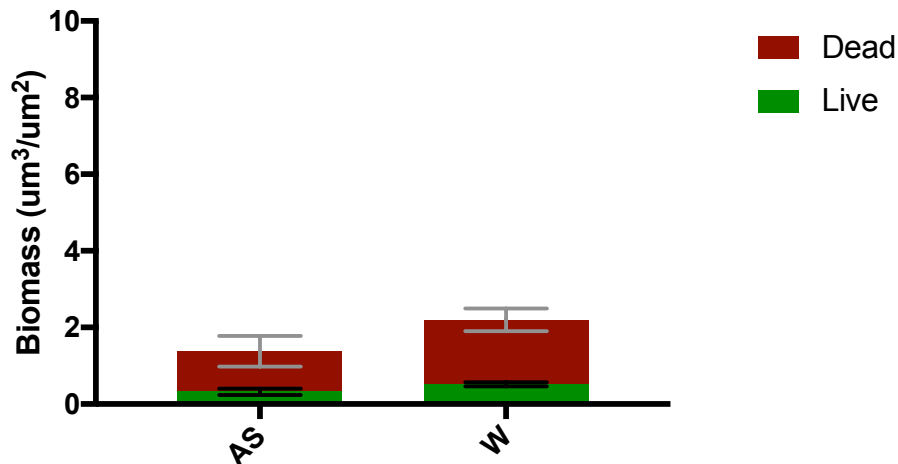
Figure 2.4.3.2: Median biomass (μ m³/μ m²) within 72 h biofilms grown on acrylic surfaces, detected by CLSM using Live/Dead stain.
Error bars represent standard deviation. All acrylic samples were imaged at five random locations, in triplicate with 2 experimental repeats.

2.4.3.4: Species distribution within biofilms detected by PNA-FISH and CLSM

S. aureus, P. aeruginosa and *C. albicans* were successfully labelled by PNA-FISH probes (Figure 2.4.3.4 A-L). PNA-FISH labelling of biofilms generated on acrylic coupons demonstrated colocalization of both *P. aeruginosa* and *S. aureus* with the representative 'commensal' oral microbes *S. sanguinis* and *A. viscosus* (Figure 2.4.3.4 D, H, L). Overall, there was limited identification of *C. albicans* (Figure 2.4.3.4 B, D), which meant assessment of the spatial distribution of this species within the biofilm was not possible.

Biofilms appeared to be predominated by *P. aeruginosa*, which tended to occupy the outer layers of the biofilm (Figure 2.4.3.4 H, L). Meanwhile the remaining bacteria were predominantly clustered in deeper biofilm regions and were not homogenously distributed throughout.

(Overleaf) Figure 2.4.3.4: Photomicrographs of PNA-FISH labelled mixed species biofilms generated on acrylic biomaterial surfaces, imaged using confocal laser scanning microscopy.

Red: universal bacterial probe

Green: *P. aeruginosa* specific probe & *C. albicans* probe

Blue: *S. aureus* specific probe

A) Universal bacterial probe only

B) *P. aeruginosa* and Yeast Traffic Light probe only (*C. albicans* indicated by arrows)

C) *S. aureus* specific probe

D) Composite, multi-channel image with all probes

E) Universal bacterial probe only

F) *P. aeruginosa* and Yeast Traffic Light probe only

G) *S. aureus* specific probe

H) Composite, multi-channel image with all probes – note the localisation of *P. aeruginosa* towards outer layers, with centralised clusters of oral bacteria and S. aureus coaggregates

I) Universal bacterial probe only

J) *P. aeruginosa* and Yeast Traffic Light probe only

K) *S. aureus* specific probe

L) Composite, multi-channel image with all probes – note coaggregates of oral bacteria and *S. aureus*
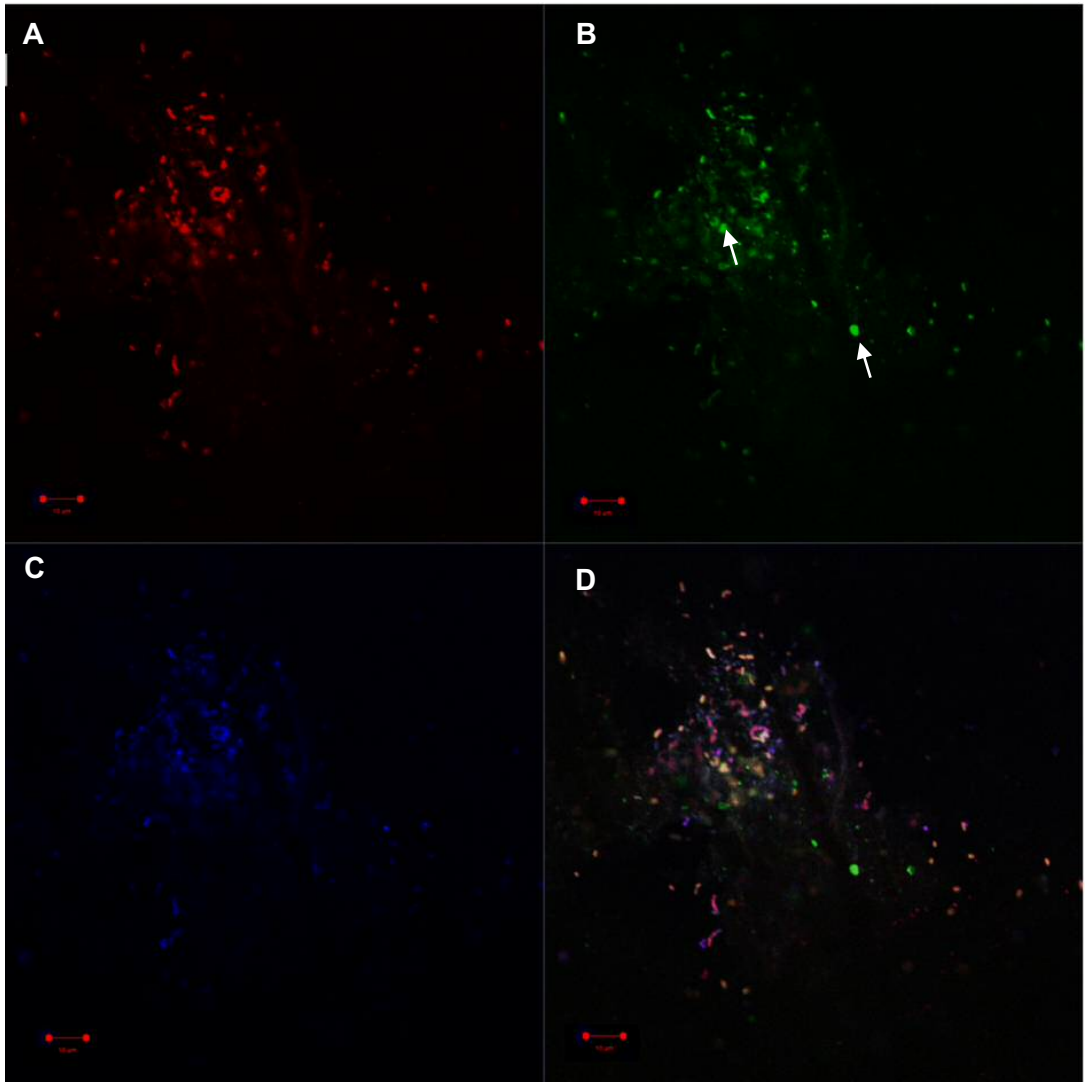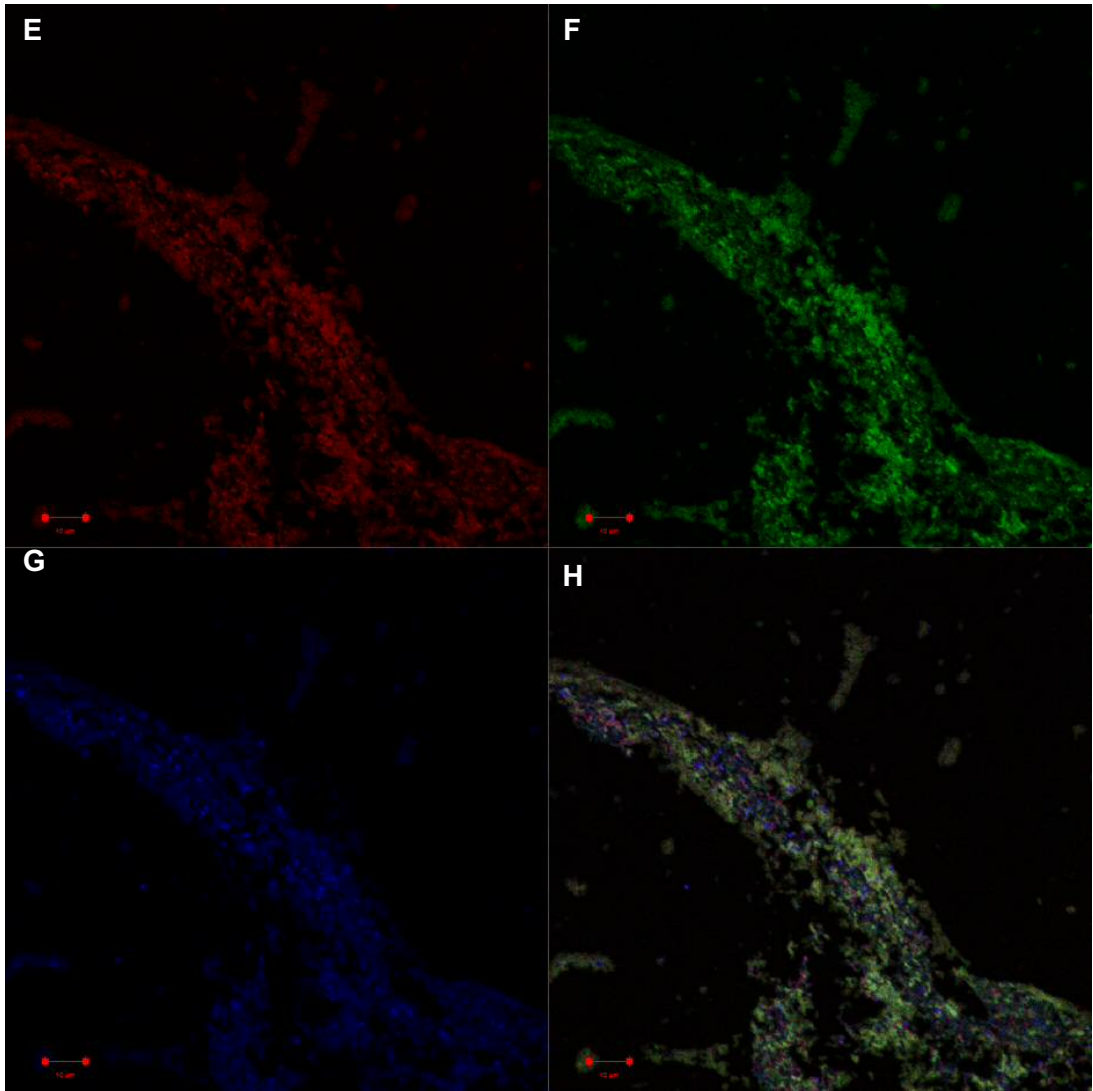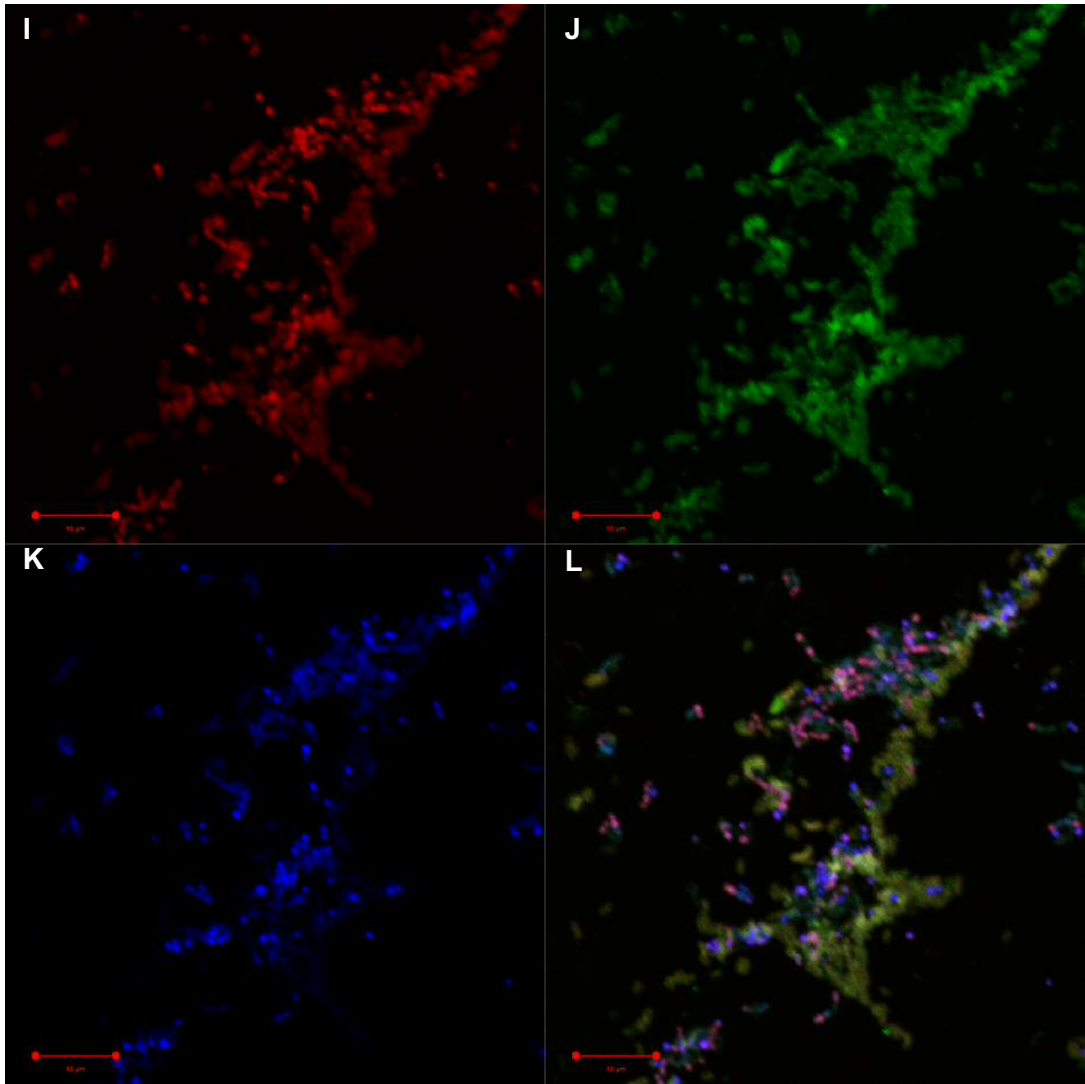
2.4.3.5: SEM imaging of biofilms

Examination of scanning electron photomicrographs at low magnification demonstrated that the majority of the acrylic coupon surface was coated with biofilm (Figure 2.4.3.5A). Images at higher magnifications (Figure 2.4.3.5B &C) revealed the morphology of biofilms more clearly, including areas of sparse coverage, which may indicate channels within the biofilm. In the most densely populated regions of the biofilm, individual microbial cells were not clearly demarcated, which may indicate extracellular polymeric substrate encasing the cells.
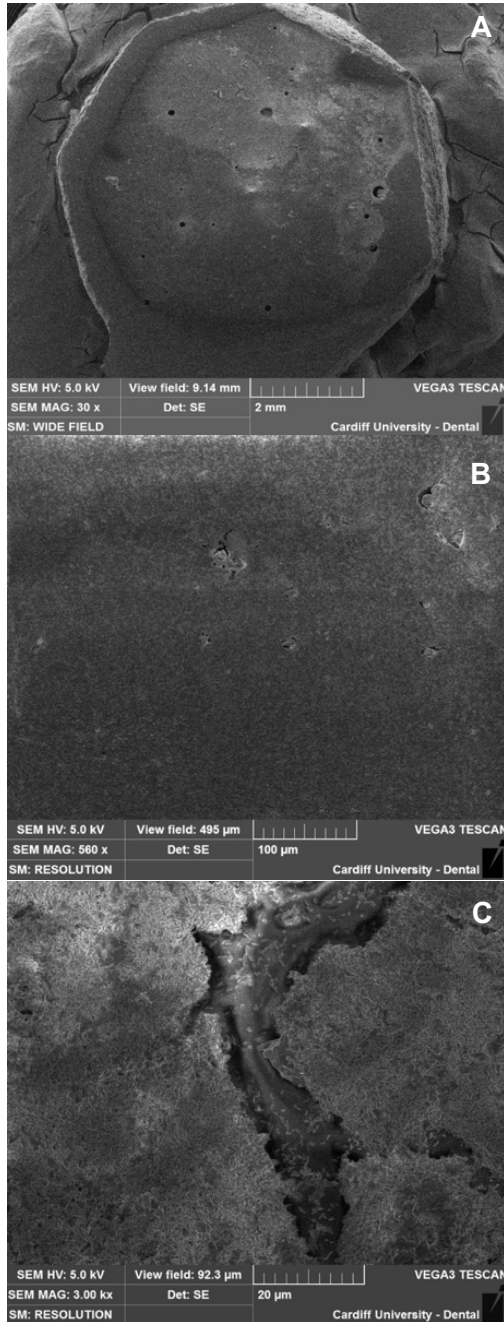
Figure 2.4.3.5: Scanning Electron Photomicrographs of biofilms on acrylic coupon surfaces

A) 30x view demonstrating biofilm distribution across acrylic coupon surface
B) 560x view demonstrating abiotic spaces within biofilm, indicating possible water channels
C) 3000x view demonstrating example of abiotic region within biofilm. Note in dense biofilm regions, microbes are not well demarcated, suggestive of extracellular polymeric substrate coating.

2.4.4: qPCR analysis of gene expression by *P. aeruginosa* and *S. aureus* - influence of oral commensal microbes

2.4.4.1: Validation and optimisation of RNA extraction method – RNA purity and concentration assessed by nanodrop

The methods of RNA extraction used resulted in different yields and quality of RNA elute from microbial biofilms (Table 2.4.4.1). The novel combination of the RNA FastPro Blue kit with a secondary Qiagen clean-up kit, both used according to the manufacturer's instructions, was the only method that produced RNA of suitable purity (determined by A260/280 and A260/230 ratios over 2.0), with an adequate RNA yield for subsequent gene expression analysis.

Table 2.4.4.1: Quantity and quality of microbial RNA eluted from biofilms using different methods of RNA extraction, as determined by Nanodrop

Method	Conc. ng/mL (Median, MAD)	A260/280 (Median, MAD)	A260/230 (Median, MAD)
Qiagen RNeasy Kit	64 (34)	1.96 (0.133)	0.345 (0.244)
Modified trizole/RNeasy method	47.5 (32)	2.0535 (0.218)	0.184 (0.104)
RNA FastPro Blue Kit	451 (155.3)	1.659 (0.089)	0.6245 (0.091)
RNA FastPro Blue + Qiagen Cleanup	204.8 (84.4)	2.029 (0.035)	2.073 (0.257)

2.4.4.2: Validation and selection of reference genes – efficiency and stability of expression

There was relatively little variation between reference gene efficiency within single, dual and mixed species biofilms for all *P. aeruginosa* primer pairs analysed (Table 2.4.4.2A) and *S. aureus* primer pairs excluding rho (Table 2.4.4.2B). Of the *P. aeruginosa* primer pairs, only proC and rpoD demonstrated reasonable efficiency (~90%) across all experimental conditions. The expression of rpoD across conditions was found to be more stable than that of proC following analysis by the Normfinder add-in for Microsoft Excel (Anderson et al. 2004), and so was selected as the reference gene for all experiments.

For *S. aureus*, 4 primer pairs showed approximately 90% efficiency, while pyk performed substantially better, with 98% overall efficiency. The gene pyk was found to be most stably expressed across all experimental conditions and so was selected

as the reference gene for subsequent qPCR experiments. However, it should be noted that the overall stability of all reference genes analysed was relatively low.

Table 2.4.4.2A: Efficiencies and stability of *P. aeruginosa* reference genes screened

Gene	Mean Primer Efficiency (SEM)			
	Single species	**Dual species**	**Mixed species**	**Overall**
proC	1.87 (0.027)	1.93 (0.019)	1.90 (0.014)	1.90 (0.013)
rpoD	1.90 (0.025)	1.91 (0.016)	1.85 (0.006)	1.89 (0.012)
fabD	1.78 (0.020)	1.83 (0.028)	1.80 (0.014)	1.80 (0.013)
ampC	1.87 (0.020)	1.86 (0.016)	1.83 (0.008)	1.85 (0.009)
pbp2	1.68 (0.004)	1.64 (0.017)	1.62 (0.007)	1.64 (0.009)

Table 2.4.4.2B: Efficiencies and stability of *S. aureus* reference genes screened

Gene	Mean Primer Efficiency (SEM)			
	Single species	**Dual species**	**Mixed species**	**Overall**
pyk	1.99 (0.030)	1.98 (0.021)	1.96 (0.015)	1.98 (0.010)
rho	1.58 (0.29)	1.54 (0.27)	1.73 (0.12)	1.61 (0.093)
16S	1.76 (0.056)	1.81 (0.057)	1.68 (0.041)	1.75 (0.034)
gyrB	1.96 (0.025)	1.89 (0.037)	1.90 (0.029)	1.90 (0.021)
rpoB	1.95 (0.014)	1.90 (0.031)	1.84 (0.017)	1.88 (0.018)
revA	1.984 (0.006)	1.92 (0.037)	1.85 (0.036)	1.90 (0.025)
ftsZ	1.92 (0.027)	1.90 (0.23)	1.83 (0.043)	1.88 (0.022)

2.4.4.3: Validation of primers – original primer efficiency

A number of primers identified from published research assessed for amplification efficiency of *P. aeruginosa*, dual species and mixed species biofilms. Despite most primers showing reasonable amplification efficiency (Table 2.4.4.3), there appeared to be poor primer specificity when melt curve analysis was undertaken. Consequently, primers were redesigned from first principles as described in section 2.3.8.8.

Table 2.4.4.3A: Primers initially screened for detection of *P. aeruginosa* gene expression

Gene of interest	Mean Primer Efficiency	SEM
rhlI	1.80	0.026
rhlR	1.84	0.018
phzA	1.78	0.017
pelA	1.89	0.010
pelD	1.71	0.035
lasR	1.89	0.023
lecA	1.80	0.047
lecB	1.69	0.057
pslA	1.93	0.017
pslD	1.91	0.010
rpoD	1.87	0.021

The primers used in *S. aureus* biofilm experiments gave no clear amplification product as determined by melt-curve analysis, and so it was not possible to calculate amplification efficiencies. Again, primers were redesigned from first principles using the process outlined in section 2.3.8.8.

2.4.4.4: Validation of primers – redesigned primer efficiency

The amplification efficiency of the newly designed primers was again assessed and combined with melt curve analysis. Overall there was a substantial improvement in primer efficiency, although this tended to vary substantially across samples and experimental conditions. As the Pfaffl method corrects for primer efficiency when calculating changes in gene expression, a less parsimonious amplification efficiency threshold of 75-125% was applied for subsequent analysis of gene expression. Despite this, 4 primer pairs fell below this threshold, and thus data for the *P. aeruginosa* genes exoS, phzG, lasI and pelD were discarded due to poor reliability.

Table 2.4.4.4A: Redesigned primers for detection of *P. aeruginosa* gene expression

Gene of interest	Mean Primer Efficiency	SEM
aprA	1.85	0.016
algG	1.75	0.009
algE	1.75	0.010
*exoS**	1.50	0.094
exoT	1.81	0.030
exoY	1.93	0.023
*phzG**	1.08	0.015
*lasI**	1.55	0.012
pslA	1.92	0.017
lasR	1.90	0.026
pelA	1.89	0.010
*pelD**	1.70	0.036
algU	1.88	0.017
exoU	1.91	0.011
lecA	1.81	0.046
*lecB**	1.67	0.072
*toxA**	1.50	0.097
phzA	1.85	0.011
rhlR	1.91	0.007
*lasB**	1.57	0.059
pslD	1.90	0.012

*amplification efficiency too low and thus excluded from further analysis.

Table 2.4.4.4B: Redesigned primers for detection of *S. aureus* gene expression

Gene of interest	Mean Primer Efficiency	SEM
icaA	1.91	0.016
icaB	1.90	0.015
icaC	1.93	0.022
icaD	1.86	0.019
fnbAB	1.96	0.030
clfA	2.06	0.020

2.4.4.5: Impact of single species or mixed species interactions on *P. aeruginosa* and *S. aureus* gene expression

The majority of Pseudomonal genes analysed demonstrated a trend of decreased expression levels in dual species and mixed species biofilms compared to single species Pseudomonal biofilms (Figure 2.4.4.5A). Only in the case of exoU, exoY, algE and aprA did the decrease in gene expression reach the threshold of statistical significance. One notable exception to this trend was lecA, which showed an almost 4-fold increase in expression, which was statistically significant. There was substantial variation in the levels of gene expression, as indicated by the wide error bars, and so any inferences made from such data must be guarded at best.

Similarly, *S. aureus* gene expression was reduced or unchanged in mixed species biofilms when compared to single species biofilms (Figure 2.4.4.5B). Several genes showed a slight increase in expression level in dual species biofilms. However, the high variability of expression and small magnitude of effect renders the presence of any biological significance in such trends improbable. In keeping with this inference, no gene analysed demonstrated any statistically significant change in expression across the different experimental conditions analysed. The corresponding Ct values for *P. aeruginosa* and *S. aureus* are displayed in Figure 2.4.4.5C and D.
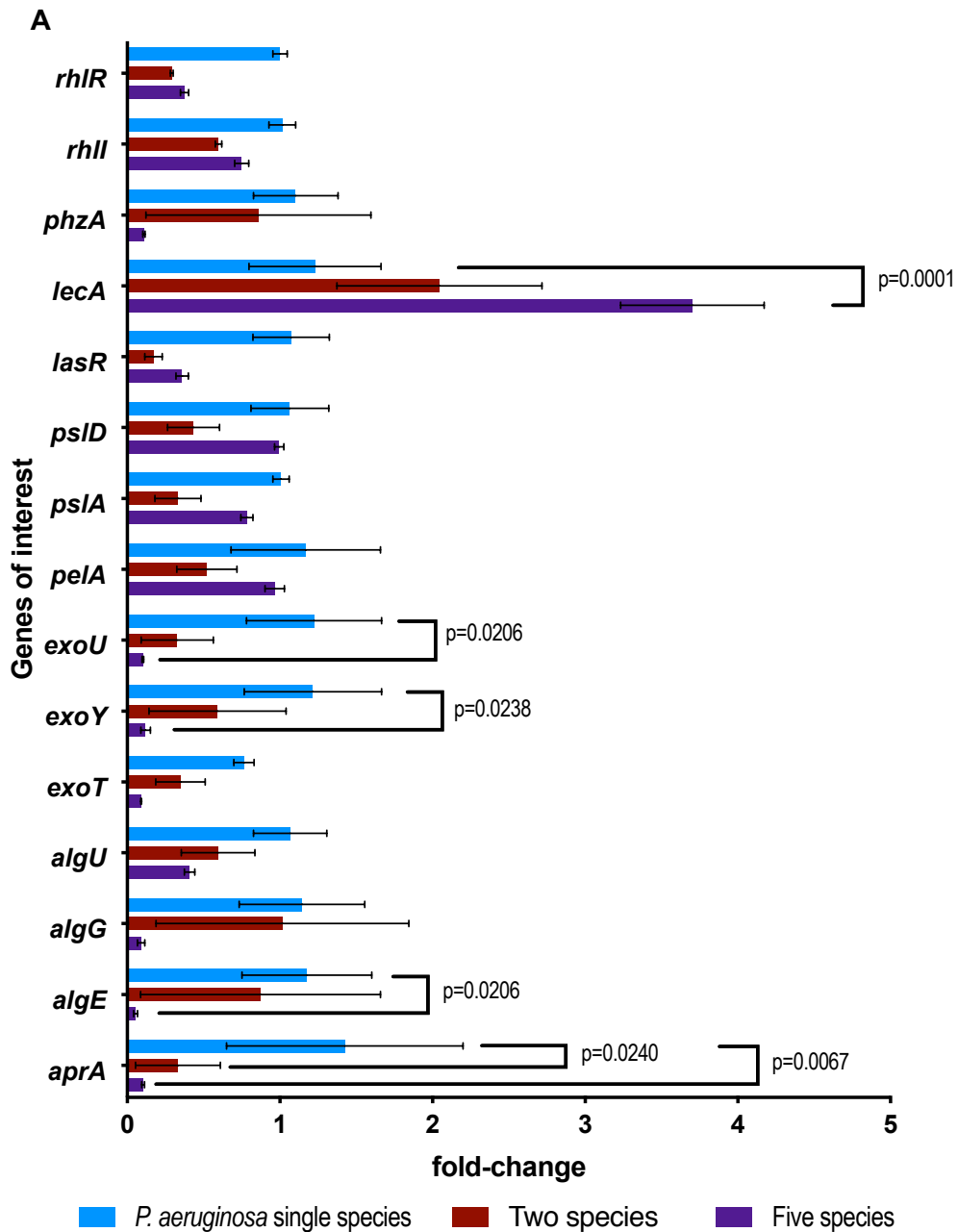
Figure 2.4.4.5A: Differential *P. aeruginosa* gene expression in single, two species (*S.aureus* and *P. aeruginosa*) and five species biofilms. Fold-change normalised to rpoD reference gene shown. All experiments were performed in duplicate wells, with 2 experimental repeats. 2-way repeated measures ANOVA was performed on the difference in fold-change for each gene examined, using *P. aeruginosa* single species biofilms as control group.

P values shown are adjusted significance using the two-stage step up method of Benjamini, Krieger and Yekutieli to control the false discovery rate (FDR). Q = 0.05.
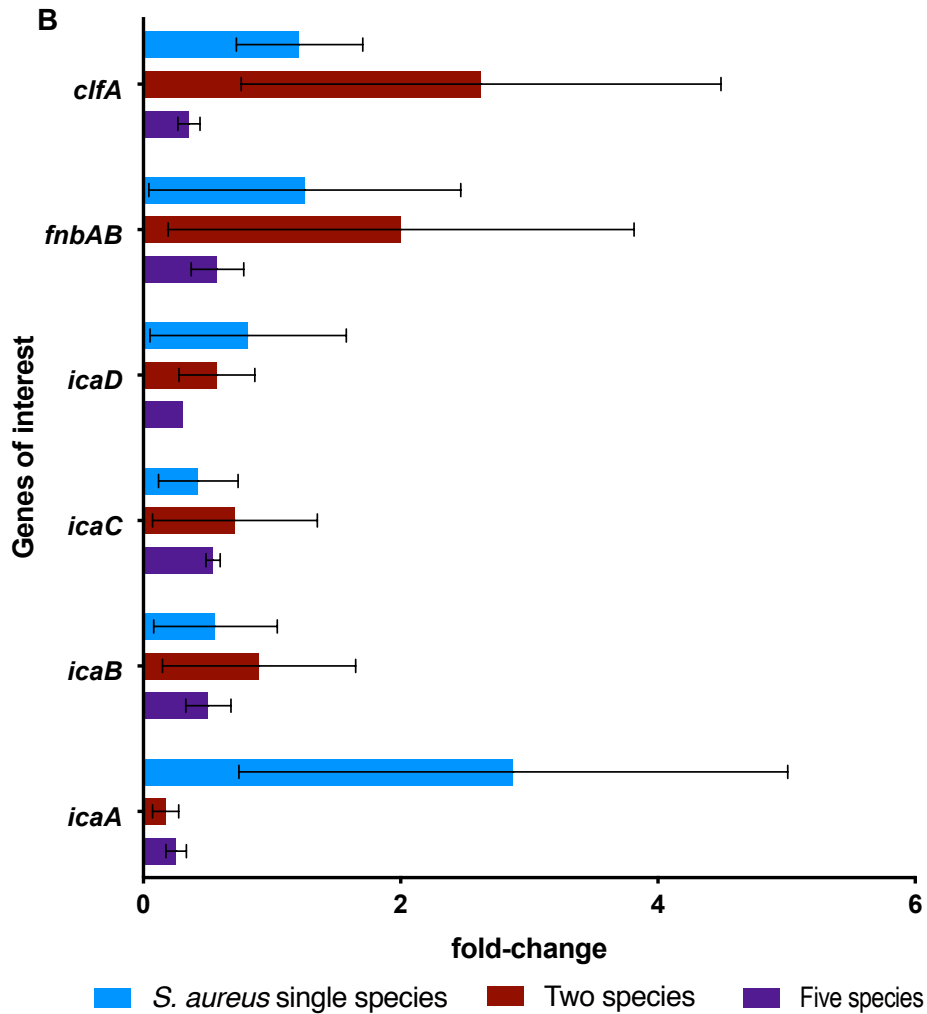
Figure 2.4.4.5B: Differential *S. aureus* gene expression in single species, two species (*S. aureus* and *P. aeruginosa)* and five species biofilms. Fold-change normalised to pyk reference gene shown. All experiments were performed in duplicate wells, with 2 experimental repeats. 2-way ANOVA was performed on the difference in fold-change for each gene examined, using *S. aureus* single species biofilms as control group. P values shown are adjusted significance using the two-stage step up method of Benjamini, Krieger and Yekutieli to control the false discovery rate (FDR). Q = 0.05.

Figure 2.4.4.5C: Differential *P. aeruginosa* gene expression in single, two species (*S.aureus* and *P. aeruginosa*) and five species biofilms. Ct values for genes shown in Figure 2.4.4.5A.

D

Genes of interest (y-axis)

Gene expression (Ct value) (x-axis)

Legend: *S. aureus* single species | Two species | Five species
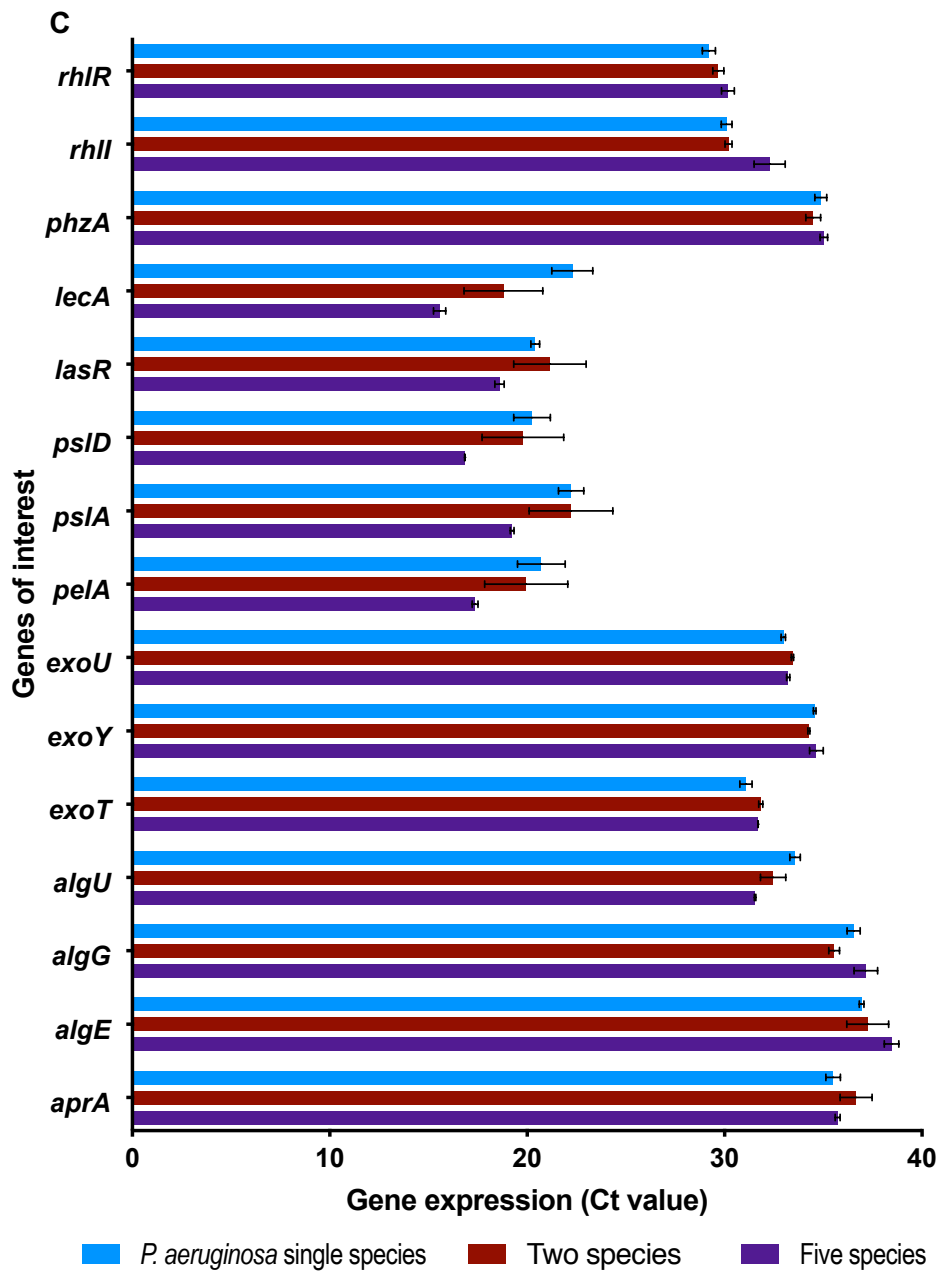
Genes (top to bottom): *clfA*, *fnbAB*, *icaD*, *icaC*, *icaB*, *icaA*
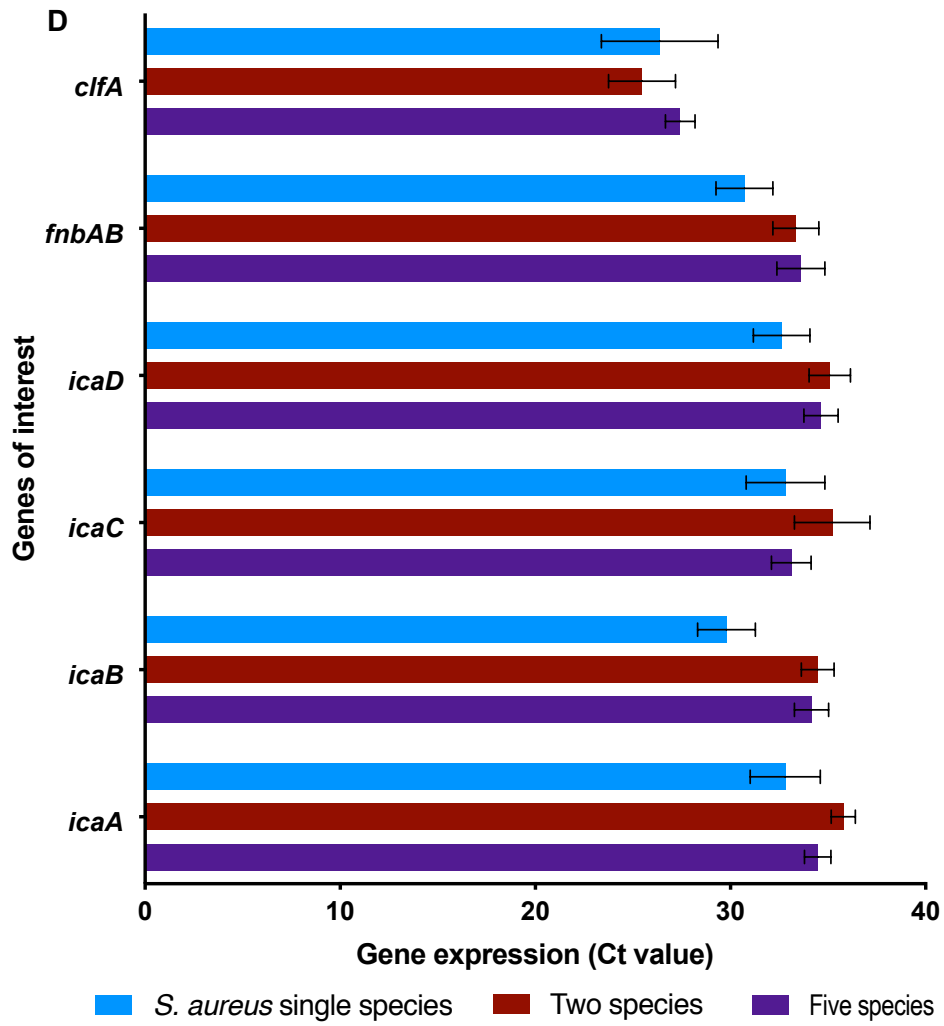
Figure 2.4.4.5D: Differential *P. aeruginosa* gene expression in single, two species (*S.aureus* and *P. aeruginosa*) and five species biofilms. Ct values for genes shown in Figure 2.4.4.5B.

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2.5: Discussion

This work package describes the development of a simple biofilm model on denture acrylic biomaterial surfaces, including both species considered to be commensal, normal oral microorganisms, and two well-established putative respiratory pathogens, which are also implicated in a wide range of ancillary healthcare associated infections. This model has undergone extensive characterisation as described within this chapter and thus provides a baseline for subsequent chapters investigating the impact of biofilm-mediated infections of tissue models and for testing of novel antimicrobial strategies.

The advantage of the batch system employed in generating such biofilms for subsequent work is primarily the high-throughput capability of the system. This is invaluable when, as is the case for Chapters 5 and 6, a wide range of experimental conditions are screened to establish maximal efficacy. However, it should be noted that this method of biofilm development suffers from important limitations that may bias the results obtained. The simplicity of this system is a double-edged sword, as gains in throughput are tempered by reduced biological relevance: a sessile inoculum which is steeped in a highly nutrient rich growth medium, without exposure to shear stress, extensive starvation, and seeded at high concentrations which may themselves impact subsequent biofilm development through population-dependent quorum sensing mechanisms. It has consequently been noted that biofilms generated by this method typically show reduced organisation and quantity of the extra-cellular polymeric substrate matrix and are less recalcitrant to physical removal (McBain AJ 2009). The impact of such observations on clinical applications regarding antimicrobial resistance and interactions with host tissues is unclear at present. It could be conversely argued that the stagnant conditions of a batch system might better simulate the sheltered, nutrient poor niche on denture-fit surfaces. While shear and compressive forces within denture-associated biofilms may be high during masticatory function, this may be interspersed by long periods of low forces. The act of replenishing media exposes biofilms to brief shear flow during movement of the biofilm sample from the nutrient depleted well to a well with fresh media. This coincides with increased nutrients, which may mimic the increase in force and nutrients periodically encountered during eating.

In keeping with previous reports, including work from our group, there was no clear impact of initial patterns of attachment and adhesion on subsequent biofilm development (Cerca et al. 2005; Morse D 2017). Species which showed higher rates of initial attachment in mixed species biofilms, such as *S. sanguinis* to acrylic surfaces preconditioned with AS (Figure 2.4.1B) were outgrown by *P. aeruginosa* by 72 h

(Figure 2.4.3B) despite an approximately 2-log reduction in initial attachment rates by the latter. This may simply reflect inherent differences in growth rates between such microorganisms. The impact of conditions for growth may also play a role, as changing media every 24 h could allow substantial build-up of toxins and waste products, for which *P. aeruginosa* is well known to harbour a high level of tolerance (Purschke et al. 2012; Gelatly and Hancock 2013).

Similarly, despite impacting initial attachment and adhesion of representative oral bacteria in mixed biofilms, there was a loss of the impact of preconditioning acrylic biomaterial surfaces with AS (Figures 2.4.1 - 2.4.3). This again is unsurprising, as only the deepest layer of cells within a biofilm actually contact the surface, and plausible coaggregative interactions between all microorganisms included in this biofilm model have been described in the introduction to this chapter.

An important consideration when assessing the validity of culture-dependent studies of biofilms is the ability to remove microbial cells from a surface (Portillo et al. 2013). Consequently, biofilms grown on acrylic surfaces were processed as if for culture, labelled with the BacLight Live/Dead stain, and the surfaces then examined using CLSM to assess both the total biomass and quantity of viable cells that remained adherent to each surface. While a substantial biomass remained following vortex processing of acrylic coupons, the majority of the retained microbes were non-viable. Importantly, there were no significant differences between either total microbial biomass or viable microbial biomass for acrylic coupons preconditioned with water or AS, which eliminates the possibility of differential recovery of microorganisms biasing the results of culture analysis in favour of either condition. A factor which remains unaddressed in these characterisation experiments is the possibility of incomplete penetration of either SYTO-9 or propidium iodine reagent in certain regions of the biofilm, which could similarly influence results. However, this method is widely employed in biofilm studies undertaken across a range of settings and fields, despite limited validation of its sensitivity and specificity in such applications (Netuschil et al. 2014).

Examination of the spatial orientation and potential co-localisation of microbial species using species specific, fluorophore-conjugated PNA probes with FISH, provides some insight into microbial interactions within biofilms. While there was considerable heterogeneity, *P. aeruginosa* was seen to preferentially occupy the outer biofilm layers in most micrographic sections (Figure 2.4.3.1 E-L), while *S. aureus* and *S. sanguinis* (identified by its classic coccal chain appearance in images) appeared to cluster more centrally. This likely reflects the aerophilic nature of *P.* aeruginosa compared with the facultative anaerobic nature of the other

microbes. It was not possible to readily identify *A. viscosus* within photomicrographic images, but the above logic suggests that this likely occupied positions within the deeper biofilm layers where oxygen tension was lowest. It should be noted when evaluating such images that the methods used to ensure hybridisation of PNA probes require harsh enzymatic treatment and prolonged, elevated temperatures. These will damage or denature biofilm structures, and may even affect cell behaviour and orientation, and so the observations gleaned from such images must be extrapolated with caution. Nevertheless, the findings corroborate previous research examining the spatial localisation of *S. aureus* and *P. aeruginosa* in wound models.

In keeping with the majority of published studies attempting to explore gene expression within biofilms; particularly in the case of *S. aureus*, extraction of sufficient quantities of good quality RNA and subsequent analysis of gene expression by qPCR was highly challenging (Cury and Koo 2007; Atshan et al. 2012; Heera et al. 2015). Consequently, extensive laboratory time, effort and resources were invested in attempting to optimise experimental conditions. This included trialling numerous methods and combinations thereof for RNA extraction, redesigning the majority of primers used from first principles, and using alternative methods of analysis for primer efficiency estimation (LinRegPCR rather than standard curves, which would have increased costs and laboratory time considerably).

An alternative approach could have been to focus on one or two target genes for *S. aureus* and *P. aeruginosa*, to ensure maximally robust data was generated. However, this would not be in keeping with the exploratory nature of this work segment, which was intended primarily to generate hypotheses for further exploration. To date, there have been few studies examining gene expression in mixed biofilm models incorporating *S. aureus* and *P. aeruginosa*. Certainly, none have evaluated the impact of incorporating these species within a biofilm model comprising normal oral microorganisms. Changes in gene expression and microbial behaviour are often strain-specific (Salama et al. 2000; Holden et al. 2004; Chugani et al. 2012). Thus, while the literature was consulted to identify target genes likely to be of relevance to colonisation of mucosal surfaces, biofilm formation and/or virulence-associated factors; it was felt that exploring a broad range of genes would provide the best insight into microbial behaviour when introduced to mixed species biofilms, and offer the greatest likelihood of identifying the pathways most strongly affected by such community interactions.

Of note, all qPCR plates demonstrated a low level of amplification of no template controls (NTCs). However, the levels noted were typically several cycles higher than gene expression levels in the template RNA wells, and subsequent melt-curve

analysis demonstrated the products responsible to have a lower melting temperature than the genes of interest. This is suggestive of primer dimer formation, rather than significant reagent contamination (Bustin and Huggett 2017).

The majority of *S. aureus* and *P. aeruginosa* genes analysed showed no statistically significant change in expression in dual species or mixed species compared to single species biofilms. However, there was an overall trend across most genes analysed towards decreased expression levels in dual and mixed species biofilms compared to when either microorganism was grown as a single species biofilm. This may in part reflect the loss of biomaterial surface area for each species (due to occupation by the other microbes) and concomitant increase in availability of alternative, adhesin-independent binding sites on each microbe's cell surface. It should also be noted that taken in conjunction with the findings of the PNA-FISH characterisation of biofilms and the localisation studies described in section 2.1.3, one reason for limited changes in gene expression in mixed compared with single species biofilms could be that there are relatively few *S. aureus* and *P. aeruginosa* cells directly interacting. As *P. aeruginosa* appears to preferentially occupy outer layers of the biofilm, while *S. aureus* tends to reside more centrally in microcolonies, only the outermost cells would potentially contact the innermost cells of *P. aeruginosa.* Although soluble signalling molecules such as those associated with quorum-sensing may exert some activity, by definition these molecules are produced in response to the immediate surrounding environment (De Kievit 2009), and so sequestration of each species within its own micro-niche could account for gene expression behaviour within mixed species biofilms which is, at the community-wide level, largely indistinguishable from monoclonal biofilms.

The pseudomonal lecA gene was increased in mixed biofilms. This encodes a glycoprotein-binding lectin receptor specific for galactoside moieties (Diggle et al. 2006). Unfortunately, expression of the lecB gene was not possible to assess due to poor primer efficiency (Table 2.4.4.4A) and specificity (data not shown). However, Pseudomonal coaggregation has been documented with oral microbes through (although not exclusively) lectin-mediated interactions (Komiyama et al. 1984, Komiyama et al. 1987). The increase in the expression of lecA could therefore be reasonably interpreted as a result of interspecies coaggregation interactions.

Limitations of the methods

It is important to emphasise that beyond the methodological challenges encountered in analysing microbial gene expression within biofilms, a number of systematic shortfalls of the experimental design and modalities employed within this work

package limit the confidence with which inferences can be drawn from the findings reported. Firstly, as has previously been mentioned, the use of a batch system to generate biofilms, while advantageous in terms of throughput, has limited relevance in healthcare associated infections, particularly to as dynamic an environment of the oral cavity and denture surface, which is constantly undergoing periods of nutrient deprivation, variable shear forces, changes in oxygen tension and temperature (when inserted or withdrawn from the oral cavity) and intermittent exposure to host defences and commensal microbiota of the oral cavity. Despite regular changes in growth media, a proportion of cells included within the "biofilms" on acrylic coupons are likely to be deposited through sedimentation from the overlying media and may confound all analyses presented.

The media used contained variable concentrations of glucose in single species biofilms; BHI contains approximately 11 mmol/L while the YNB used was supplemented with 100 mmol/L glucose. Physiological levels of fasting glucose in saliva are typically less than 1 mmol/L glucose, although this increases dramatically during meals (Bhattacharrya et al. 2018). Similarly, levels of electrolytes are much higher in growth media compared with oral fluids. Both factors are likely to have a substantial influence on biofilms development and may have affected the biofilm development seen. For mixed species biofilms, FAB was used as the growth medium. This contains no glucose and a very different concentration of other nutrients and ions compared with either BHI or YNB. This change in growth conditions may have exerted a stronger influence on growth rates and biofilm formation than interspecies interactions, and thus may have masked the true interactions between microbes.

Growth curves were not undertaken for the studied microorganisms. This may have influenced adhesion and biofilm assays as different growth rates during the 90 min initial incubation period might have influenced the levels of adhesion and subsequently biased biofilm composition. The strains employed, with the exception of *A. viscosus* were not originally isolated from the oral cavity, nor were the representative pathogenic strains originally isolated from pneumonia patients. Given the high degree of variability in behaviour possible between strains, the results of this work must be supported by replication in more biologically relevant strains. Similarly, because only one strain for each species was examined, it is not clear if this behaviour is generalisable across the species. Replication with a range of biologically relevant strains would be an important feature of any future work. Another aspect of future work, given the importance and ubiquity of *C. albicans* on denture surfaces, would be to ensure culture recovery methods employed in characterisation of the

biofilm model allow the detection of *C. albicans*. Simply adding an antimicrobial, such as ciprofloxacin which has good activity against *P. aeruginosa* could achieve this. Any imaging method is essentially destructive to the biofilm, alters and dehydrates the ultrastructure (in the case of SEM), or is actively cytotoxic (in the case of CLSM). The use of qPCR generates bias in results due to the restrictive nature of this technique. While more unprejudiced methods exist, which offer a more global picture of gene expression, such as microarrays or RNA sequencing, significant further work would be required to ensure robust and relevant biofilm generation, and consistent high-quality RNA extraction was undertaken prior to implementing these far more expensive methods. With the sheer multitude of RNA extraction methods available, each tailored to a specific species or even strain, this would likely require separate methods (or at least substantial modifications of a single method) for *P. aeruginosa* and *S. aureus*, as the challenges posed by the radically different biofilm matrix and cell wall composition may preclude a universally applicable workflow. Given the redundancy of gene expression, and the complexity of regulatory networks governing protein translation, even the most fastidious study of gene expression must be proceeded by an evaluation of resultant protein production to demonstrate biological relevance. This was – due to both financial and time constraints – beyond the scope of this body of work to examine.

Despite the limitations outlined above, this work package demonstrates the development of a mixed microbial biofilm on acrylic biomaterial surfaces which successfully incorporates putative respiratory pathogens. The extensive characterisation included provides a baseline for modelling biofilm-mediated infections in the following chapter, while the reliable generation of biofilms with a high-throughput capacity allows subsequent investigation of novel anti-biofilm strategies, such as the use of microwave frequency electric field described in Chapter 6. The methods used within this section could easily be applied to biofilm models of increased complexity, or even '*ex vivo*' models generated either by seeding biofilms with microbes recovered from the oral cavity by a number of means, or by the use of a removable oral device worn to generate a more clinical relevant biofilm, such as the enamel chips used in Palmer et al. (2001).

CHAPTER 3: HOST-PATHOGEN INTERACTIONS IN AN ACRYLIC BIOFILM ORAL INFECTION MODEL

3.1: Introduction

The diversity of microbial communities within the oral cavity, combined with complex immune responses driven by both local and systemic factors has led to the fields of microbiology and immunology, in many respects, developing in silos. More recently, recognition has grown of the importance of host-pathogen interactions in understanding biofilm-mediated infections (Stappenbeck and Virgin 2016); the ultimate goal being the development of personalised medicine, with individualised treatments based on a comprehensive understanding of both host and microbial factors (Wilson ID 2009). This has also been identified as a key area for improved patient care in dentistry (Zarco et al. 2012) and respiratory medicine (Rogers and Wesselingh 2016). Clearly integration across a range of medical and related fields would be necessary to truly deliver holistic personalised healthcare.

In the late 1990s, organotypic 3D tissue models of human epidermis, dermis and mucosa emerged as an alternative to animal models in research. These models were originally repurposed from biopsy-derived tissue sheets grown for treatment of burns and to be used as intra-oral grafts (Moharamzadeh et al. 2007). Such models afforded a number of advantages over animal testing; particularly higher throughput capacity at reduced cost, increased biological relevance and reliability for translation to clinical applications, and a means of avoiding the ethical quandaries of animal testing. While initially employed to test commercial products such as cosmetics for toxicity, corrosivity and other adverse effects, within a decade tissue models were being used as a means of investigating host-pathogen interactions in infection.

3.1.1: The use of 3-dimensional tissues to model infection

A number of groups have examined the pathogenesis of microbial infection using organotypic tissue models, with a diverse range of microbes, tissues, techniques and applications.

One of the most commercially successful applications of such model tissues has been in the production of epidermis, or dermis-epidermis (full-thickness) skin models. Such tissue models have gained wide acceptance for the testing of toxicity (Pampaloni et al. 2009), corrosion (Fentem et al. 1998), phototoxicity (Lelièvre et al. 2007) and irritation (Spielmann et al. 2007) of both pharmaceutical and cosmetic products. Subsequently, further protocols have been developed to investigate percutaneous absorption (Rozman et al. 2009) and to assess the effect of nanomaterials (Lee et al. 2009).

Early models of mucosal infection soon followed the increasing use of *in vitro* tissues. Of note, one of the first of such applications focussed on oral (and cutaneous) candidiasis (Korting et al. 1998; Schaller et al. 1998). This work helped to characterise the role of secreted aspartic proteinases (SAPs) in *Candida* virulence, through a combination of histopathological examination of sectioned infected tissues and qPCR analysis. The applicability of infection modelling was validated by the close morphological similarities between *in vitro* tissues and clinical biopsy samples from individuals with candidiasis. However, more recent analyses comparing cancer cell-derived tissue models (such as the SkinEthic reconstituted human oral epithelium model used in this chapter) with normal human keratocyte-based models has demonstrated the closer *in vivo* likeness of the latter over the former (Yadev et al. 2011).

More recently, an organotypic model of gingival tissue has been established to mimic periodontal pockets (Bao et al. 2015a). These tissues were grown in a perfusion bioreactor, to which the researchers subsequently added a hydroxyapatite disc supporting a 24 h mixed species biofilm including 11 microorganisms to represent both normal oral bacteria and periodontal pathogens. In addition to biofilm composition with and without the presence of the tissue models, the production of a number of important cytokines by the tissue models in response to infection was evaluated. In a subsequent paper, the same group reported the secreted proteome of both periodontal tissue models and microbial biofilms during infection (Bao et al. 2015b). The power of tissue models to drive hypothesis generation is thus likely to increase due to the enhanced capabilities of modern, 'omics' techniques to naively explore host-microbiome interactions in a high-throughput, reproducible and clinically relevant setting.

3.1.2: Interactions between the oral mucosa and denture acrylic biofilms

Much of the available literature exploring denture acrylic biofilms in oral mucosal infection has focussed heavily on the influence of *Candida* species, particularly *C. albicans* (Schaller et al. 1998; Schaller et al. 1999; Schaller et al. 2002; Bartie et al. 2004; Mostefaoui et al 2004; Moyes et al. 2010*)*. This is unsurprising, as oral candidiasis is a frequently encountered clinical problem in denture-wearing patients. Recent work from our research group (Morse et al. 2018) evaluated the impact of single species candidal biofilms or mixed species biofilms containing both *C. albicans* and representative commensal oral bacteria in denture acrylic biofilm tissue infection models. Interestingly, the degree of tissue damage (characterised by quantification of lactate dehydrogenase release) induced by *C. albicans* monospecies biofilms was

reduced compared to mixed bacterial/*Candida* biofilms in keratinocyte-only tissue models, but these results were equivocal in full-thickness tissue models. It was speculated that such differences may reflect the protective nature of the highly keratinised surface presented by full-thickness mucosal models. Additionally, the influence of other factors, such as the production of antimicrobial peptides which has been reported in full-thickness, but not keratinocyte-only, tissue models cannot be excluded.

The potential influence of putative respiratory pathogens within denture-associated biofilms has yet to be explored through oral mucosal tissue models. However, evidence from experiments evaluating microorganisms such as *P. aeruginosa* and *S. aureus* on mucosal models offers some insights into the potential impact that introducing these species to the denture microbiome may confer.

Tsang and colleagues (1994) examined the impact of a clinical isolate of *P. aeruginosa* in a resected adenoid tissue model. It was found that 8 h infection with *P. aeruginosa* led to substantial epithelial cell damage, and importantly that adherence by microbial cells was enhanced in areas of epithelial damage. *P. aeruginosa* appeared to preferentially bind to mucus, damaged epithelial cells which had extruded from the surface, and the basement membrane rather than the intact epithelial surface. These findings have subsequently been replicated and expanded upon, with a multifactorial range of mechanisms being shown to coalesce in facilitating enhanced *Pseudomonas* adherence and pathogenesis (Reviewed in Crabbé et al. 2014).

Co-infection of skin models by *S. aureus* and *P. aeruginosa* has been found to result in distinct patterns of localisation; with *S. aureus* tending to remain in the superficial epidermis, while *P. aeruginosa* invades into the lower dermis by 72 h (Shepherd et al. 2009). While limited evidence has shown that *P. aeruginosa* and *S. aureus* localise to occupy distinct spatial domains (DeLeon et al. 2014); there is a dearth of research evaluating each species segregation and behaviour within tissues using contemporary imaging techniques outside of the Shepherd et al. study.

There has otherwise been limited use of *in vitro* organotypic 3-D tissue models to study *S. aureus* and *P. aeruginosa* infections; although a number of studies have evaluated various antimicrobial agents or disinfection methods (Schaller et al. 2004; Shepherd et al. 2011; Crabbé et al. 2017; Zheng et al. 2019). The majority of research continues to rely on either direct use of animal studies, or of *ex vivo*, biopsy-based animal tissue models (Wardenburg et al. 2007, Zhao et al. 2010, Anderson et al. 2012). Some researchers have employed *ex vivo* human tissue models (Coolen et al. 2008), which arguably confer greater clinical validity to their *in vivo* counterparts.

However, standardisation is more challenging due to increased biological variability with the former, which may reduce experimental power to detect effects. As tissue engineering techniques improve further, it is likely that laboratory-generated tissue models will increase in popularity in microbiological (and a range of other) research.

3.2: Aims and Objectives

The primary aims of this research Chapter were to:

1. Develop and characterise an oral mucosal denture-acrylic biofilm infection model using standard histological techniques and PNA-FISH
2. Examine the impact biofilms containing putative respiratory pathogens on tissue viability and inflammatory markers
3. Evaluate the impact of host and microbial interactions on the expression of a range of virulence-associated genes of putative respiratory pathogens *S. aureus* and *P. aeruginosa*

Hypotheses

- Denture-associated biofilm species composition will affect tissue damage, with two species pathogenic biofilms causing the highest levels of damage
- Microbes in tissue infections will show different invasive potential and occupy different niches within tissues
- Gene expression by *S. aureus* and *P. aeruginosa* in tissue infections will be modulated by the presence of oral commensal species

3.3: Materials and Methods

3.3.1: Generation of acrylic biofilms

Single, two, three and five species biofilms were generated on acrylic coupons preconditioned with artificial saliva using the methods described in section 2.3.8.5. Only *S. aureus* and *P. aeruginosa* single species biofilms were evaluated. Two species biofilms consisted of a mixed inoculum of *S. aureus* and *P. aeruginosa*, while 3 species biofilms consisted of the representative commensal species *C. albicans*, *S. sanguinis* and *A. viscosus*.

3.3.2: Preparation of SkinEthic Reconstituted Human Oral Epithelium (RHOE) full-thickness tissue models

Keratinocyte 3-dimensional oral mucosal tissue models were obtained from EpiSkin (Lyon, France). The keratinocytes were TR146 cells, which were originally isolated from a neck node in a 67-year-old female patient with a buccal squamous cell carcinoma. Upon receipt, tissues were aseptically removed from the manufacturer's agar maintenance medium, with care taken to avoid damage to the tissues. Tissues were then placed in a cell-culture plate using the provided inserts each housing a polycarbonate membrane, with 0.4 µm pores facilitating nutrient exchange with the cells from the surrounding media. Immediately after adding the models to each insert, 2 ml of the proprietary cell culture medium was added, ensuring that tissues were situated at the air-liquid interface. Tissues were then incubated for 12 h at 37ºC in 5% (v/v) CO_2 before infection.

3.3.3: Infection of tissue models by acrylic biofilms

Cell-culture plates were prepared with 2 ml of cell culture medium and incubated for 1 h at 37ºC in 5% (v/v) CO_2 to ensure equilibration of the culture medium prior to transfer of the tissues. Tissues were then transferred to new medium. Biofilms prepared as described in section 2.3.8.5 on acrylic coupons for 72 h were briefly rinsed in PBS to remove non-adherent cells, followed by inversion and gentle placement on tissue models using sterile forceps. Internal controls were prepared by inverting sterile acrylic coupons which had been preconditioned with artificial saliva onto tissues. In addition, one triplicate set of tissues were cultured under the same conditions without the addition of any acrylic coupons, to act as a negative control. Immediately upon infection, tissues were again incubated for 12 h at 37ºC in a 5% (v/v) CO_2 atmosphere.

Figure 3.3.3: Flow diagram of experimental input and analyses

3 species biofilm (*S. sanguinis*, *A. visocosus* and *C. albicans*) 6 experimental replicates per run, each experiment conducted a total of 3 times

Single species biofilm (*P. aeruginosa* and *S. aureus* only) 6 experimental replicates per run, each experiment conducted a total of 3 times

2 species biofilm (*P. aeruginosa* with *S. aureus*) 6 experimental replicates per run, each experiment conducted a total of 3 times

Mixed (5) species biofilm 6 experimental replicates per run, each experiment conducted a total of 3 times

1) SkinEthic tissues infected with acrylic biofilms for 24 h

2) Supernatant removed for cytokine profiling by FACS

4a) Histological evaluation

3) Infected tissues sectioned

4b)Virulence gene expression analysed by qPCR

Negative controls for tissue infections were: acrylic only, tissue-only control

3.3.4: Processing of infected tissues

After 12 h incubation of biofilm-infected tissues, tissues were removed from the incubator and each acrylic coupon was aseptically removed, taking care to minimise disturbance to the tissue surface. The cell-culture insert supporting each tissue was inverted and the polycarbonate membrane removed using a sterile scalpel and forceps. Each tissue model was then bisected along its diameter, with one half added to a screw cap pathogen lysis tube (FastPro Blue, MP Biomedicals, Santa Ana, CA, USA) containing 500 µg glass beads (500 – 750 µm in size) and 1 ml proprietary lysis buffer. These tubes were stored on ice prior to further processing.

One ml of spent medium from each tissue culture well was transferred by sterile pipetting to 1.5 ml microcentrifuge tubes cooled on ice for further use.

3.3.4.1: Cryogenic preparation of tissues for histological processing

The remaining half of each tissue model was coated in optimal cutting temperature compound (OCT) embedding medium then transferred to a cork insert in correct orientation for cryo-sectioning. Each tissue was then 'snap-frozen' by immersion in a beaker containing 100 ml isopentane, cooled to -160ºC in a liquid nitrogen flask for 1 min. Upon completion of tissue fixation by 'snap-freezing', tissues were stored at -80ºC prior to further use.

3.3.4.2: Microbial RNA extraction

RNA extraction from infected tissue models was undertaken using the FastPro Blue kit (MP Biomedicals, Santa Ana, CA, USA) followed by further treatment using the Qiagen RNeasy clean-up kit (Qiagen, Manchester, UK) as described in Section 2.3.8.6. Similarly, cDNA synthesis was undertaken using the methods described in Section 2.3.8.7. Resultant cDNA was stored at -20ºC prior to qPCR experiments.

3.3.4.3: Lactate dehydrogenase (LDH) assay of tissue supernatant

Spent cell culture medium from tissue models collected at the time of processing (as described in Section 3.3.4) was analysed using the Pierce Lactate Dehydrogenase Assay (Thermofisher Scientific, Loughborough, UK) in a 96-well microtitre plate, according to the manufacturer's protocol for cell-mediated cytotoxicity. In addition to experimental controls, 100 µl of cell culture medium was added in triplicate to assay wells, to control for background absorbance.

3.3.4.4: Histological processing and staining

Samples prepared according to Section 3.3.4.1 were sectioned in 10 µm increments by a trained histopathologist, using a cryostat (Thermofisher Scientific, Loughborough, UK). Upon sectioning, samples were mounted onto glass slides and first examined using Haematoxylin and Eosin staining completed using the pathology laboratory's automated processing equipment. Additional Gram staining was conducted by immersion in crystal violet for 60 s, Lugol's iodine for 60 s, acetone decolourisation for 5 s and counterstaining with carbol fuchsin for 30 s (washed with running tap water between each step). Further sections were then processed for PNA hybridisation and immunohistochemistry (IHC) labelling.

3.3.4.5: PNA-FISH, Immunohistochemistry and CLSM imaging

Sectioned tissues were placed carefully onto charged glass slides (Superfrost Plus) and circumscribed with a wax pen (Dako) to contain subsequent reagents. The mounted tissues were immersed in 10% neutral-buffered saline for 30 min to fix samples, followed by washing in PBS for 10 min (x3). Excess moisture was removed from the slides using absorbent paper, taking care not to disturb the tissues.

To each slide, 100 µl each of lysozyme (10 mg/ml) and protease K (0.1 mg/ml) was added, followed by incubation at 37ºC for 30 min. Slides were then washed with 100 µl PBS (x2), and the excess liquid drained.

Subsequently, mouse monoclonal pan-cytokeratin primary antibody (Santa Cruz Biotechnology, Wembley, UK) was diluted 10-fold with PBS, and 100 µl added to each slide. Slides were then incubated at room temperature whilst protected from light for 1 h. Slides were washed with 100 µl PBS for 5 min (x2), and the excess liquid drained. An Alexa-488 fluorophore-conjugated goat anti-mouse secondary antibody was diluted 10-fold in PBS, and again, 100 µl added to each sample. Samples were incubated at room temperature for a further 1 h whilst protected from light, followed by washing with 100 µl PBS (x2), and the excess liquid drained.

PNA probes were used to label *P. aeruginosa*, *S. aureus* and *C. albicans*, in addition to a universal bacterial probe within tissues, following the method outlined in Section 2.3.8.3.1. Control slides were prepared with single species smears of each microbe fixed and hybridised using the same methods as tissue samples.

After PNA probe hybridisation and washing of samples in PBS, the excess liquid was drained from the surface of each sample. A 1 µg/ml solution of Hoechst 33258 was prepared in PBS, and 20 µl added to each sample to counterstain cell nuclei. Samples were incubated at room temperature for 10 min whilst protected from light. Samples were then washed with PBS for 5 min (x2), and excess moisture removed using absorbent paper, taking care not to disturb the mounted tissues.

A drop of Vectashield mounting medium (H1100) was added to each sample, and 0.17mm thick (#1.5) glass coverslips mounted to each slide. Coverslips were sealed using clear nail varnish and protected from light prior to imaging.

CLSM imaging was undertaken as described in section 2.3.8.3.2, with the additional parameters for the pan-cytokeratin and Hoechst 33258 stains outlined in Table 3.3.4.5.

Table 3.3.4.5: Excitation and Emission Spectra for Immunohistochemistry stains

Target species	Fluorophore	Excitation (nm)	Wavelength	Emission Frequency (nm)
Pan-cytokeratin	Alexa 488	488		500-550
Hoechst 33258	Hoechst 33258	405		410-480

3.3.4.6: Bacterial gene expression analysis by qPCR

Gene expression levels were analysed for *S. aureus* and *P. aeruginosa* using the optimised primer pairs described in Section 2.3.8.8. Initial screening of reference genes and evaluation of primer efficiencies for microbes within tissue models was performed using LinRegPCR as previously described in Section 2.3.8.8.1, with stability of reference genes assessed using the NormFinder plugin for Microsoft Excel. All qPCR reactions were undertaken using the reagents and conditions described in Section 2.3.8.8.2. Similarly, the Pfaffl method for changes in gene expression was used, with a threshold of 75-125% for primer efficiency applied. Reactions which failed to meet this threshold were excluded from subsequent analysis.

3.3.4.7: Evaluation of tissue cytokine profiles by CBA using FACS analysis

Spent tissue culture medium collected from tissues (see Section 3.3.4) was diluted 1:5 in sterile PBS in a 1.5 ml microcentrifuge tube. This 'tissue supernatant' was then analysed for a panel of 6 pro-inflammatory cytokines (Table 3.3.4.7), using the proprietary multiplex cytometric bead array kit from BD Biosciences (Wokingham, UK) according to the manufacturer's protocol for supernatant samples.

Samples were analysed using a flow-assisted cell sorted (BD FACSCanto II, BD Biosciences, Wokingham, UK). The setup beads provided with the Human Inflammatory Cytokines Kit were used to calibrate the experimental parameters. Subsequently, standard curves were performed using the provided samples, to cover an 8-log range, with an additional negative sample. All samples were processed using medium speed and a total of 10000 observations per run. All samples were processed in a single experimental batch, to avoid the problem of variation between runs.

Experimental data was analysed using the FACsuite software (BD Biosciences, Wokingham, UK) after manual noise-filtering and cytometric bead assignment to the

clusters observed. Subsequent analysis against the standard curve was undertaken according to the manufacturer's instructions.

Table 3.3.4.7: Cytokines detected in the multiplex cytometric bead array kit (according to assay handbook)

Cytokine measured	Putative biological function
IL-12p70	Induction of Th1 cells from naïve T cells, stimulates production of IFN-γ and TNF-α, inhibits angiogenesis
TNF	Pyrexia, acute phase response stimulation, chemotaxis of neutrophils, stimulation of phagocytosis by macrophages
IL-10	Induces B cell proliferation and production of antibodies; inhibits lipopolysaccharide-mediated induction of other pro-inflammatory cytokines
IL-6	Acute phase response, stimulates prostaglandin E_2 production, stimulates neutrophil production
IL-1β	Pyrexia, stimulates prostaglandin and collagenase release, role in wound-healing through fibroblast growth induction
IL-8	Neutrophil chemotaxis, stimulates phagocytosis, promotes angiogenesis, stimulates histamine release by mast cells

3.3.5: Statistical analyses

Fold-change in gene expression between experimental groups was normalised to reference genes using the Pfaffl method. Subsequently, 2-way ANOVA was performed on the Δ fold-change for each gene, using single species biofilm infections as the control group for both *P. aeruginosa* and *S. aureus.* Post-hoc correction for multiple comparisons was applied using the two-stage step up method of Benjamini, Krieger and Yekutieli to control the false discovery rate (FDR). The Q value selected was Q = 0.05.

Statistical analysis of cytokine profiles from spent tissue model culture medium was undertaken using Microsoft Excel and Graphpad Prism 7.0. Analysis was undertaken using One-way ANOVA tests with post-hoc Dunnett test for multiple comparisons; the PMMA only, uninfected tissues were selected as controls.. The significance threshold was set at p<0.05.

3.4: Results

3.4.1: Light microscopic analysis of tissue damage resulting from biofilm infection

Visual inspection of photomicrographs demonstrated minimal perturbation of normal tissue morphology in uninfected controls (Figure 3.4.1 1A & B), although there was some evidence of breakdown within the epithelial strata, particularly superficially. It was unclear if this was related to processing artefact or represented true tissue damage. Infection by 3 species 'commensal' biofilms resulted in minimal disruption of epithelial integrity (Figure 3.4.1 2A), although there was some evidence of invasion by *C. albicans* hyphae (Figure 3.4.1 2B). *S. aureus* single species biofilm infection of tissues led to a greater disturbance of tissues (Figure 3.4.1 3A & B), with separation of the tissues from the underlying polycarbonate membrane and within cell strata. Infection of tissues with *P. aeruginosa* single species biofilms resulted in obliteration of normal tissue morphology, with destruction of cells and acantholytic changes within tissues (Figure 3.4.1 4A & B). In contrast to *S. aureus,* which showed limited invasion of tissues, *P. aeruginosa* was visible throughout the tissues and even spread beyond the most basal layer of cells.

When tissues were infected by two species 'pathogenic' biofilms containing both *P. aeruginosa* and *S. aureus*, there was again obliteration of normal tissue morphology (Figure 3.4.1 5A & B). However, there was a tendency for increased integrity of tissues compared to *P. aeruginosa* infections alone, with less evidence of cell destruction in the form of acantholytic spaces. Similarly, infection by 5 species mixed species biofilms containing both the representative commensal microbes and pathogens resulted in highly destructive changes within the tissues, but the level of tissue obliteration and invasion varied between lesser (Figure 3.4.1 6A) and greater (Figure 3.4.1 6B) than *P. aeruginosa* alone.

[Overleaf] Figure 3.4.1: Histologically stained cross sections of biofilm infected epithelial tissue models visualised by light microscopy (x630)

1) Negative (uninfected) control; **A)** Hematoxylin and eosin; **B)** Gram stain

2) 'Commensal' biofilm infections; **A)** Hematoxylin and eosin; **B)** Gram stain

3) *S. aureus* biofilm infections; **A)** Hematoxylin and eosin; **B)** Gram stain

4) *P. aeruginosa* biofilm infections; **A)** Hematoxylin and eosin; **B)** Gram stain

5) Dual species 'pathogenic' biofilm infections; **A)** Hematoxylin and eosin; **B)** Gram stain

6) Mixed species biofilm infections; **A)** H&E; **B)** Gram stain

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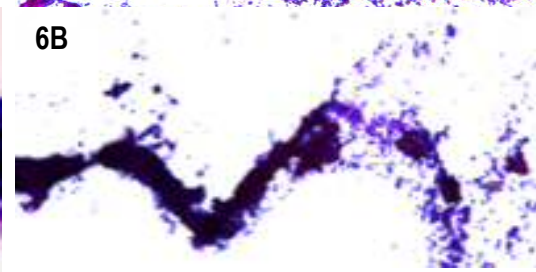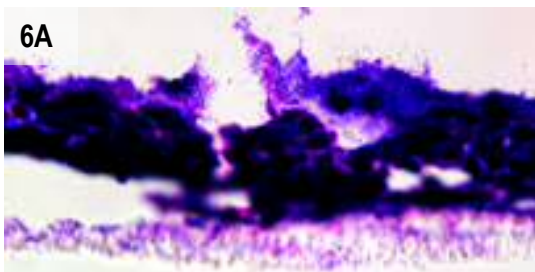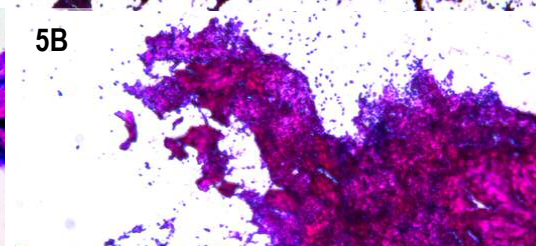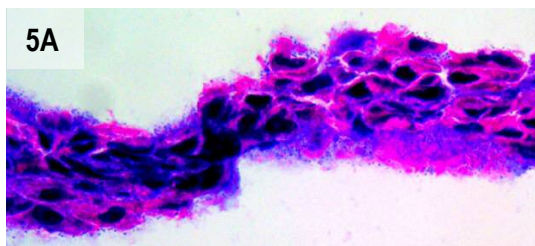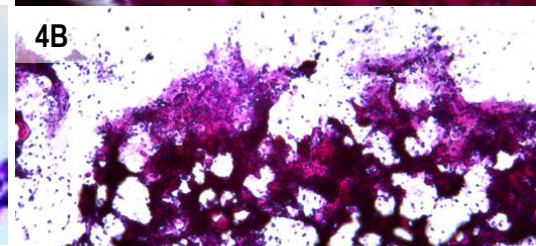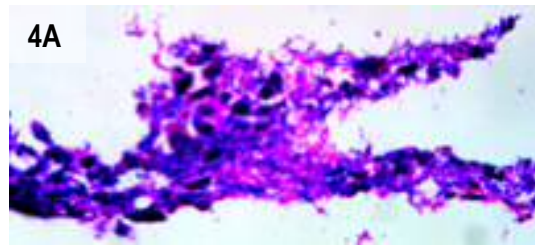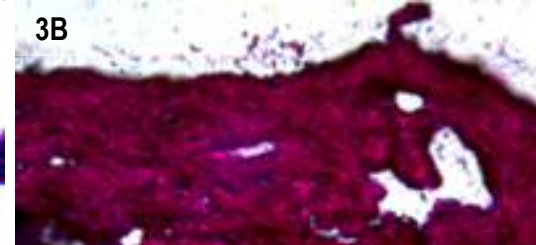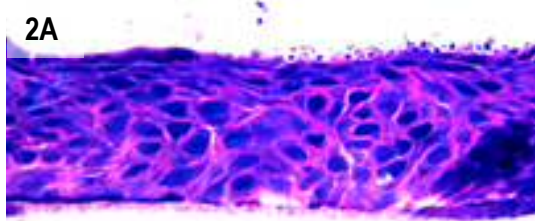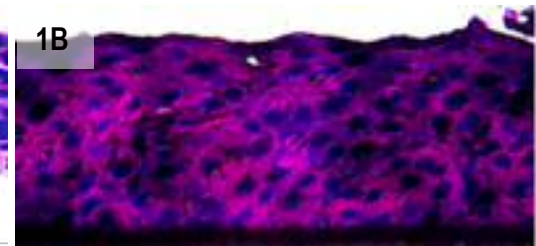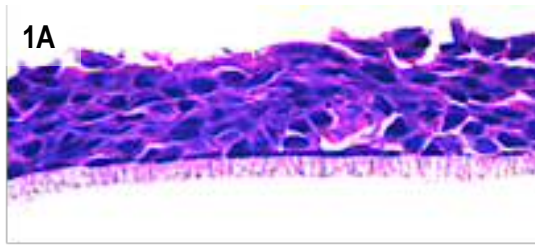
3.4.2: CLSM analysis of species-specific infection patterns using PNA-FISH

To analyse the distribution of the representative pathogenic microbes during biofilm infection of tissue models, examination of PNA-FISH labelled microbes within infected tissues was undertaken using CLSM. In keeping with the observations in Section 3.4.1 using histological stains, uninfected tissues showed a uniform structure with minimal interruption of cells in a stratified organisation (Figure 3.4.2 A).

Tissues infected with a 3 species 'commensal' biofilm consisting of *S. sanguinis*, *A. viscosus* and *C. albicans* demonstrated minimal disruption to tissues, with disturbance of the most superficial epithelial cell layer only (Figure 3.4.2 B). Infection of tissues by *P. aeruginosa* single species biofilms led to loss of normal tissue morphology and deformation of normal epithelial cell morphology (Figure 3.4.2 C). Both cell outlines and nuclei appeared flattened and elongated. *P. aeruginosa* could be seen invading close to the basal layer of cells within tissues. Infection by *S. aureus* single species biofilms led to limited disruption of tissues, although there was greater reduction in cross-sectional thickness compared to control or tissues infected by 3 species 'commensal' microorganisms (Figure 3.4.2 D). This was similar in appearance to tissues examined by light microscopy after histological staining (Figure 3.4.1 3A). The *S. aureus* PNA probe was not identified by CLSM, and there was limited identification of the universal bacterial probe within these samples, so it was not possible to make inferences about the level of tissue invasion in such biofilm infections. Two species 'pathogenic' biofilm infection of tissues was not possible to examine, as all sections were unable to be visualised using CLSM. This may have been due to loss of samples from slide surfaces as a by-product of processing. However, the isolation of this to 'pathogenic' biofilm infections only suggests that such an event occurred secondary to massive tissue destruction and loss of integrity of the samples. Infections of epithelial tissues by mixed biofilms (Figure 3.4.2 E) resulted in disturbance of tissue morphology and acantholytic body formation. However, the altered cell morphology observed in *P. aeruginosa* single species biofilm infection was not seen. This morphological change was observed in all *P. aeruginosa* infected tissues examined. However, the underlying mechanisms behind such effects are unclear. In mixed biofilm infections of tissues, *P. aeruginosa* was seen to be dispersed throughout tissues. Of note, *S. sanguinis*, identifiable by the typical 'string of cocci' morphology, was also evident throughout the tissues, alongside singular coccal microorganisms presumed to be *S. aureus*. Whether this apparent invasivity is simply passive movement of microbes throughout tissues which have lost integrity secondary to *P. aeruginosa* invasion or reflects an actual change in microbial behaviour within mixed biofilms containing pathogens is not clear.

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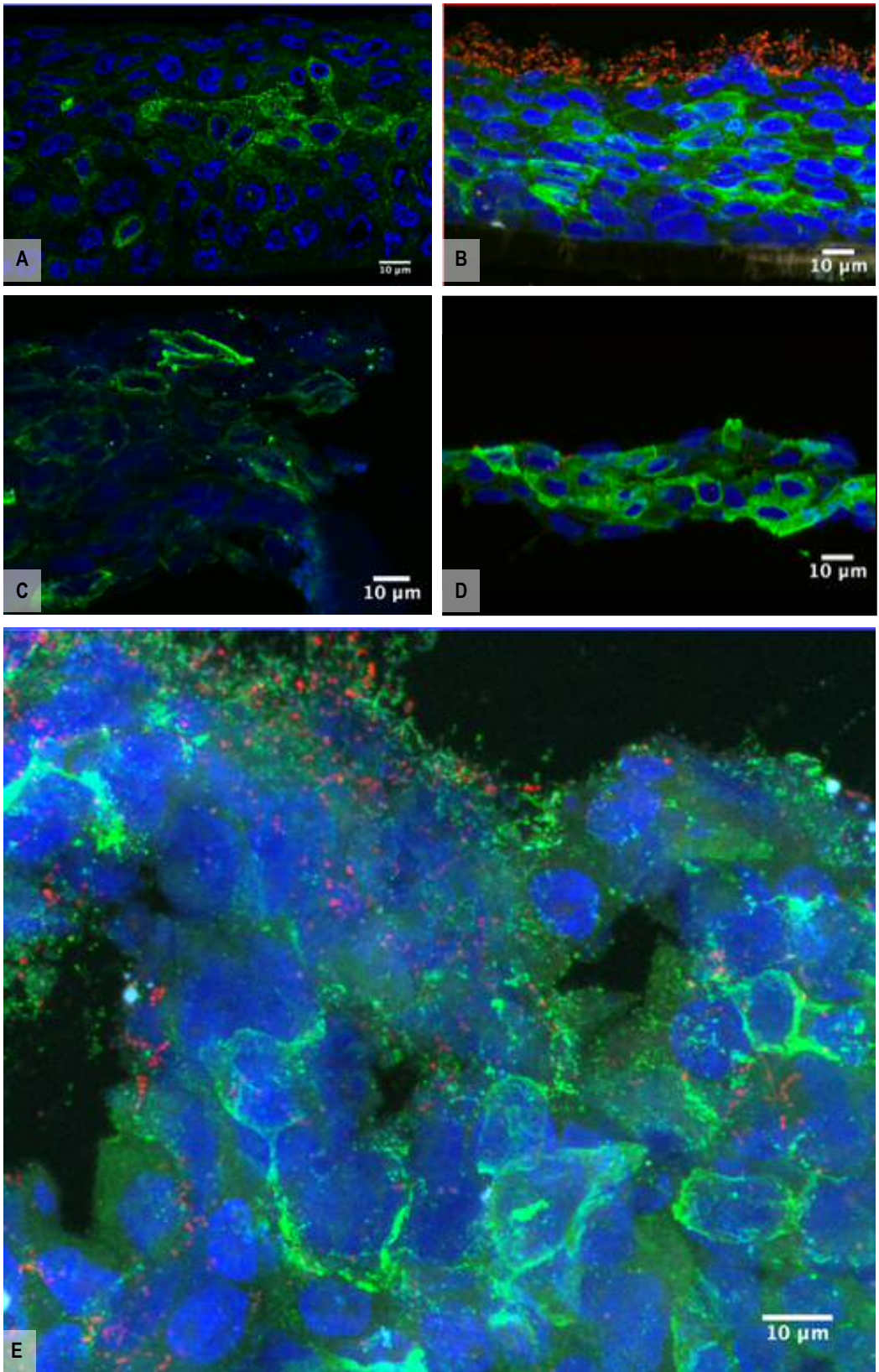
Figure 3.4.2: CLSM photomicrographs of tissue models labelled by IHC, infected by biofilms of varying composition, with constituent microbes labelled by PNA-FISH

A) Negative (uninfected) control
B) 'Commensal' biofilm infection
C) *P. aeruginosa* biofilm infection
D) *S. aureus* biofilm infection
E) Five species mixed biofilm infection

Epithelial cells within tissues are labelled by pancytokeratin (green) with nuclei counterstained using Hoechst 33258 (blue).

'Commensal' bacteria labelled with universal bacterial PNA probe (red) and *C. albicans* with Yeast Traffic Light system (green, approximately 10x larger than bacteria). *S. aureus* PNA probe failed to be detected, therefore *S. aureus* appears red and difficult to differentiate from 'commensal' bacteria. *P. aeruginosa* labelled with species specific PNA probe (green).

3.4.3: Lactate Dehydrogenase (LDH) release in response to different biofilm microorganisms

LDH release from epithelial tissue models infected with microbial biofilms for 24 h was broadly consistent with the pattern of tissue damage seen in microscopic analysis (Figure 3.4.3.1). There was a low level of LDH release seen in tissues overlaid with uninfected acrylic coupons, and a similarly low level of LDH release in tissues infected with 'commensal' biofilms on acrylic coupons. An increase in LDH release was seen in tissues overlaid with acrylic coupons supporting *S. aureus* single species biofilms, but this was highly variable, as can be seen by the wide error bars, and did not reach the threshold of statistical significance. In tissues infected by *P. aeruginosa* single species biofilms, there was a statistically significant increase in LDH production compared with uninfected acrylic coupons. This effect was also evident compared with 3 species 'commensal' biofilm infections, although this was not statistically significant. Two species pathogenic biofilm infection of tissues resulted in further LDH release, which was statistically significantly greater than the response seen against both uninfected acrylic coupons and 3 species 'commensal' biofilm infections. Infection by mixed biofilms containing both 'commensal' microorganisms and pathogens resulted in an intermediate level of LDH release, which was similar in magnitude to *S. aureus* single species biofilms.

When LDH release was analysed as fold change (relative to uninfected acrylic coupons), the difference in magnitudes of effect of species composition in infective biofilms became more apparent (Figure 3.4.3.2). There was a greater than 20-fold increase in LDH release in the two species pathogenic biofilm tissue infections, compared with an approximately 8-fold increase in both *S. aureus* single species biofilms and mixed species biofilms. The increase in tissue LDH release in response

to infection with *P. aeruginosa* single species biofilms was approximately 17-fold. In contrast, infection by 3 species 'commensal' biofilms on acrylic coupons led to a less than 1-fold increase in tissue LDH release.
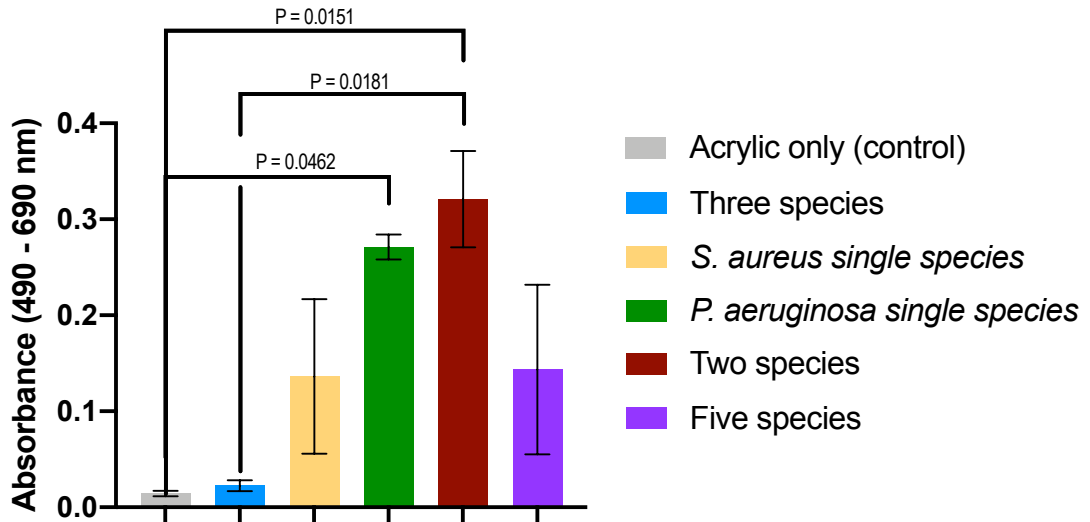
Figure 3.4.3.1: LDH release in biofilm-infected tissue models (measured by spectrophotometric absorbance at 490 nm minus background absorbance at 680 nm)
Acrylic coupon, uninfected control
Experiments performed in triplicate with 2 experimental repeats. Mean values shown. Error bars represent standard error of the mean.
Adjusted P value following One-way ANOVA with Tukey post-hoc correction shown.
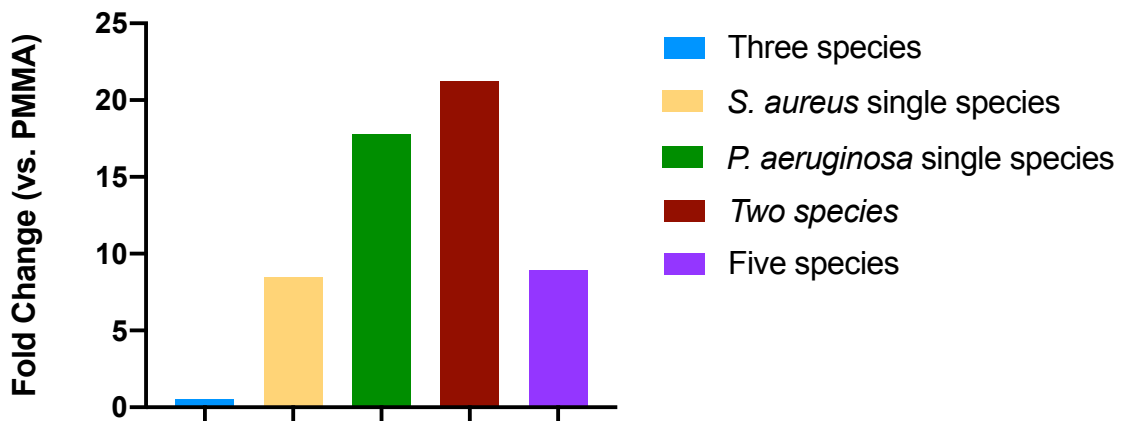
Figure 3.4.3.2: Fold-change in LDH release in tissue models

Calculated from the data presented in figure 3.4.3.1, normalized to acrylic coupon, uninfected control.

3.4.5: Gene expression profiles of biofilm-associated *P. aeruginosa* and *S. aureus* following tissue infection

There was a statistically significant upregulation in the expression of the Pseudomonal genes algE, which is involved in alginate secretion and aprA, which encodes alkaline protease (Figure 3.4.5A), while expression of RhlR, which represses transcription of quorum sensing genes was significantly decreased. However, the magnitude in fold-change for RhlR and algE was of questionable biological significance, both demonstrating an approximately two-fold change in expression levels. In contrast, aprA expression was upregulated over 200-fold in mixed species biofilm infections compared with single species infections. The majority of Pseudomonal genes analysed demonstrated a trend of increased expression levels in two and five species mixed biofilm infections compared to single species infections, in opposition to the findings seen when evaluating the same biofilms in Section 2.4.4.5.

As was seen in Section 2.4.4.5, *S. aureus* gene expression showed no significant differences between single, two and five species mixed biofilm infections of epithelial tissue models (Figure 3.4.5B). There was a high degree of variability in expression between experimental repeats, which may reflect the technical challenges of extraction sufficient quantity and quality of RNA from *S. aureus* biofilms.
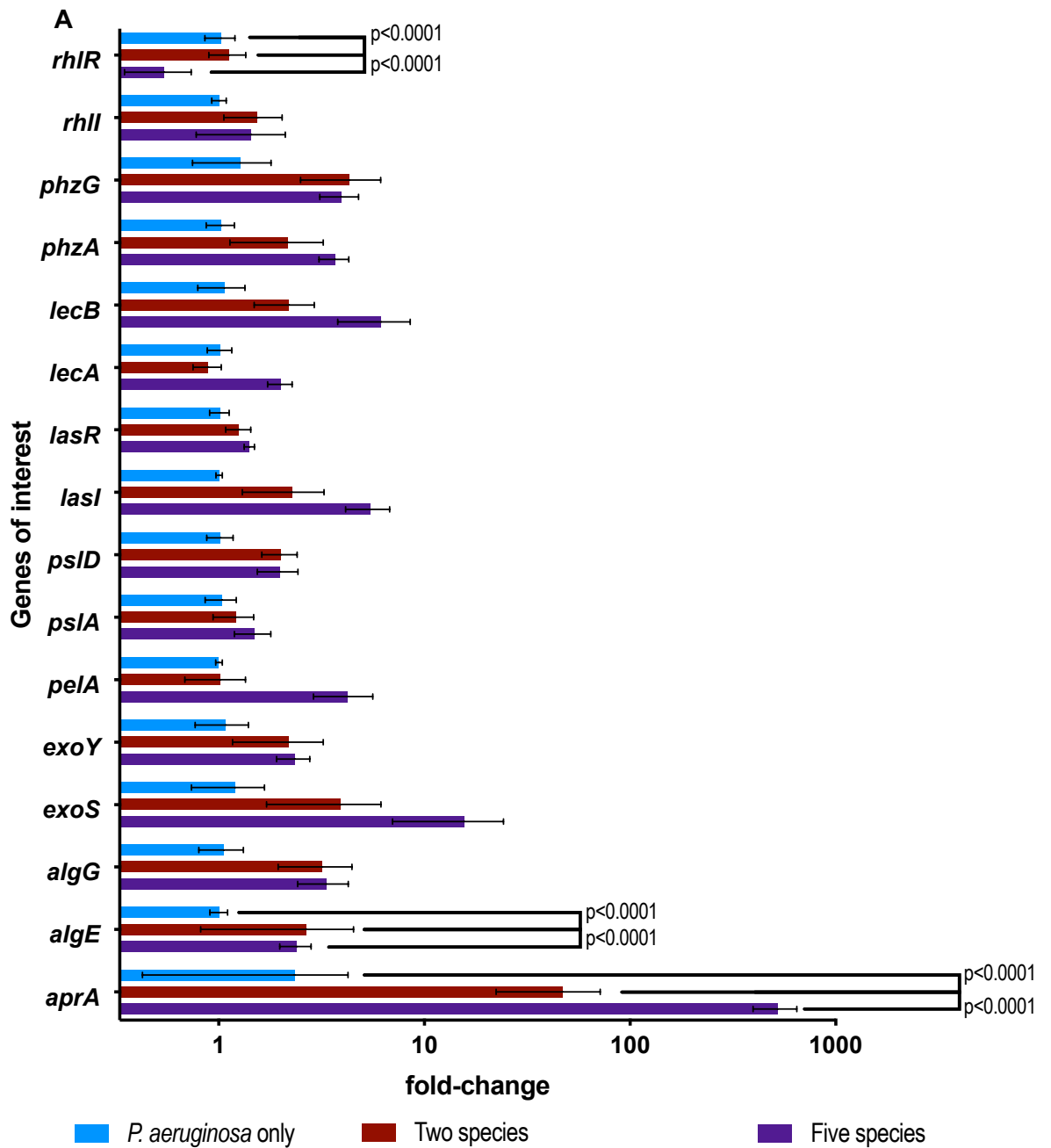
Figure 3.4.5A: Differential *P. aeruginosa* gene expression in single, dual and mixed species infections. Fold-change normalised to rpoD reference gene shown. All experiments were performed in duplicate wells with 2 experimental repeats. 2-way repeated measures ANOVA was performed on the difference in fold-change for each gene examined, using *P. aeruginosa* single species infections as control group.

P values shown are adjusted significance using the two-stage step up method of Benjamini, Krieger and Yekutieli to control the false discovery rate (FDR). Q = 0.05.
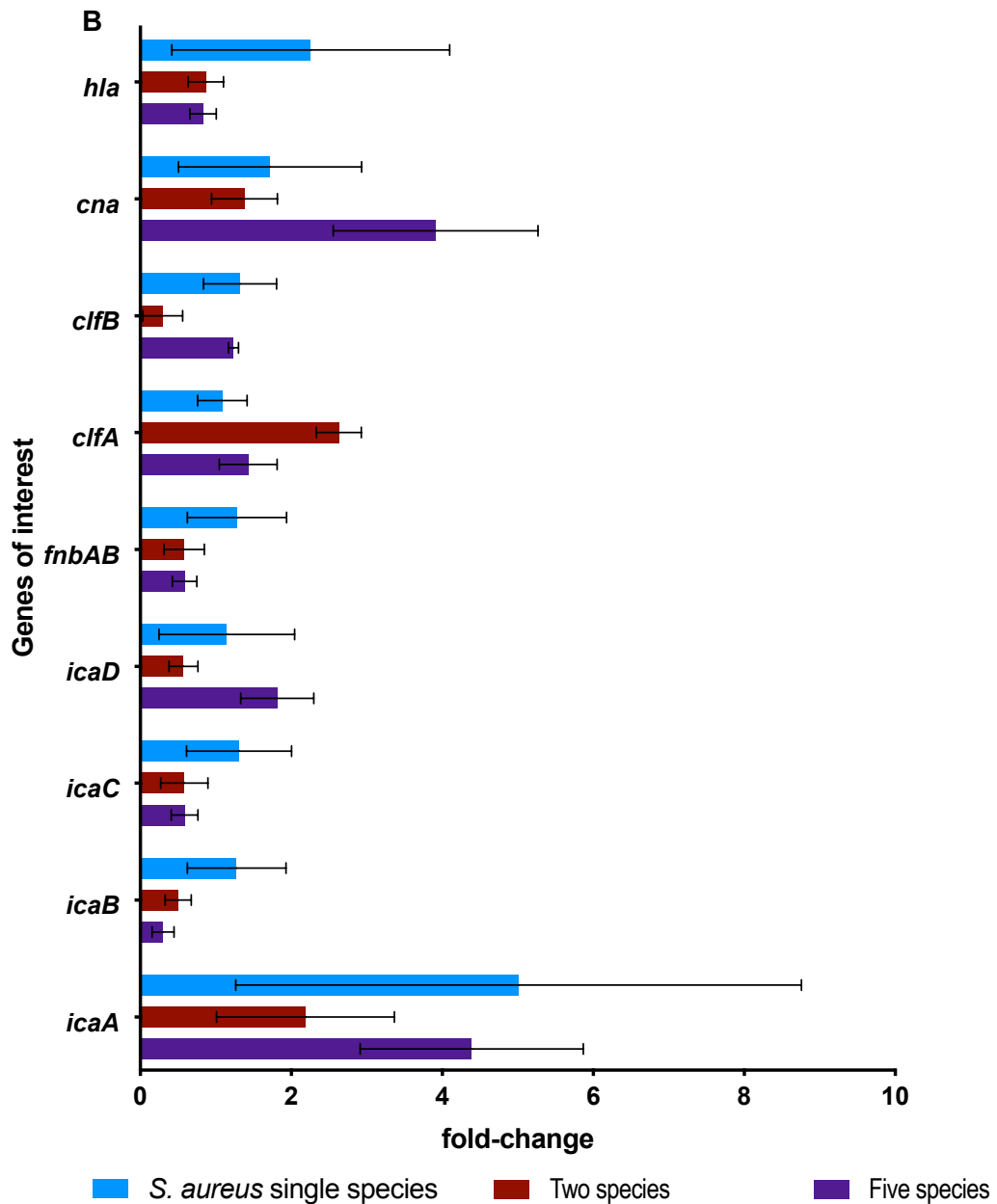
Figure 3.4.5B: Differential *S. aureus* gene expression in single species, two and five species infections. Fold-change normalised to pyk reference gene shown. All experiments were performed in duplicate wells with 2 experimental repeats. 2-way repeated measures ANOVA was performed on the difference in fold-change for each gene examined, using *S. aureus* single species infections as control group. P values shown are adjusted significance using the two-stage step up method of Benjamini, Krieger and Yekutieli to control the false discovery rate (FDR). Q = 0.05.

C
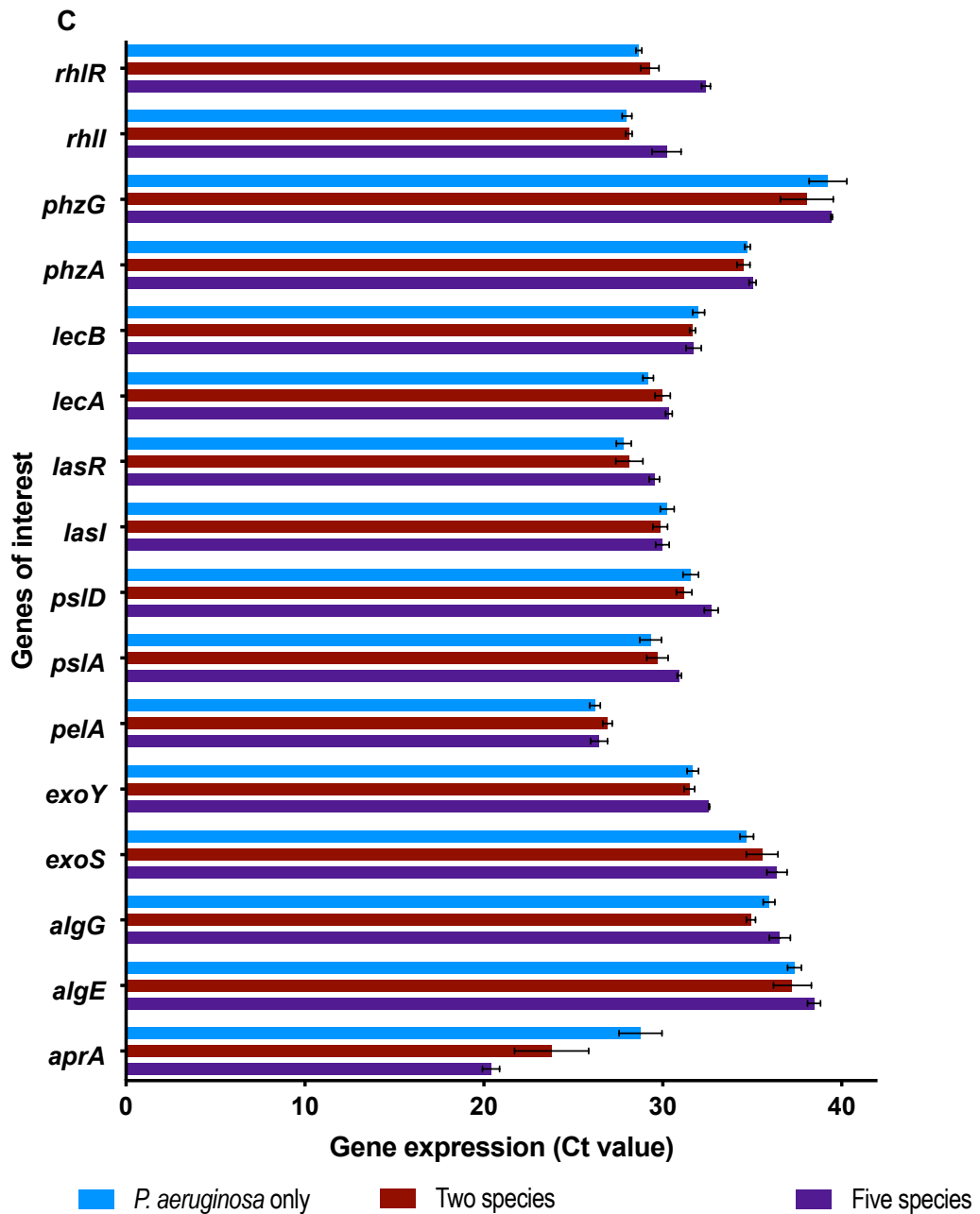
Figure 3.4.5C: Differential *P. aeruginosa* gene expression in single, two species (*S.aureus* and *P. aeruginosa*) and five species biofilms. Ct values for genes shown in Figure 2.4.4.5A.
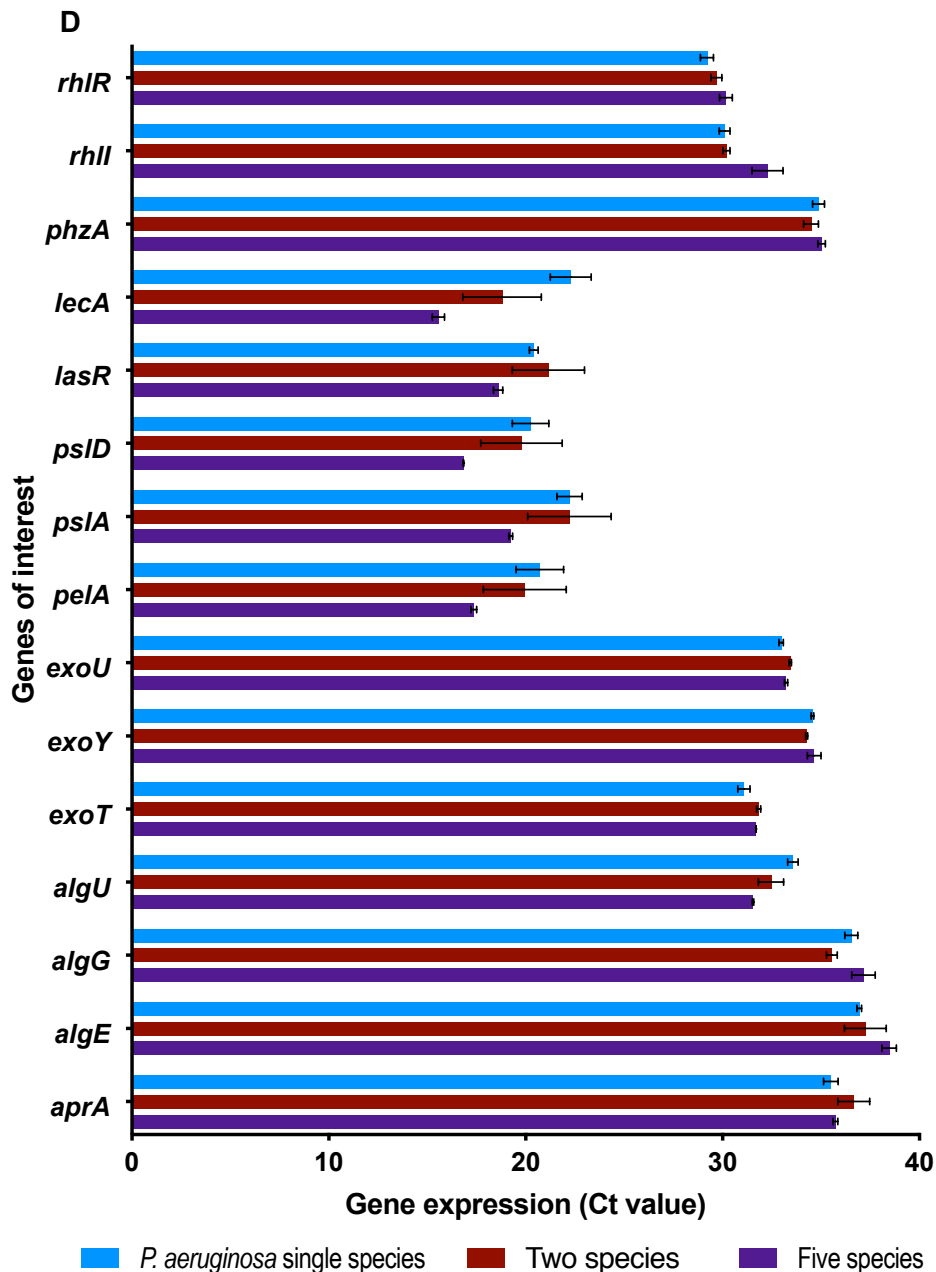
Figure 2.4.4.5C: Differential *P. aeruginosa* gene expression in single, two species (*S.aureus* and *P. aeruginosa*) and five species biofilms. Ct values for genes shown in Figure 2.4.4.5A.

3.4.6: Cytokine expression by epithelial tissue models in response to denture-acrylic associated biofilm infections

There was no significant difference in the expression levels of IL-12p70, TNF, IL-10 or IL-8 between experimental groups (Figure 3.4.6). Surprisingly, IL-6 levels were decreased in *P. aeruginosa,* two or five species biofilm infections. This may be due to rapid obliteration of the epithelial tissues as seen in Figure 3.4.1, rather than a reflection of cell behaviour in response to such pathogens. The expression of IL-1β was significantly increased only in two species biofilm infections compared to control,

but the magnitude of this difference was small (less than 2-fold) and highly variable. The biological relevance of this change is therefore questionable.
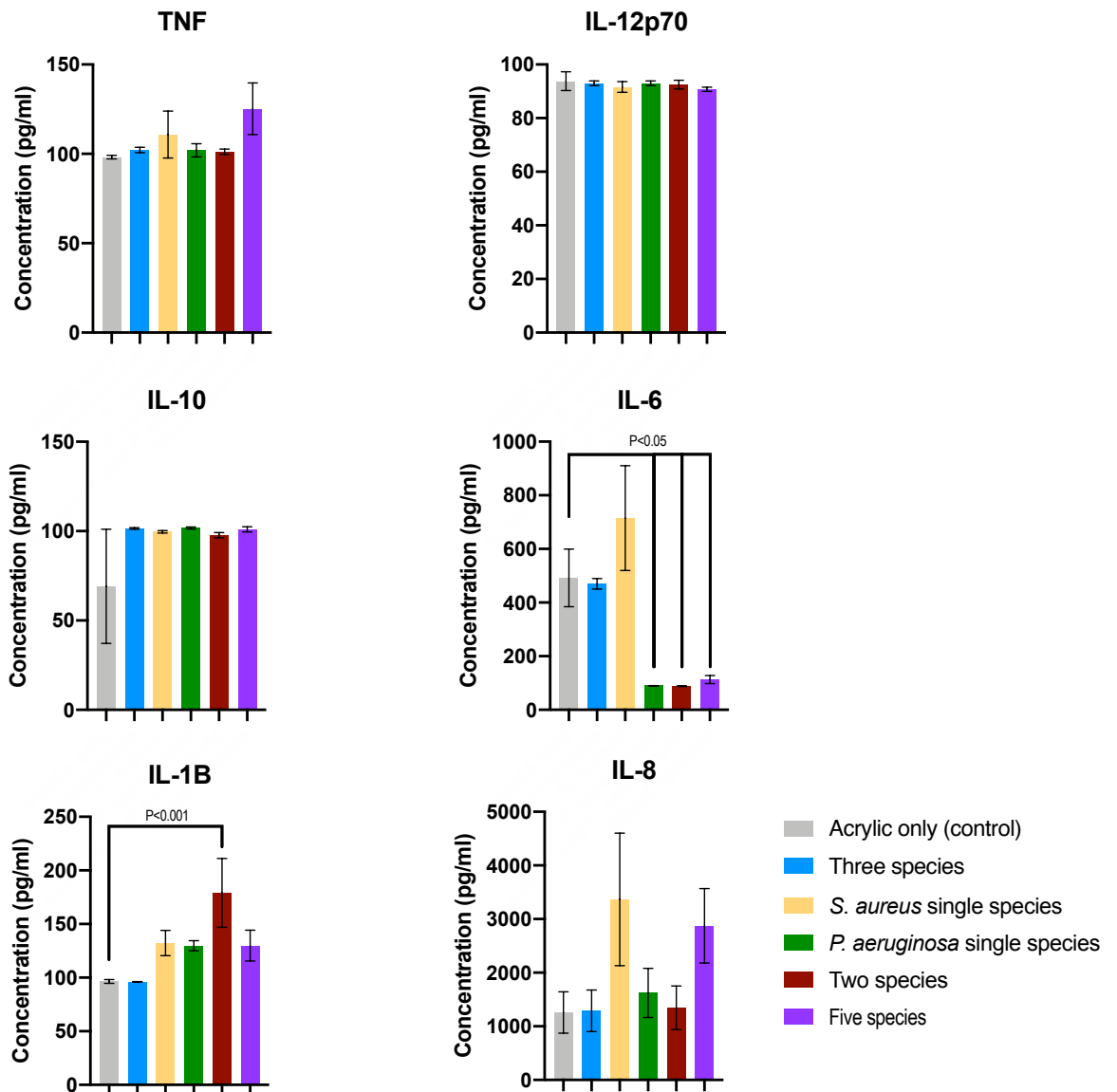
Figure 3.4.6: Cytokine expression levels of tissues in response to denture-acrylic associated biofilm infections

Mean values shown. Error bars indicate standard error of the mean. Each experiment was performed in triplicate with 2 experimental repeats.

One-way ANOVA was performed with Dunnett test to compare each biofilm infection with PMMA only (control) group. Note – logarithmic scale

3.5: Discussion

In this chapter, the model denture-acrylic associated biofilms developed in the previous chapter were used to explore the impact of putative respiratory pathogens on oral mucosal tissue damage and inflammation. Furthermore, the impact of interactions within microbial communities and with host tissues on the expression of key microbial biofilm and virulence-associated genes was explored. This involved the development of a novel RNA extraction process which enabled effective recovery of RNA from microbial biofilms.

Examination of haematoxylin and eosin stained infected tissue sections by light microscopy demonstrated a low level of tissue damage sustained following 24 h contact with PMMA resin in the absence of microbial biofilms (Figure 3.4.1). This problem is well documented in the literature (Bhola et al. 2010; Gautam et al. 2012) and is likely due to leaching of monomeric materials which failed to polymerise during the curing process. Tissue disruption and chronic, low-grade inflammation of the oral epithelium has been proposed as a potential mechanism by which colonisation of the oral cavity by atypical, potentially pathogenic species may occur (Scannapieco et al. 2001). Therefore, the observation that denture-acrylic resins induce such changes is important. The 3 species 'commensal' biofilm infections demonstrated minimal tissue disruption overall, although as seen in Figure 3.4.1.2B, increased perturbation of tissues occurred in association with candidal hyphae. This observation was corroborated by the close similarity in LDH levels induced by both acrylic resin and 3 species 'commensal' biofilm infections. The LDH enzyme is a cytoplasmic protein present in all cells. Release of LDH into the extra-cellular environment occurs following membrane permeabilization, which is a key event in apoptosis and necrosis (Kumar et al. 2018). Thus, the level of LDH in tissue supernatants is a reasonable surrogate marker for the level of cell damage.

Biofilm infections with *P. aeruginosa* alone appeared to result in extensive tissue damage, as might be expected from such a virulent microorganism, but the addition of *S. aureus* did not appear to impact the level of local damage seen, which was in keeping with the relatively low levels of tissue damage noted in *S. aureus* single species biofilm infections (Figure 3.4.1.3A/B). Interestingly, although microscopic analysis shows considerable tissue damage in response to five species mixed biofilm infections, the level of LDH release was reduced at least twofold in five species mixed biofilm infections compared with *P. aeruginosa* single or two species infections. This may be simply due to the reduced numbers of *P. aeruginosa* present in such biofilms but may also reflect a reduction in binding sites available due to occupation by commensal species, offering a degree of protection from *P. aeruginosa.*

Analysis of tissue infections by PNA-FISH and IHC confirmed the presence of extensive tissue damage following *P. aeruginosa* infection (Figure 3.4.2C) with microbial invasion seen throughout the full thickness of the tissue section. Again, minimal tissue disturbance in response to 3 species 'commensal' biofilm infections and infection with *S. aureus* biofilms was noted. It was not possible to find intact tissues for imaging on sections of two species biofilm infections. This may be due to obliteration of tissues but may be artefactual loss of tissues from the slides as a result of the extensive and aggressive preparation methods required to enable PNA probe hybridisation. Mixed species tissue infections showed similar levels of microbial invasion to *P. aeruginosa* infected tissues, but the total bioburden visible was increased. Importantly, tissue invasion in such samples was not limited to the representative pathogens. *Streptococcus sanguinis* was clearly recognisable by its characteristic 'string of pearls' morphology deep within the tissue section (Figure 3.4.2E). Another finding of interest in both light and confocal photomicrographs is the absence of candidal hyphae in mixed species infections, potentially due to the inhibitory interactions between *P. aeruginosa* and *C. albicans* discussed in section 2.1.3.

Microscopic analysis of tissue infections is a convenient and effective technique to assess both the response of tissues to infection, and the biogeographical distribution of infective microbes. The highly invasive nature of *P. aeruginosa* in such infections is illustrated here. However, a number of limitations must be considered when evaluating such images. Firstly, while care has been taken to ensure representative samples have been included, the distribution and species composition of biofilms at the tissue surface is heterogeneous. Consequently, the effects of multispecies biofilm infections are challenging to capture effectively. Additionally, the sectioning and subsequent processing of tissues can introduce artefacts. It is possible that some of the observed tissue damage and invasion is actually introduced during sectioning. Processing artefact is a particular concern for PNA-FISH and IHC treated samples, as many of the reagents and conditions used are harsh, and a degree of disruption is required to permit effective hybridisation of the PNA probes. This is mitigated by the presence of control samples for comparison, and the use of LDH and cytokine analysis to corroborate tissue damage with molecular markers of cell death and inflammation.

Tissue cytokine responses (summarised in Figure 3.4.6) to microbial infections are surprising; particularly the reduced expression of IL-6 in response to biofilms containing *P. aeruginosa.* Previous research has demonstrated a marked increase in IL-6 expression in airway epithelial cell-lines (Kube et al. 2001) and corneal epithelial

cell-lines (Zhang et al. 2005) in the presence of *P. aeruginosa*. It is possible that in the present work, rapid obliteration and killing of epithelial tissues occurred in response to infection with *P. aeruginosa* biofilms. In addition, the pseudomonal aprA gene encodes the enzyme alkaline protease. This enzyme can degrade a number of immune proteins and cytokines (Bardoel et al. 2012), and so may be directly responsible for the apparent reduction in IL-6 noted. This eventuality seems likely when taken in the context of the tissue obliteration observed in photomicrographs and the high levels of LDH. The regulation of cytokine expression is a complex process with many interdependent interactions between different cell and tissue types. The tissue models used lacked immune cells, which have critical and divergent roles in orchestrating the cytokine response to infection in a context-dependent manner. For instance, neutrophils, recruited at an early stage of infection can induce further release of pro-inflammatory cytokines by release of alpha defensins (Van Wetering et al. 2002). Conversely, in longer-term infections T-regulatory cells can contribute to mucosal tolerance by altering the behaviour of surround immune and epithelial cells (Sharma and Rudra 2018). Consequently, interpretation of cytokine expression in a reductionist, keratinocyte-only tissue model is limited.

Microbial gene expression levels, displayed in Section 3.4.5, is likely influenced both by intra and interspecies community interactions, and interactions with host cells. The challenges inherent to RNA extraction from microbial biofilms, particularly *S. aureus,* have been discussed in Section 2.5. This may explain the high degree of variability in gene expression seen in Figure 3.4.5B, although this may also relate to variation inherent to the heterogeneous environment in biofilms, with microorganisms close to the surface contacting the epithelial tissues and experiencing a drastically different environment to those microbes in deeper biofilm layers distant to the tissues. While changes in the levels of rhlR and algE were of questionable relevance given the small magnitude of difference in expression level, aprA, which encodes the enzyme alkaline protease was upregulated over 200-fold in mixed species biofilms. This enzyme has well documented roles in immune evasion (Laarman et al. 2012; Pel et al. 2014). However, a role for this protein in inter-species interactions has not been documented to date. It could be that alkaline protease plays a role in competition during infection by degrading microbial proteins and allowing *P. aeruginosa* to dominate tissue invasion, or the aprA gene may have other functions in quorum sensing pathways or the regulation of other virulence factors.

In contrast to *S. aureus*, *P. aeruginosa* is a highly motile microorganism, and so it might be expected that location within the biofilm is of limited consequence. Certainly, the impact of interaction with tissues appears to outweigh that of intermicrobial

interactions, as the trends in gene expression noted in Figure 2.4.4.5A are largely reversed in tissue infections (Figure 3.4.5A). This finding is in keeping with the observation that invasion of tissues appears to be instigated by *P. aeruginosa* almost exclusively among the microbes tested. Other species are only seen in deeper tissue layers when present in biofilms containing *P. aeruginosa.* This may reflect 'microbial hitchiking', described in infections with *S. aureus* and *C. albicans* (Schlect et al. 2015), although as seen in Figure 3.4.2E, this is more likely an opportunistic process secondary to increased tissue permeability following damage induced by *P. aeruginosa*, as close associated of microbes is not clearly seen.

Limitations of the methods

This work uses both reductionist biofilm models and tissue models, which limits the ecological validity of the findings. The influence of changes in microbial community composition, or even among different strains of each species cannot be accounted for. Similarly, keratinocytes are closely associated with an underlying dermis rich in fibroblasts, while epithelial tissue is also intimately connected to dendritic cells and circulating polymorphonucleocytes. Cytokines released from epithelial tissues typically initiate a zymogen cascade, whereby leukocytes attracted by chemotaxis release further cytokines and chemoattractants, which have reciprocal effects on epithelial tissue behaviour and signalling. Replication of such intricate signal networks would necessitate the use of animal models, although such approaches are not without limitations. Similarly, humoral immune components including complement and antibodies are absent. Both the lack of effective clearance of microbes and lack of regulatory components, particularly from T-regulatory cells which are critically important in modulating inflammatory signals at mucosal sites, may explain the elevation in cytokine levels seen across all experimental conditions. Further work should seek to employ a more physiologically relevant model or consider the use of animal models to ensure such factors are accounted for.

The lactate dehydrogenase assay undertaken did not include a positive control. It is possible that reduced LDH levels seen in some conditions are actually due to proteolytic degradation of the enzyme by microbes rather than true reductions in levels. Incorporation of a positive control with a known concentration of LDH enzyme with each experimental inoculum along the tissue models could have alleviated this issue. However, the trends in assay result are in keeping with what might be expected, with the two representative pathogens causing greater release of LDH, alongside increased tissue destruction seen microscopically.

Despite the above caveats, this work has demonstrated the utility of the simple denture-acrylic biofilm model incorporating respiratory pathogens developed in Chapter 2 in exploring the impact of such microorganisms on the oral epithelial environment. In particular, the role of *P. aeruginosa* in driving inflammation and invasion within the oral epithelium has been highlighted. An avenue that certainly warrants further exploration is the role of aprA in mixed species infections, as this may reveal as-yet undiscovered functions for this gene which govern microbial behaviour and inter-species interactions. Further work could seek to confirm thi finding across a range of strains, particularly clinically relevant strains associated with pneumonia. Subsequently, development of a more biologically relevant tissue model incorporating fibroblasts and immune cells may provide a more realistic insight into host-microbial interactions. Alternatively, animal models could be employed. Additionally, an *'ex vivo'* approach to modelling biofilm infections could be applied, using a PMMA insert worn on the denture-fit surface or as a separate appliance to develop a clinically relevant biofilm to subsequently seed infections in tissue models. This approach has been applied to the study of Candida biofilms in rodents (Nett et al. 2010). Once a robust assay has been developed, use of RNA-sequencing approaches to perform unrestricted explorations of gene expression in host-microbial interactions might identify novel proteins of interest. Subsequent confirmation and characterisation of such proteins might yield new diagnostic markers or therapeutic targets. Such models could also be used to identify tissue biomarkers that are indicative of infection by respiratory pathogens, through evaluation of a wider range of cytokines at different time points. Early identification of respiratory pathogens in at-risk individuals could allow for effective interventions to reduce pneumonia risk.

CHAPTER 4: ANALYSIS OF THE DENTURE MICROBIOME - ASSOCIATION WITH HOST CYTOKINE PROFILES AND PNEUMONIA STATUS - A PILOT STUDY

4.1: Introduction

The human microbiome can be defined as 'the collective genomes of the microbes (composed of bacteria, bacteriophage, fungi, protozoa and viruses) that live inside and on the human body' (Yang J 2012). As a host-microbial ecosystem, the oral cavity comprises some of the most diverse and rich microbial communities (Huttenhower et al. 2012). This is largely due to its continuity with both the gastrointestinal tract and the external environment, and the range of different niches formed by its complex assemblage of tissues.

There is a substantial body of evidence implicating the oral microbiome as a reservoir for putative respiratory pathogen (PRP) colonisation in susceptible populations (Fourrier et al. 1998, Russell et al. 1999, Didilescu et al. 2005). The link between pneumonia and oral PRP carriage is clearest in ventilator-associated pneumonia (VAP). A recent Cochrane systematic review found evidence to support the effectiveness of oral care in reducing VAP incidence in mechanically ventilated intensive care patients (Hua et al. 2016). However, the effect size was modest, with high risk of bias in most included studies, and heterogeneity in oral care interventions provided. As is frequently found in research examining interventions for VAP, the reduced incidence of pneumonia did not translate into a significant reduction in mortality. This is likely a reflection of the overall small effect size of such treatments in the context of a multifaceted care regime, coupled with high mortality levels amongst the included intensive care patients.

Research at the School of Dentistry, Cardiff has previously demonstrated that PRP colonisation of dental plaque occurs prior to colonisation of endotracheal tubes, a preliminary step in demonstrating a definitive causal link between perturbation of the oral microbiome and respiratory infection in mechanically ventilated patients (Sands et al. 2017). However, it is unclear if changes in oral microbial communities are driven solely by altered systemic health and prolonged contact with healthcare institutions, or also influenced by the presence of the artificial biomaterial surface of the endotracheal tube. In the case of the latter, endotracheal tubes and dentures may both act as a nidus for PRP colonisation.

4.1.1: The denture-associated microbiome and pneumonia - summary of the evidence

Building on evidence linking the oral microbiome and pneumonia, recent research has found that denture biomaterial surfaces are frequently colonised by PRPs. The first study to consider a potential link between dentures, risk of oropharyngeal colonisation, and subsequent pneumonia, was a cross-sectional study evaluating oropharyngeal colonisation by PRPs (in this case focussing on Gram negative bacilli) in elderly subjects, stratified by increasing reliance on care institutions (Valente et al. 1978). In this study, a number of potential risk factors for Gram negative bacilli colonisation of oropharyngeal tissues were evaluated, including poor oral hygiene and the presence of dentures. Neither the presence of dentures nor poor oral hygiene were found to be significantly associated with PRP colonisation.

Subsequently, there was a dearth of research into the presence of PRPs associated with dentures until the work of Russell et al. in 1999, some 21 years later. The authors used selective culture to identify 8 known PRPs: *Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenzae, Klebsiella pneumoniae, Serratia marcescens, Proteus mirabilis* and *Escherichia coli* from dental plaque, denture biofilms and buccal mucosal swabs in elderly patients in a chronic care facility, compared with a dental outpatient control group. Chronic care patients had poorer oral and denture hygiene, and a higher number of comorbidities compared to outpatient control subjects. Importantly, PRPs were detected in 25% of chronic care patients. In 14.3% of participants, PRPs comprised more than 12% of the total recovered aerobic colonies. Notably, *P. aeruginosa* and *S. aureus* were the most frequent isolates recovered and constituted the greatest proportion of PRPs within each positive sample. While frequency of PRP colonisation was equivalent between both chronic care and dental outpatients, all dental outpatients demonstrated low levels of colonisation (defined as <1% PRP colonies within each sample).

In the following decade, a number of reports emerged detailing the presence of PRPs in the denture-associated microbiome of frail, elderly individuals in institutionalised care settings (Sumi et al. 2002, Senpuku et al. 2003, El Solh et al. 2004, Daniluk et al. 2006, Sumi et al. 2007). Furthermore, it was found that there was a high correlation between the community composition of denture biofilms and bacteria colonising the oropharynx, including a number of PRPs (Sumi et al. 2003). However, with the exception of *S. aureus*, microbes were identified at genus, rather than species level. Consequently, only guarded inferences can be made about the recovered isolates representing PRPs and by extension, pneumonia risk.

It was not until 2016 that the first report emerged using molecular techniques to identify and quantify PRPs in denture biofilms (O'Donnell et al. 2016). Here, the authors used targeted qPCR to identify a panel of 9 PRPs on the denture surfaces of patients attending Glasgow Dental Hospital. Of the 130 total participants, PRPs were identified on the dentures of 84 (64.6%) individuals. The high levels of PRPs identified in this study compared to others, likely reflected the use of a sonicating water bath, leading to more effective removal of microbes (compared to swabs used in previous studies). The results also underpin the increased sensitivity of molecular microbiological techniques over conventional culture. Of note, the authors found high levels of *S. aureus* (most prevalent; 51.2% of all samples) and *P. aeruginosa* (third-most prevalent; 11.5% of all samples), with lower levels of *S. pneumoniae* (6.9% of all samples). Such pathogenic species are conventionally more typically associated with hospitalised patients or those in long-term institutionalised care, rather than individuals in a community setting.

The increased sensitivity and accuracy in microbial identification achieved using contemporary molecular approaches is evident in the higher PRP recovery rates and species-level identification demonstrated in the above study. The use of culture-independent methods represents an important step forward in understanding the association between the denture-associated microbiome and pneumonia. Such techniques provide an advantage in identifying PRPs, many of which are challenging to isolate and grow using conventional methods (Nolte 2008, Gadsby et al. 2016). However, any targeted approach offers only limited insight into the composition of the entire microbial community. Consequently, atypical or rare PRPs may be missed in such studies. Furthermore, our understanding of infectious diseases has progressed beyond traditional concepts of pathogenicity into a 'post-Koch' era (Stacy et al. 2016), wherein functional changes across a community, rather than the presence or absence of an individual pathogenic species, are increasingly recognised as key in driving many diseases. This conceptual change has been widely accepted in gut microbiome research. Indeed, gut microbiome studies using contemporary 'omics' methodologies to profile microbial communities; their collective metabolic and functional activities, were pivotal in expanding our understanding of the importance of the complex microbial populations that reside in the gastrointestinal tract, both for normal function and development, and in contributing to a diverse range of local and systemic diseases (Kinross et al. 2011). As the oral cavity is continuous with the gut, and therefore part of the gastrointestinal tract, it is surprising that there is a comparative deficit in similar research aiming to explore interactions between host tissues and the oral microbiome.

4.1.2: Community profiling of the microbiota by metataxonomic sequencing

The above studies have demonstrated the presence of PRPs in the oral cavity and on denture surfaces in a variety of populations. However, reliance on a targeted approach to identify frequently isolated respiratory pathogens is highly reductionist and does not account for the complexity of the oral microbiome. These studies do not address population shifts that may result in respiratory pathogen colonisation, nor allow for novel putative pathogens to be identified. In addition, the role of opportunistic pathobionts may be overlooked (Mazmanian et al. 2008). It is known that most microbially-mediated diseases are characterised by a change in microbial biodiversity (reduction; in all gastrointestinal diseases investigated to date), with overgrowth of one or several species (Petersen and Round 2014). However, in periodontal disease one of the most ubiquitously implicated pathogens, *Porphyromonas gingivalis*, rarely exceeds 5% of the total microbial bioburden. Reports have shown that at relatively low levels *P. gingivalis* is able to modulate the host immune response, while simultaneously orchestrating more virulent, invasive behaviour from the surrounding microbiota, to induce the connective tissue damage characteristic of the disease (Hashishengalis et al. 2011). Clearly there are already well-established cases in which a more nuanced view of interactions within microbial communities is needed to understand aetiopathogenesis of such diseases.

The advent of Next Generation Sequencing (NGS) - and related fields of genomics, transcriptomics, and metabolomics - has revolutionised our insight into the importance of host-microbial ecosystems in health and disease (van Baarlen et al. 2013). Rapidly decreasing costs and error rates, with concomitant improvements in sequencing accuracy, genome coverage, and read depth are leading to an explosion in studies relying upon such methodologies (Voelkerding et al. 2009, Park and Kim 2016). Sequencing technologies rely on the polymerase chain reaction (PCR) to amplify either random genome fragments (*e.g.* whole genome shotgun sequencing) or targeted genes (*e.g.* 16S metataxonomic sequencing). The exponential amplification generated during PCR means that a relatively small starting quantity of bacterial DNA is required, improving sensitivity and throughput capability.

While a number of competing technologies exist, the majority of researchers rely primarily on Illumina sequencing platforms for 16S metataxonomic profiling studies. This is largely due to Illumina platforms offering a good compromise between cost per run, run time, read depth, coverage and error-rates (Liu et al. 2012, Van Dijk et al. 2014). Illumina sequencing relies on 'sequencing by synthesis' (Figure 4.1.2). Briefly; after ligation to adapter sequences, target single-stranded genomic DNA is amplified using a proprietary 'bridge-amplification' method to generate clusters of

identical, clonal sequences. Subsequently, sequencing proceeds in a manner analogous to Sanger sequencing, by addition of a specific, fluorescently labelled, reversible chain-terminating nucleotide. This nucleotide blocks the binding site for DNA polymerase, meaning no further reaction can proceed. A fluorescent camera detects those sequence clusters which are positively fluorescent, before the terminators are enzymatically cleaved, and another nucleotide can be added. Using this iterative approach, a reverse-strand sequence is obtained for the entire length of the target DNA. The strict complementarity of bases means that the original gDNA sequence can be inferred by substituting the complementary base with the sequenced base (*i.e.* adenine in the reverse-strand indicates thymine in the original, complementary gDNA strand). These reactions occur in a parallel manner, with millions of reactions occurring simultaneously, allowing unprecedented high-throughput analysis; reducing both time and monetary costs, and allowing much improved coverage and read depth than has previously been achievable (Bentley et al. 2008).

Metataxonomic sequencing differs from metagenomic sequencing in that the former relies solely upon the bacterial 16S ribosomal RNA gene (or internal transcribed spacer (ITS) gene in fungi), rather than the entire genome (Marchesi and Ravel 2015). This gene is highly conserved across bacteria and archaea, with the exception of 9 "hypervariable" regions, each spanning 1-200 base pairs, which show remarkable heterogeneity between species. Consequently, the 16S rRNA gene is an ideal candidate to allow genus and often species-level discrimination of bacteria (Yang et al. 2016). Universal primers can be designed to span one or more hypervariable regions, annealing with the conserved sections of the gene.

The latest advances in Illumina technology has permitted paired-end reads of up to 300 base pairs (bp) to be obtained with relatively low error rates. This means that greater portions of the 16S gene can be sequenced, offering improved species-level discrimination. However, many closely related species still present a challenge. An important example is the respiratory pathogen *Streptococcus pneumoniae*, which is a frequent isolate in sputum samples from patients with community-acquired pneumonia (CAP) (Welte et al. 2012). *Streptococcus pneumoniae* is a member of the Mitis group of streptococci and features a genome almost completely identical to other members, such as *Streptococcus mitis* and *Streptococcus pseudopneumoniae.* Both species are considered normal oral commensals but cannot be distinguished from their pathogenic counterpart by 16S sequencing alone, due to their very recent phylogenetic divergence (Sholz et al. 2012). In lieu of a metataxonomic sequencing approach, the autolysin gene (lytA) has been demonstrated to provide high specificity

and sensitivity in delineating *S. pneumoniae* from its commensal counterparts, and is routinely targeted in a multiplex, TaqMan qPCR assay in diagnostic laboratories around the UK (Gadsby et al. 2015).

Despite such limitations, metataxonomic sequencing represents the best available method for profiling diverse microbial communities where limited DNA quantity can be obtained. As costs decrease further, and sequencing technology continues to improve, whole-genome sequencing (WGS) will allow further exploration, with higher resolution of species and even individual strains, alongside important insights into genome function, which may help predict antimicrobial resistant isolates or identify potential novel therapeutic targets.
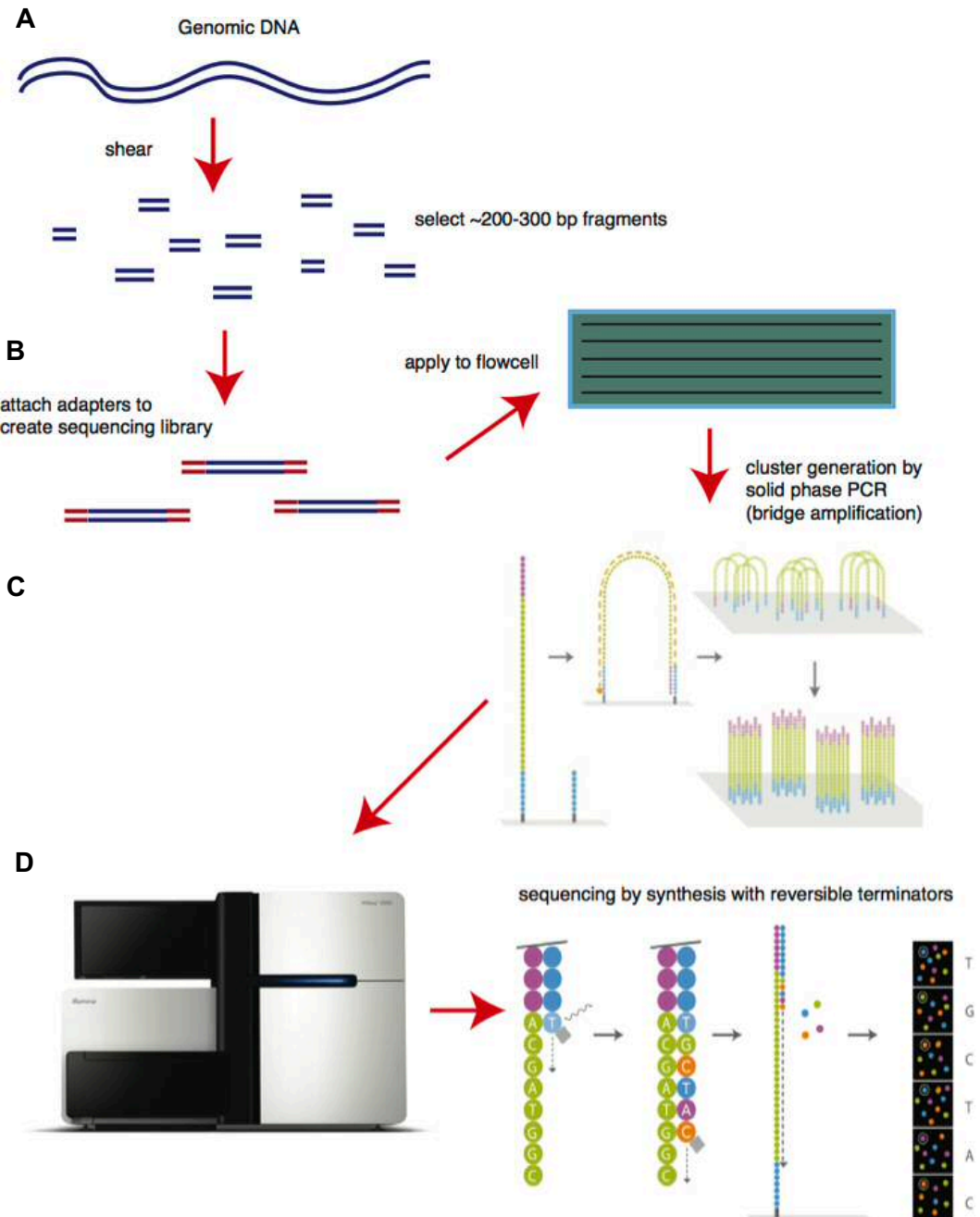
Figure 4.1.2: Illumina next generation sequencing workflow:

A - Bacterial genomic DNA is enzymatically cleaved into small (≤300 BP) fragments.
B - These fragments are ligated to adapter sequences which attach to a flowcell device coated with complementary oligonucleotides.
C - PCR "bridge" amplification generates clusters of identical gDNA amplicons.
D - Sequencing proceeds by the addition of a single fluorescently-labelled, reversible terminator nucleotide. A fluorescent camera detects any clusters positive for this nucleotide. The terminator is then washed away, and the next fluorescent nucleotide added. The process continues iteratively until the entire length of the strand has been sequenced.
Image from: https://bitesizebio.com/13546/sequencing-by-synthesis-explaining-the-illumina-sequencing-technology/

4.2: Aims

The principle aims of this work package were to:

1. Compare the denture-associated oral microbiota in respiratorily healthy care home residents with hospitalised pneumonia patients.

2. Compare the incidence of two target PRPs - *Staphylococcus aureus* and *Pseudomonas aeruginosa* - recovered by conventional microbiological culture between cohorts.

3. Evaluate the potential of pro-inflammatory salivary cytokines as biomarkers for oral PRP colonisation and pneumonia status.

Hypotheses

- There will be an increased abundance of PRPs in pneumonia patients compared with care home residents (detected by both culture dependent and independent methods)

- There will be a reduction in microbial diversity in pneumonia patients compared with care home residents

- Proinflammatory salivary cytokines will be raised in patients with a high oral PRP load

- Proinflammatory salivary cytokines will be raised in pneumonia patients compared with care home residents

4.3: Materials and Methods

4.3.1: Ethical approval

The approved study documents are presented in in Appendix I. This study was sponsored by Cardiff University. Following internal peer-review by the PhD supervisory team, ethical approval was obtained from Wales REC 6; reference number 16/WA/0317. Local R&D approval was obtained from the Cardiff and Vale University Health Board for recruitment of patients from the University Hospital Wales (UHW) and University Hospital Llandough (UHL) sites. As all included care homes were private institutions, no local approval was required. However, each home was provided with a letter of access to sign, which was countersigned by a university representative from the contracts department and the PhD student. This letter evidenced permission to enter the premises for the purposes of the study during pre-established dates and provided a formal record to each care home.

4.3.2: Participant recruitment

For this pilot study, the initial intention was to recruit 100 participants, comprising 50 care home residents and 50 hospitalised pneumonia patients. However, due to challenges in recruiting from respiratory wards, this was amended to 35 patients per cohort (70 in total). In addition to microbial samples, demographic information, relevant medical history and smoking status were recorded for each participant. A brief dental history and examination was undertaken. Information pertaining to oral health, such as denture cleaning habits, denture hygiene, Newton's classification of palatal inflammation (Newton 1962) and a Decayed, Missing and Filled Teeth score (Klein et al. 1938) was also included as part of the assessment for each participant. Both participants and caregivers were asked about oral and denture care, including who was normally responsible for this. All information was recorded on a pre-formulated data collection sheet.

4.3.2.1: Selection of recruitment sites

Suitable hospital wards at both University Hospital Wales (UHW) and University Hospital Llandough (UHL) were identified through discussion with consultants and R&D leads for each location. Care homes were selected from a list used in a prior study conducted by colleagues in the School of Dentistry, as these sites had shown interest in research participation previously. In total, 6 hospital wards were included for recruitment: a general medical ward with a focus on respiratory medicine, 2 geriatric general medical wards, a medical-decisions ward (in UHW); a respiratory

ward and medical decisions ward (in UHL). Of the 24 care homes approached for inclusion in the study, 7 agreed to participate.

4.3.2.2: Inclusion and exclusion criteria

Care home residents were deemed eligible for inclusion in this study if they were over 18 years of age, had capacity to consent (assessed on a case-by-case basis), and wore a removable denture (complete or partial) of any material.

Pneumonia patients could be included if they met the above inclusion criteria and had a confirmed diagnosis of pneumonia, with evidence of focal consolidation on chest x-ray. If radiographic signs were inconclusive, the opinion of the treating consultant was sought to confirm the diagnosis.

Individuals who lacked capacity to consent, refused to participate in the study for any reason, were receiving palliative end-of-life care or had taken part in another study in the preceding 6 months were excluded. Also excluded were individuals who were severely immunosuppressed (*e.g.* chemotherapy recipients) or immunocompromised; and patients with oro-pharyngeal or lung malignancy. Care home patients who had been diagnosed with any form of respiratory infection in the preceding 30 days were excluded due to potential effects on the oral microbiota and salivary cytokine profiles.

4.3.3: Sample collection and immediate processing

A total of 7 samples were collected from each patient (Figure 4.3.3). Foam squares measuring 2x2 cm were dampened in phosphate buffered saline (PBS) solution (0.9% w/v) and pressed against the denture-fit surface, dorsal tongue and denture-bearing palate, for 30 s each. The foam was then transferred to blood agar, imprinting the recovered microorganisms onto the agar surface. It was left in contact with the agar surface for at least 1 min, before sequential transfer to CHROMagar™ Candida (CHROMagar, Paris, France), Mannitol Salt agar and Pseudomonas agar, to culture *Candida* species, staphylococci and Pseudomonades, respectively. Sterile swabs were similarly steeped in PBS prior to sampling from the same sites, before placement in Amies transport medium to preserve cell-viability and thus DNA integrity during transit. Finally, a sterile cotton salivette was placed in the buccal sulcus of each participant for at least 1 min, to collect unstimulated saliva, before recovery into a sterile container. Salivettes were centrifuged twice for 5 min at $3000 \times g$ to recover decellularised saliva, which was transferred to microcentrifuge tubes and stored at -20°C until further use.

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Wherever possible, all samples were obtained at least 1 h after participants had eaten, this was to ensure minimal contamination by foodstuffs, and to assess normal salivary flow. However, in some cases it was not clear when participants had last eaten, or for pragmatic reasons, less than 1 h had elapsed prior to sample collection.
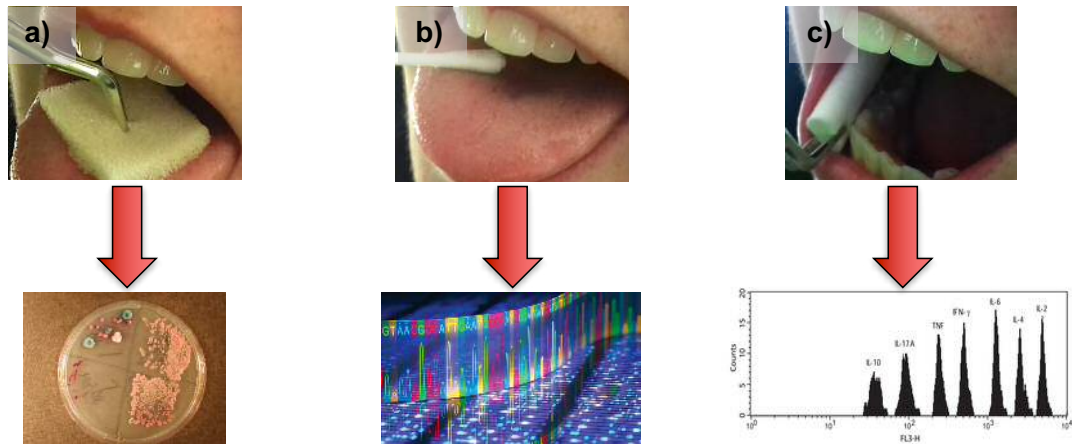
Figure 4.3.3: Sampling methods for this study.

a) Foam squares pressed against the dorsal tongue for 30 s then immediately imprinted on to agar plates (CHROMagar™ Candida shown here).

b) Sterile cotton swabs passed gently over the dorsal tongue using a rolling motion in a standardised manner, before placement into Amies transport medium and subsequent extraction of bacterial genomic DNA for metataxonomic sequencing.

c) A cotton salivette, placed aseptically into the buccal sulcus for 1 min to collect unstimulated saliva for cytokine assessment by flow cytometry.

4.3.4: Screening of imprint cultures by conventional microbiological techniques

Following imprinting of foam squares on to agar surfaces to inoculate any recovered microorganisms, agar plates were cultured for 48 h aerobically, at 37˚C to allow colony growth. Secondary isolation of morphologically distinct colonies for each site on selective agars was undertaken, using the plate streak method with a flame-sterilised metal inoculation loop. Isolates presumptively identified as *S. aureus* and *P. aeruginosa* were further tested by biochemical methods. A representative 'sweep' of blood agar colonies was collected and frozen on polypropylene beads in a glycerine-based cryopreservative (Microbank™ Bacterial and Fungal Preservation System, Pro-Lab Diagnostics, Birkenhead, UK).

4.3.4.1: Identification of presumptive *Candida* species using CHROMagar™ Candida

CHROMagar™ Candida contains a proprietary chromogenic mix which enables differentiation of *C. albicans* (green)*, C. tropicalis* (metallic blue) and *C. krusei* (pink). This medium can also indicate the presence of *C. glabrata* (mauve-brown), but several other species may appear similar in colour, reducing specificity. Colonies which differed in colour, or in the case of cream or mauve-brown, any obvious morphologically distinct colonies, were isolated and subsequently cultured on Sabouraud Dextrose Agar (SDA) aerobically for a further 48 h at 37ºC. Representative colonies were emulsified in sterile distilled water on a glass slide surface, then heat fixed. Subsequently, fixed slides were Gram-stained by immersion in crystal violet for 60 s, Lugol's iodine for 60 s, acetone decolourisation for 5 s and counterstaining with carbol fuchsin for 30 s (washed with running tap water between each step). Glass 0.17mm thick (#1.5) coverslips were mounted onto the stained slides, which were examined by light microscopy, using a x100 oil-immersion lens. A control slide was prepared using a *C. albicans* reference strain (ATCC 90028) with each batch of staining. Any colonies identified as *Candida*-positive by CHROMagar and exhibiting yeast cell or hyphal morphology under microscopic examination were subcultured for 48 h, then stored on frozen microbank beads for future use.

4.3.4.2: Identification of presumptive *Staphylococcus* species

Colonies on MSA plates after 48 h aerobic growth were assessed based on morphological characteristics and mannitol metabolism. MSA contains the indicator phenol-red, which changes to a yellow colour in the presence of the acidic by-products produced following the breakdown of mannitol by *S. aureus*. Each distinct colony was inoculated on tryptone-soy agar (TSA), and cultured aerobically for a further 48 h at 37˚C. After culture, colonies were heat-fixed on to the surface of a glass slide and Gram-stained using the method described in 4.3.4.1. A control slide was prepared with each batch, using *S. aureus* CTC 6571. Isolates identified as Gram-positive cocci were selected for further biochemical testing.

Presumptive *S. aureus* were identified using catalase and coagulase tests. Colonies were sampled using a sterile, plastic inoculation loop and steeped in 20% (w/v) hydrogen peroxide solution. Production of gas bubbles, indicating enzymatic breakdown of hydrogen peroxide into water and oxygen, was interpreted as a positive test result. Such catalase-positive isolates were tested for coagulase activity using a Staphaurex™ kit (Staphaurex Plus Latex Agglutination Test, Fisher Scientific UK Ltd, Loughborough, UK), according to the manufacturer's instructions. The test solution,

containing fibrinogen-coated latex beads, was dropped on to the test card surface, alongside a control solution containing a latex bead suspension, without fibrinogen. A plastic inoculation loop was used to transfer test colonies onto the card, and the bacterial colonies were mixed thoroughly to emulsify them in the latex solution for 30 s. Latex agglutination, visible as 'clumping' of the test solution, confirmed the presence of *S. aureus* isolates. Isolates which tested catalase-positive, but coagulase-negative, were identified as coagulase-negative staphylococci (CoNS). All staphylococcal isolates were frozen on cryo-beads, as described in section 4.3.4.

4.3.4.3: Presumptive identification of *Pseudomonas* species

Colonies present on Pseudomonas agar after 48 h aerobic growth were identified based on morphological distinction and Gram-stained as described in 4.3.4.1. A control slide was prepared with each batch, using *P. aeruginosa* ATCC 12923. Isolates identified as Gram-negative bacilli were selected for further biochemical testing.

Presumptive Pseudomonades were confirmed using the oxidase test (Sigma Aldrich, Gillingham, UK). A sterile strip of filter paper impregnated with tetramethyl-p-phenylenediamine was dampened with distilled water, before application of a single test-isolate colony using a disposable, plastic loop. Generation of indophenol resulting in a purple/blue colouration of the paper strip was interpreted as oxidase positive. A positive control (*P. aeruginosa* ATCC 12923) and negative control (*S. aureus* ATCC 6571) were included in every test batch.

4.3.4.4: Phenotypic antimicrobial susceptibility profiling of microbial cultures

Antimicrobial susceptibility of *S. aureus* and *P. aeruginosa* isolates was determined against clinically relevant antimicrobials (Table 4.3.4.4) using the EUCAST disc diffusion method (EUCAST 2017). Overnight cultures of test bacteria were prepared in Mueller-Hinton Broth (MHB), incubated aerobically at 37°C. Samples were centrifuged for 5 min at 5000 x g to pellet the suspended bacteria, and the supernatant removed. The cell-pellet was resuspended in 0.9% (w/v) PBS to a 0.5 McFarland standard, determined by photospectrometric absorbance at 600 nm wavelength. An absorbance of 0.08-0.1 indicated an approximate inoculum density of 1-1.5x10^8 cells per ml.

Standardised inocula were spread evenly across Mueller-Hinton Agar (MHA) plates using sterile cotton swabs to produce a lawn of bacteria. Filter-paper discs impregnated with a known concentration of a relevant antimicrobial agent were placed on the inoculated plates within 15 min of inoculation, at a set distance, using

an 8-cartridge antimicrobial susceptibility testing disc dispenser (Oxoid™, Basingstoke, UK). MHA plates were then incubated aerobically for 24 h at 37 °C, prior to measurement of any zones of clearing - where no bacterial growth occurred - using a digital calliper. Antimicrobial susceptibility or resistance of test isolates was determined using breakpoint tables published by the British Society of Antimicrobial Chemotherapy (BSAC 2015; Table 4.3.4.4).

Table 4.3.4.4: Antimicrobial susceptibility testing of cultured PRPs according to BSAC guidance (BSAC 2015)

Species tested	Antimicrobial	Test method	Breakpoint (zone diameter: mm)		
			S[1]	I[2]	R[3]
S. aureus	Amoxicillin	EUCAST disc-diffusion method on Mueller Hinton Agar	≥26	-	≤25
	Co-Amoxiclav		≥26	-	≤25
	Gentamicin		≥20	-	≤19
	Fusidic acid		≥30	-	≤29
	Erythromycin		≥20	17-19	≤16
	Clindamycin		≥26	23-25	≤22
	Cefoxitin		≥22	-	≤21
P. aeruginosa	Amikacin	EUCAST disc-diffusion method on Mueller Hinton Agar	≥22	16-21	≤15
	Gentamicin		≥18	-	≤17
	Imipenem		≥23	17-22	≤16
	Pipericillin-Tazobactam		≥25	-	≤24
	Ciprofloxacin		≥23	13-22	≤12
	Ceftazidime		≥24	-	≤23

4.3.5.1: DNA extraction from oral swabs

Microbial swabs were aseptically transferred to 10 ml glass Bijou bottles containing 1 ml of PBS by cutting the swab head using flame-heated scissors. The swabs were vortexed vigorously at high speed for 1 min, and the resultant cell suspension transferred to a 1.5 ml microcentrifuge tube, cooled on ice, by pipetting. Cell suspensions were centrifuged for 1 min at 13300 RPM and the supernatant removed by pouring. The cell pellet was then resuspended in 1 ml of Qiagen PuraGene proprietary cell suspension solution (Qiagen, Manchester, UK). Subsequently, bacterial DNA extractions were performed using the Qiagen PuraGene kit, following

the manufacturer's protocol for Gram-positive bacteria, with DNA elution in 20 µL of Tris-buffered water.

4.3.5.2: Validation of DNA extraction by PCR and agarose gel electrophoresis

To ensure DNA extraction from microbial swabs was successful, random samples were amplified by PCR universal bacterial 16S rRNA gene primer pair 27f (GTGCTGCAGAGAGTTTGATCCTGGCTCAG) and 1492r (CACGGATCCTACGGGTACCTTGTTACGACTT) (Eurofins Genomics LLC, Kentucky, USA) (Dalwai et al. 2007, Zuanazzi et al. 2010). All PCR reagents were purchased from Promega (Southampton, United Kingdom). In a microcentrifuge tube the following reagents were combined: 5 µL 5× GoTaq Green FlexiBuffer solution, 0.5 µL of dNTPs (0.2 mM), 1.25 µL of each of the forward and reverse primers (0.5 µM), 1 µL of MgCl$_2$ (1 mM), and 0.25 µL (1.25 Units) of *Taq* Polymerase; dissolved in 14.75 µL sterile, nuclease free water. The total reaction volume of 25 µL was reached by adding 1 µL of bacterial genomic DNA. Negative controls contained 1 µL of nuclease free water in place of DNA. PCR amplification was undertaken in a thermocycler (G-Storm, Gene Technologies Ltd, Braintree, UK) with denaturation at 95°C for 1 min, then 30 cycles of: 94°C, 50°C for 45 s each and 72°C for 90 s. Finally, samples were held at 72 °C for 5 min. Following completion of the PCR amplification, samples were stored at 4 °C until ready for gel electrophoresis.

For visualisation of PCR products by gel electrophoresis, a 1% (w/v) agarose gel was prepared, with the addition of 10% (v/v) SafeView™ solution (NBS Biologicals, Cambridgeshire, UK). Ten µl of each product was loaded into the gel, with a 1 Kbp molecular weight ladder to allow an estimate of PCR product size. Electrophoresis was performed at 70 V/cm^2 for 40 - 60 min in 0.5× Tris Borate EDTA (TBE) buffer. After completion of electrophoresis, PCR products were visualised using the BioRad GelDocIT UVP system (Bio-Rad Laboratories Ltd, Watford, UK). A single, well-demarcated, positive band of approximately 1500 bp size was considered evidence of successful extraction and amplification of gDNA.

4.4.5.3: Identification of *Streptococcus pneumoniae* by TaqMan qPCR

Due to the near identical 16S sequences of the Mitis group streptococci, including *S. pneumoniae, S. mitis* and *S. sanguinis,* species-level identification of *S. pneumoniae* by metataxonomic sequencing was not possible. As this microorganism is responsible for a high proportion of CAP cases in the UK, a species-specific TaqMan™ assay, targeting the autolysin-encoding gene *lytA* was employed. The primers used were:

(Forward primer sequence: ACGCAATCTAGCAGATGAAGCA,

Reverse primer sequence: TCGTGCGTTTTAATTCCAGCT,

Probe sequence: YY-TGCCGAAAACGCTTGATACAGGGAG-BHQ1).

The primer and probe design used was published as part of a diagnostic, multiplex qPCR detection set of common respiratory pathogens (Gadsby et al. 2015). Oligonucleotides were prepared as a single tube mixture at a 20× stock concentration of 2 µM by Life Technologies Ltd. (Paisley, UK).

In each reaction, 10 µL of 2x TaqMan Fast Advanced MasterMix (Applied Biosystems™, California, USA) was combined with 2 µL of nuclease free water; 2 µL of the oligonucleotide primer/probe mixture and a 6 µL aliquot of genomic DNA extracted as per section 4.3.5. PCR was undertaken using a QuantStudio 6 Flex instrument (Applied Biosystems™, California, USA). Thermocycler parameters were: an initial degradation stage at 95°C for 5 min, followed by 45 cycles of 95°C for 45 s and 60°C for 45 s. All reactions were run in duplicate in BrightWhite PCR 96 well-plates sealed carefully using Optical adhesive seals (PrimerDesign, Camberley, UK). In addition to a no-template control, *S. pneumoniae* ATCC 49619 was used as a positive control for each assay.

4.4.5.4: Confirmation of lytA assay sensitivity and specificity

To confirm similar sensitivity and specificity to that reported in the original paper, a standard curve was performed using *S. pneumoniae* ATCC 49619. In addition, two Mitis group streptococci - *S. gordonii* ATCC 10558[T] and *S. sanguinis* ATCC 7863 were used to confirm specificity of the assay. Test microorganisms were cultured on blood agar in 5% CO_2 at 37 °C for 48 - 72 h. Subsequently, a single colony was selected using a sterile loop and added to 10 ml of brain-heart infusion medium, with further incubation overnight in the same conditions. A standardised inoculum was prepared to approximately 1×10^8 cells/ml from this overnight culture using a spectrophotometer to measure optical density at 600 nm. DNA extraction was then performed using the procedure detailed in section 4.3.5.1.

Following extraction, seven 10-fold serial dilutions of genomic DNA were performed, to a lower limit of 10 cells/ml. Each dilution was prepared for qPCR as above and added to a 96 PCR well-plate (BrightWhite PCR, PrimerDesign, Camberley, UK) in triplicate. A no-template control was prepared using nuclease free water in place of gDNA.

4.4.5.5: Metataxonomic sequencing of bacterial 16s rRNA genes

Library preparation and next generation sequencing of genomic DNA samples was conducted by Research and Testing Laboratories (RTL, Texas, USA) using the primers 28f and 519r, which cover the V1-V3 hypervariable regions of the 16S rRNA gene, on an Illumina Miseq platform. Each DNA sample nucleotide concentration was estimated using a nanodrop (GE Genequant 1300 spectrophotometer, GE Lifesciences, Amersham, UK). A volume of 50 μL of each gDNA sample was aliquoted and packaged in dry-ice for transfer to RTL.

Staff at RTL used a two-step amplification process for samples according to the laboratory standard operating procedures. Initial preamplification was undertaken using the Illumina i5 sequencing primer with 28f 16S primer and Illumina i7 sequencing primer with the 519r 16S primer. Sequences for these primers can be seen in Table 4.3.5.5.

Table 4.3.5.5: Primers used for 16S amplification of bacterial genomic DNA

Primer	Sequence	Reference
Illumina i5	5'-CGTCGGCA GCGTCAGATGTGTATAAGAGACAG-3'	Oligonucleotide sequences © 2018 Illumina, Inc. All rights reserved.
Illumina i7	5'-GTCTCGT GGGCTCGGAGATGTGTATAAGAGACAG-3'	
28f	5'-GAGTTTGATCNTGGCTCAG-3'	Fan et al. (2012)
519r	5'-GTNTTACNGCGGCKGCTG-3'	

PCR amplification was undertaken using Qiagen HotStarTaq reagents (Qiagen Inc, California, USA). To 1 μL of template genomic DNA the following were added: 1 μL of each primer (at a concentration of 5 μM); 10 μL of 10×PCR buffer; 2 μL of dNTPs (at a concentration of 200 μM of each dNTP); 0.5 μL of HotStarTaq polymerase, and 9.5 μL of nuclease free water, to give a total reaction volume of 25 μL. An ABI Veriti thermocycler (Applied Biosystems, California, USA) was used to perform the reactions with the following thermal cycling parameters: 95°C for 5 min, followed by 25 cycles of 94°C for 30 s, 54°C for 40 s and 72°C for 1 min. A final cycle of 72°C for 10 min was undertaken, with samples stored at 4°C before further use.

After determining the amplicon concentration, a second PCR was performed, with sample volumes dependent on the amplicon concentration. The primers used in this second stage PCR were designed based on the Illumina Nextera PCR primers:

Forward - 5'-AATGATACGGCGACCACCGAGATCTACAC[i5index]TCGTCGGCAGCGTC-3' and Reverse - 5'-CAAGCAGAAGACGGCATACGAGAT[i7index]GTCTCGTGGGCTCGG-3'.

Thermocycling parameters were as used for the first PCR, but only 10 cycles were employed.

The eGel system (Life Technologies, New York, USA) was used for visualisation of PCR products. Products were then pooled in equimolar concentrations, then size-selected in two rounds using Agencourt AMPure XP (BeckmanCoulter, Indiana, USA) in a 0.7 ratio for both rounds to ensure cDNA fragments of appropriate length were pooled. Size-selected pools were quantified using a Quibit 2.0 fluorometer (Life Technologies, New York, USA) and loaded on an Illumina MiSeq (Illumina, Inc. California, USA) 2x300 flow cell at 10 pM. Sequencing was performed according to the MiSeq Reporter Metagenomics Workflow – Reference Guide (Illumina, Inc. 2014).

4.3.5.6: Sequence Analysis

Sequencing data was provided as paired FASTQ files for each sample. Generation of 16S rRNA gene sequences was undertaken using the open-source software MOTHUR (Schloss PD et al. 2009). The Illumina MiSeq standard operating procedure was followed throughout. Paired end reads were first assembled with the make.contigs command. This command combines the data from the paired FASTQ files and provides a quality score for each file. Each contig was then filtered using the screen.seqs command, using the parameters: maxn = 0, maxambig = 0, maxhomop = 5, maxlength = 605. Reads were subsampled to 675 which resulted in the exclusion of 3 samples (2 from pneumonia patients, 1 from a care home resident).

Rare OTUs (<10 reads) were excluded from further analysis and any operational taxonomic units (OTUs) with less than 98% coverage or 97% sequence identity to a known bacterial species were categorised to genus level only. After manual scanning, OTUs that would not be expected to occur in the oral cavity were re-examined using the NCBI BLASTn database. Where changes were made through BLASTn, the highest scoring species assignment was selected and OTUs reclassified accordingly, using the threshold of at least 98% coverage and 97% sequence identity.

4.3.5.7: Analysis of salivary cytokines by cytometric bead array using flow-assisted cell sorting

Saliva was obtained as described in section 4.3.3 and analysed for a panel of 6 pro-inflammatory cytokines (Table 4.3.5.7), using a multiplex cytometric bead array kit (Human Inflammatory Cytokines Kit, BD Biosciences, Wokingham, UK). The manufacturer's protocol for supernatant samples was followed, including the recommended standard curve parameters.

A flow-assisted cell sorter (BD FACSCanto II, BD Biosciences, Wokingham, UK) was calibrated using the proprietary setup beads to achieve appropriate laser power. All samples were processed at medium speed, with a total of 10000 observations per run. Samples were analysed in a single batch to eliminate inter-run variability.

Table 4.3.5.7: Assay sensitivity for cytokine detection in the multiplex cytometric bead array kit (according to assay handbook)

Cytokine measured	Assay sensitivity (pg/ml)
IL-12p70	1.9
TNF	3.7
IL-10	3.3
IL-6	2.5
IL-1β	7.2
IL-8	3.6

Data was analysed using the FACsuite software (BD Biosciences, Wokingham, UK). Imported data was manually noise-filtered, and cytometric bead identities manually assigned to the observed clusters. A standard curve was generated, and samples interpolated against this using a 5-parameter logistic regression model, as per the manufacturer's instructions.

4.3.6: Statistical analyses

Statistical analysis was conducted using R (R Core Team 2013), SPSS 21 and Microsoft Excel. Simple descriptive summary statistics were undertaken on participant demographic data and oral health scores. Age, Charlson Index (Charlson et al. 1987), Denture Hygiene Score, Newton's Classification (Newton 1962) and DMFT scores (Klein et al. 1938) were treated as continuous variables. The remaining variables were analysed as categorical variables. Distribution of data was assessed

by visual inspection of histograms, the Kolgomorov-Smirnov test of normality (alpha set to p<0.05) and inspection of Q-Q plots and detrended Q-Q plots. To assess any difference between participant cohorts at baseline, the Kruskal-Wallis test was undertaken on nonparametric data, while two-way ANOVA with Bonferroni correction was used to analyse normally distributed continuous data. Categorical variables were analysed using the Chi-Squared (x^2) goodness of fit test.

Alpha diversity was measured by the Chao2 and Inverse Simpson Indices. Alpha diversity indices were compared using the Kruskal-Wallis test and Median K-tests.

Multivariate logistic regression was employed to assess the impact of demographic and oral health variables on Chao and Inverse Simpson indices using a forward stepwise approach. Beta diversity was evaluated from UniFrac (Unique Fraction metric; Lozupone and Knight 2005) Weighted distance matrices, analysed by principle component analysis using non-metric multidimensional scaling (NMDS) ordination. All diversity indices were generated using the vegan package in R (Oksanen et al. 2015). Permutational multivariate analysis of variance (PERMANOVA) was performed on this transformed data to determine the degree of phylogenetic dissimilarity between participant cohort samples for each oral site.

Heatmaps were created to demonstrate the relative abundance of the top 30 genera and species present in each oral site, with participants clustered by a dendrogram according to the degree of similarity in OTU profiles.

PRP species were assigned to 9 groupings: enterococci, *Acinetobacter* spp. *Enterobacteraciae*, *Haemophilus* spp., *Klebsiella* spp., *P. aeruginosa*, *Serratia* spp., *S. aureus*, and *E. coli*. The percentage relative abundance of PRP species was calculated and analysed using the Two-Stage Linear Step up Procedure of Benjamini, Krieger and Yekutieli (2001) to control the false discovery rate, with Q-value set at 0.05. Fold differences between participant cohorts' PRP relative abundance were calculated for each oral site, normalised to care home residents.

Metataxonomic data was also used to explore community diversity and species richness. The Chao$_2$ index (Chao 1984) uses the number of rare OTUs (singletons and doubletons) and the total number of unique OTUs to estimate species richness. It is represented by the formula:

$$S_{Chao2} = S_{obs} + \frac{q_1^2}{2q_2}$$

S_{obs} is the total number of species (OTUs) identified

q_1 is the number of singletons (OTUs identified only once in a sample)

q_2 is the number of doubletons (OTUs identified twice in a sample)

(Gotelli and Colwell 2011).

The Inverse Simpson index (Simpson 1949) reflects community diversity (how evenly distributed the OTUs are within each sample). This index is derived from the calculated probability that any 2 individuals selected at random from each sample will represent the same OTU. Interpretation of Simpson Index values can be counterintuitive however, as increasing Simpson Index represents decreasing diversity. To allow more intuitive interpretation, the reciprocal or inverse of this value is used, so that increasing community diversity will lead to an increasing index value. Percentage relative abundance was converted to decimal data, and Linear discriminant analysis of Effect Size (LEfSe) conducted using the open access galaxy module (Segata et al. 2011), with circular cladograms generated from this analysis. A summary of the LEfSe algorithm is presented in Figure 4.3.6. Each column in the data matrix represented a sample, while each row contained a normalised OTU value. Samples were separated into classes by pneumonia status. Step 1 employed the non-parametric Kruskal-Wallis test to evaluate if the OTU values were differentially distributed between the participant cohorts. In step 2, those OTUs which differed significantly between cohorts were analysed using the Wilcoxon rank test to identify OTUs which followed the same trend in distribution within each class. The results of step 2 generated vectors, which were used to construct a Linear Discriminant Analysis (LDA) model, ranking OTUs by their relative difference in abundance, providing an effect-size linked ranking system.

Statistical analysis of salivary cytokine profiles was undertaken using Microsoft Excel and Graphpad Prism 7.0. Normality was assessed using the Kolgomorov-Smirnov test with an alpha of 0.05. Subsequent analysis was undertaken using Mann-Whitney U tests with Bonferroni correction applied post-hoc. The significance threshold selected for Bonferroni-adjusted test results was $p < 0.05$.
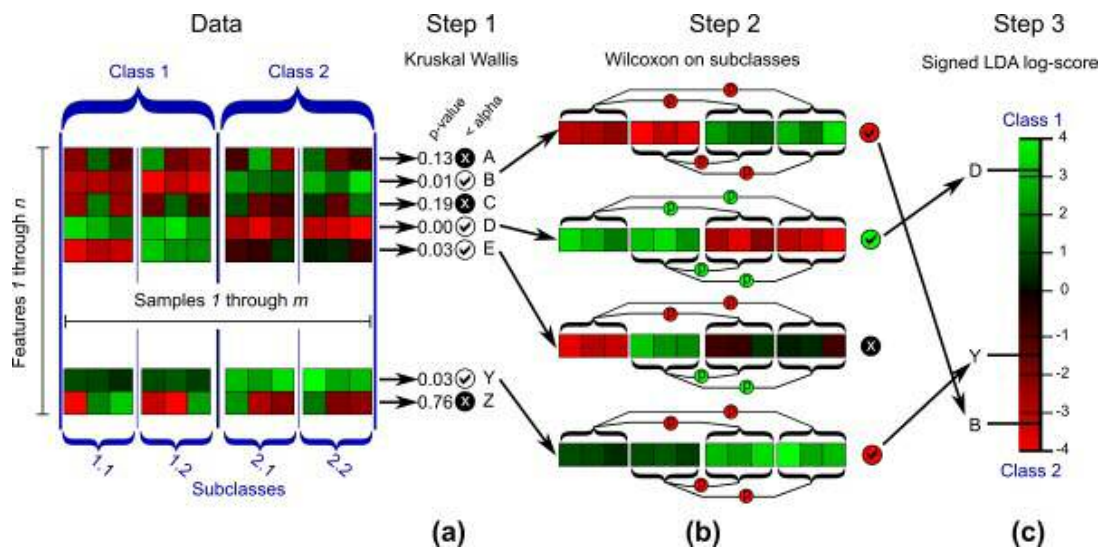
Figure 4.3.6: LeFSe stepwise statistical algorithm (reproduced from Segata et al. 2011):

For this study: Class 1 = pneumonia patients; class 2 = care home residents. Alpha = 0.05.

4.4: Results

4.4.1: Study participant demographics

A total of 61 participants were recruited for the study, comprising 35 care home residents and 26 pneumonia patients.

A summary of participant demographics, denture hygiene and basic oral health indices, is provided in Table 4.4.1. The mean age of respiratory ward patients was 84 years; a mean difference of 4 years younger compared to care home residents (P=0.0006). However, the clinical significance of this difference is questionable. All pneumonia patients received antibiotics based on British Thoracic Society protocol. Excluding cases of allergy, co-morbid conditions contraindicating their use, or blood/sputum cultures demonstrating resistant microorganisms, the first-line treatment for pneumonia patients was IV or oral Amoxicillin and Clarithromycin as deemed necessary by the treating clinician. In contrast, only 4 care home residents had received antibiotics in the preceding 6 months. All other demographic data and clinical findings were similar between included participants. A summary of the study methods is provided in Figure 4.4.1.

```
┌─────────────────────┐                              ┌─────────────────────┐
│ Care home           │                              │ Pneumonia patients  │
│ residents (n=35)    ├──────────────┬───────────────┤ (n=26)              │
└─────────────────────┘              │               └─────────────────────┘
                                     │
                                     ▼
┌─────────────────────┐   ┌──────────────────────────┐   ┌─────────────────────┐
│ 35    sample   sets │   │ Imprint cultures from    │   │ 26    sample   sets │
│ completed as planned│   │ dorsal tongue,           │   │ completed as planned│
│                     │   │ denture-bearing palate   │   │                     │
│                     │   │ and denture-fit surface  │   │                     │
│                     │   │ (to detect S. aureus,    │   │                     │
│                     │   │ P. aeruginosa & C.       │   │                     │
│                     │   │ albicans                 │   │                     │
└─────────────────────┘   └──────────────────────────┘   └─────────────────────┘
                                     │
                                     ▼
┌─────────────────────┐   ┌──────────────────────────┐   ┌─────────────────────┐
│ 35    sample   sets │   │ Swabs from dorsal        │   │ 26    sample   sets │
│ completed as planned│   │ tongue, denture-bearing  │   │ completed as planned│
│ (105 total)         │   │ palate and denture-fit   │   │ (78 total)          │
│ 2 samples failed to │   │ surface for bacterial    │   │ 6 samples failed to │
│ amplify for         │   │ DNA extraction and       │   │ amplify for         │
│ sequencing          │   │ sequencing / qPCR (to    │   │ sequencing          │
│                     │   │ detect S. pneumoniae)    │   │                     │
└─────────────────────┘   └──────────────────────────┘   └─────────────────────┘
                                     │
                                     ▼
┌─────────────────────┐   ┌──────────────────────────┐   ┌─────────────────────┐
│ 16    sample   sets │   │ Saliva sample via        │   │ 8     sample   sets │
│ completed as planned│   │ salivette for cytokine   │   │ completed as planned│
│ (19       samples   │   │ analysis                 │   │ (18       samples   │
│ inadequate   saliva │   │                          │   │ inadequate   saliva │
│ volume)             │   │                          │   │ volume)             │
└─────────────────────┘   └──────────────────────────┘   └─────────────────────┘
```

Figure 4.4.1: Participant sampling and analysis for this study

Sampling methods are shown in the order performed.

Table 4.4.1: Summary participant information

	Care home residents (n=35)			Pneumonia patients (N=26)		
Mean Age (S.D.)	88 (7.6)			84 (8.2)		
Gender (%)	15% Male, 85% Female			15% Male, 85% Female		
Antibiotics in last 90 days (%)	15% (none at time of sampling)			100% (all at time of sampling)		
Smoking History (%)	15% current smokers 54% ex-smokers 31% never smoked			8% current smokers, 65% ex-smokers, 27% never smoked		
Mean Charlson Comorbidity Index# (S.D.)	5.52 (0.97)			5.125 (2.11)		
Mean DMFT score* (S.D.)	Decayed	Missing	Filled	Decayed	Missing	Filled
	1.60 (2.00)	24.97 (5.33)	2.28 (4.38)	1.80 (1.48)	24.31 (5.77)	3.60 (3.06)
Complete or Partial Denture (%)	63% Complete 27% Partial (10% of participants had no denture in one arch)			60% Complete 15% Partial (25% of participants had no denture in one arch)		
Acrylic or Cobalt Chromium Denture (%)	91% Acrylic, 9% Cobalt Chromium			92% Acrylic, 8% Cobalt Chromium		
Mean Denture Cleanliness Index+ (S.D.)	1.83 (1.11)			1.58 (1.19)		
Mean Newton Index$ (S.D.)	0.89 (0.53)			1.10 (0.80)		

\# Charlson Comorbidity Index scores a number of physiological measures and diseases to provide estimate of 10-year survival. The Maximum score (highest mortality risk) is 33. A score of 7 or greater indicates a predicted 10-year survival rate of 0%.

* A DMFT score is indicative of Decayed, Missing, Filled Teeth. Absence of a tooth, or the presence of any dental restoration or caries scores 1 point. The maximum score is 28. Wisdom teeth were not included in this score.

\+ Denture Cleanliness Index scores denture cleanliness from 0 (pristine denture surfaces) to 4 (damaged dentures).

$ Newton Index scores palatal inflammation from 0 (normal, healthy mucosa) to 3 (grossly erythematous, swollen mucosa).

4.4.1.1: Reasons for participant exclusion

A detailed screening log was not kept as it was impossible to ascertain the total number of eligible individuals across all locations. However, common reasons for participant exclusion were noted and are summarised in Table 4.4.1.1.

Table 4.4.1.1: Common reasons for exclusion from this study

Reason	Comment
Declined to participate	Many individuals declined as they felt too unwell to participate or did not wish to partake for personal reasons.
Questionable mental capacity to provide valid consent	Often, nurses or carers would rule out individuals deemed to lack sufficient mental capacity (either formally assessed or in their professional opinion) to provide valid consent for the study. In some instances, after discussion with individuals about the nature of the study, it was evident that they lacked capacity to consent (unable to retain, understand or communicate the information provided). In any cases where capacity was considered questionable, participants were excluded.
Not wearing denture	A number of pneumonia patients had avoided wearing their denture since the onset of the disease, as they felt it affected their breathing, or found it uncomfortable. A small number of care home residents also did not routinely wear their dentures.
Medical exclusion criteria	Many participants were receiving palliative care, most frequently for malignancies. Some respiratory ward patients were under investigation for lung cancers. A small number of participants were severely immunocompromised or immunosuppressed.
No clear diagnosis (Respiratory ward only)	Frequently, no clear diagnosis was available for respiratory ward patients, or a definitive diagnosis established only after patients had recovered from the pneumonia, sometimes over a week following admission.

4.4.2: Analysis of microorganisms cultured from imprints

Foam imprints from each of the 3 oral sites were sequentially transferred to Blood Agar, then selective agars as described in section 2.3.4.1-3. Recovery rates were expressed as percentages for each oral site and participant cohort.

4.4.2.1: Recovery rates of targeted microorganisms from selective agars

The overall recovery of *Candida* species across all oral sites in all participants was 159 out of 183 total samples (87%). Presumptive *S. aureus* isolates were recovered in 31 out of 183 samples (17%), while *P. aeruginosa* was recovered in 16 out of 183 samples (9%). There was no difference in recovery rates from each oral site, nor between the participant cohorts (p>0.05).

The target species recovery rates for each participant cohort and oral sites are summarised in Table 4.4.2.1 and Figure 4.4.2.1.

Table 4.4.2.1 – Recovery rates N (%) of target microorganisms by culture

	Microbial species	*Candida* species			*Staphylococcus aureus*			*Pseudomonas aeruginosa*		
	Oral site	Tongue	Palate	Denture	Tongue	Palate	Denture	Tongue	Palate	Denture
Participant cohort	**Overall (n=61)**	54 (88)	51 (83)	(54) 88	13 (21)	8 (13)	10 (16)	6 (10)	4 (7)	6 (10)
	Care home (n=35)	31 (89)	29 (83)	32 (91)	8 (23)	4 (11)	7 (20)	3 (9)	1 (3)	3 (9)
	Respiratory ward (n=26)	23 (88)	22 (85)	22 (85)	5 (19)	4 (15)	3 (11)	3 (11)	3 (11)	3 (11)
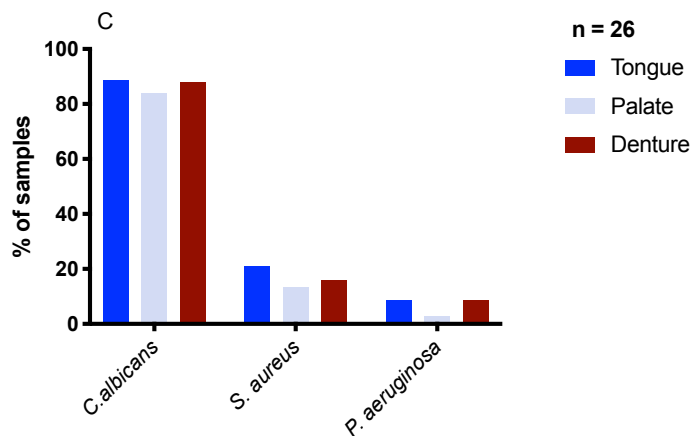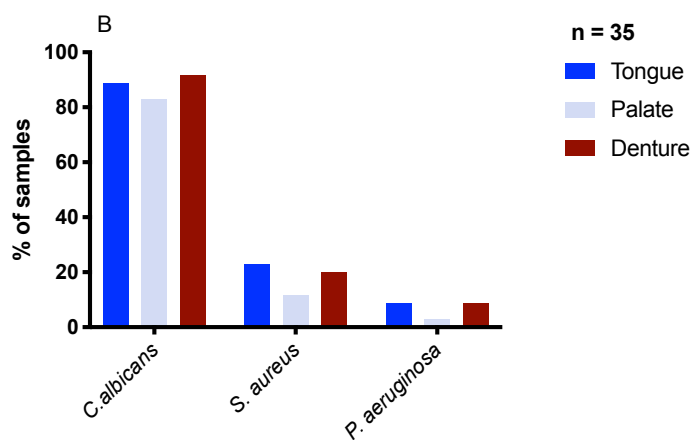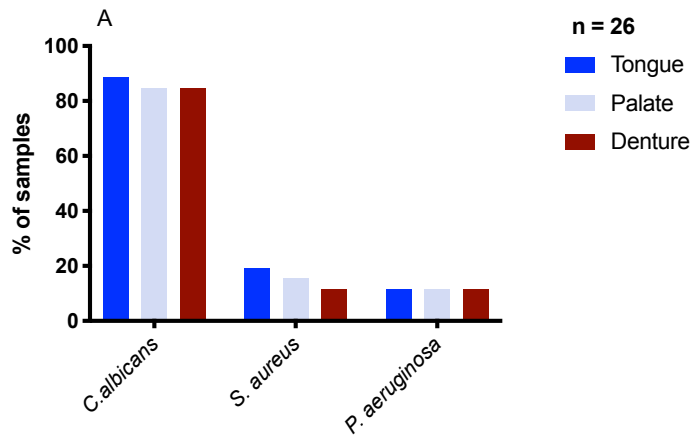
Figure 4.4.2.1 – Recovery rates of target microorganisms by culture

a) Overall – combined recovery of microbes in all study participants

b) Care home residents only

c) Respiratory ward patients only

4.4.2.2: Antimicrobial susceptibility testing of recovered species

After confirmation of isolate identity by biochemical testing and phenotypic examination, the antimicrobial susceptibility of *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates was tested against a panel of relevant antimicrobials. A summary of test isolate resistance rates for each participant cohort is presented in Table 4.4.2.2.1 and Table 4.4.2.2.2.

Table 4.4.2.2.1: Antimicrobial resistance rates (%) among cultured isolates of *S. aureus*

	Care Home Residents (n = 22 isolates)	Respiratory Ward Patients (n = 10 isolates)	Overall (n = 32 isolates)
Amoxicillin	27.27	0	18.75
Co-Amoxiclav	0	0	0
Gentamicin	4.55	0	3.13
Fusidic acid	13.64	20	15.63
Erythromycin	18.18	50 (plus 10 intermediate)	28.13 (plus 3.13 intermediate)
Clindamycin	13.64	50	25
Cefoxitin	4.55	0	3.13

Table 4.4.2.2.2: Antimicrobial resistance rates (%) among cultured isolates of *P. aeruginosa*

	Care Home Residents (n = 7 isolates)		Respiratory Ward Patients (n = 13 isolates)		Overall (n = 20 isolates)	
Amikacin	0 (plus intermediate)	57.40	7.69 (plus intermediate)	92.31	5 (plus intermediate)	80
Gentamicin	0		0		0	
Imipenem	14.29 (plus intermediate)	28.57	0		5 (plus intermediate)	10
Pipericillin-Tazobactam	85.71		46.15		60	
Ciprofloxacin	0 (plus intermediate)	28.57	84.62		55 (plus intermediate)	10
Ceftazidime	85.71		0		30	

4.4.3: Analysis of metataxonomic sequencing data

Prior to quality control, a total number of 3,658,008 sequence reads were generated from a total of 178 samples (including 3 negative controls provided as a 'dummy' sample set). Eight samples did not amplify; 2 from care home residents and 6 from pneumonia patients. After removing all reads with ambiguous base calls, a total of 914,503 sequence reads remained. Operational taxonomic units (OTUs) were sub-sampled to 675 reads, which led to a further 3 samples with low read counts being discarded. A summary of sequence reads and quality is presented in Table 4.4.3.

Table 4.4.3: Sequence data and quality after processing data using the MOTHUR pipeline

	Start	End	Number of Bases	Ambiguous base calls	Polymer	Number of Sequences
Minimum	1	35	35	0	2	1
2.5%-tile	1	492	492	0	4	91451
25%-tile	1	528	528	0	5	914503
Median	1	535	535	2	5	1829005
75%-tile	1	544	544	5	5	2743507
97.5%-tile	1	561	561	14	7	3566558
Maximum	1	602	602	268	300	3658008
Mean	1	530.109	530.109	3.59424	5.2611	

In total, there were 924 unique OTUs identified, spanning 16 bacterial phyla. Removal of OTUs with less than 10 sequence reads reduced this number to 218 unique OTUs (23.6%), comprising 9 phyla (Figure 4.4.3A&B) and 82 genera.

All oral sites in both participant cohorts were predominated by bacteria from the *Streptococcus* genus, comprising between 36-61% of all OTUs detected. There were more PRPs present in the 10 most abundant genera from respiratory ward patients' samples (Figure 4.4.3C). In tongue samples, the Pseudomonas, Klebsiella and Escherichia/Shigella genera were heavily represented in pneumonia patients, with minimal presence in care home residents' samples (Figure 4.4.3C). Palate samples demonstrated similar trends although Pseudomonas genera were similarly represented in both cohorts. Denture samples showed high abundance of Klebsiella, Escherichia and Haemophilus genera in pneumonia patients, none of which had high abundance in care home residents. There was a similar, low level of Pseudomonas genera in both cohorts.

A

Tongue

- 69% Firmicutes
- 14% Bacteroidetes
- 8% Proteobacteria
- 6% Fusobacteria
- 3% Actinobacteria
- 0.1% TM7

Palate

- 77% Firmicutes
- 8% Bacteroidetes
- 9% Proteobacteria
- 4% Fusobacteria
- 0.8% Actinobacteria
- 0.1% TM7
- <0.1% Spirochaetes

Denture

- 82% Firmicutes
- 8% Bacteroidetes
- 5% Proteobacteria
- 3% Fusobacteria
- 2% Actinobacteria
- 0.1% TM7
- <0.1% Spirochaetes

B

Tongue

- 56% Firmicutes
- 6% Bacteroidetes
- 34% Proteobacteria
- 1% Fusobacteria
- 3% Actinobacteria

Palate

- 57% Firmicutes
- 6% Bacteroidetes
- 32% Proteobacteria
- 1% Fusobacteria
- 3% Actinobacteria
- <0.1% Spirochaetes

Denture

- 60% Firmicutes
- 8% Bacteroidetes
- 29% Proteobacteria
- <0.5% Fusobacteria
- 3% Actinobacteria

Figure 4.3.3: Distribution of bacterial phyla in oral samples from care home residents and respiratory ward patients

A Care home samples   **B** Pneumonia patient samples

196
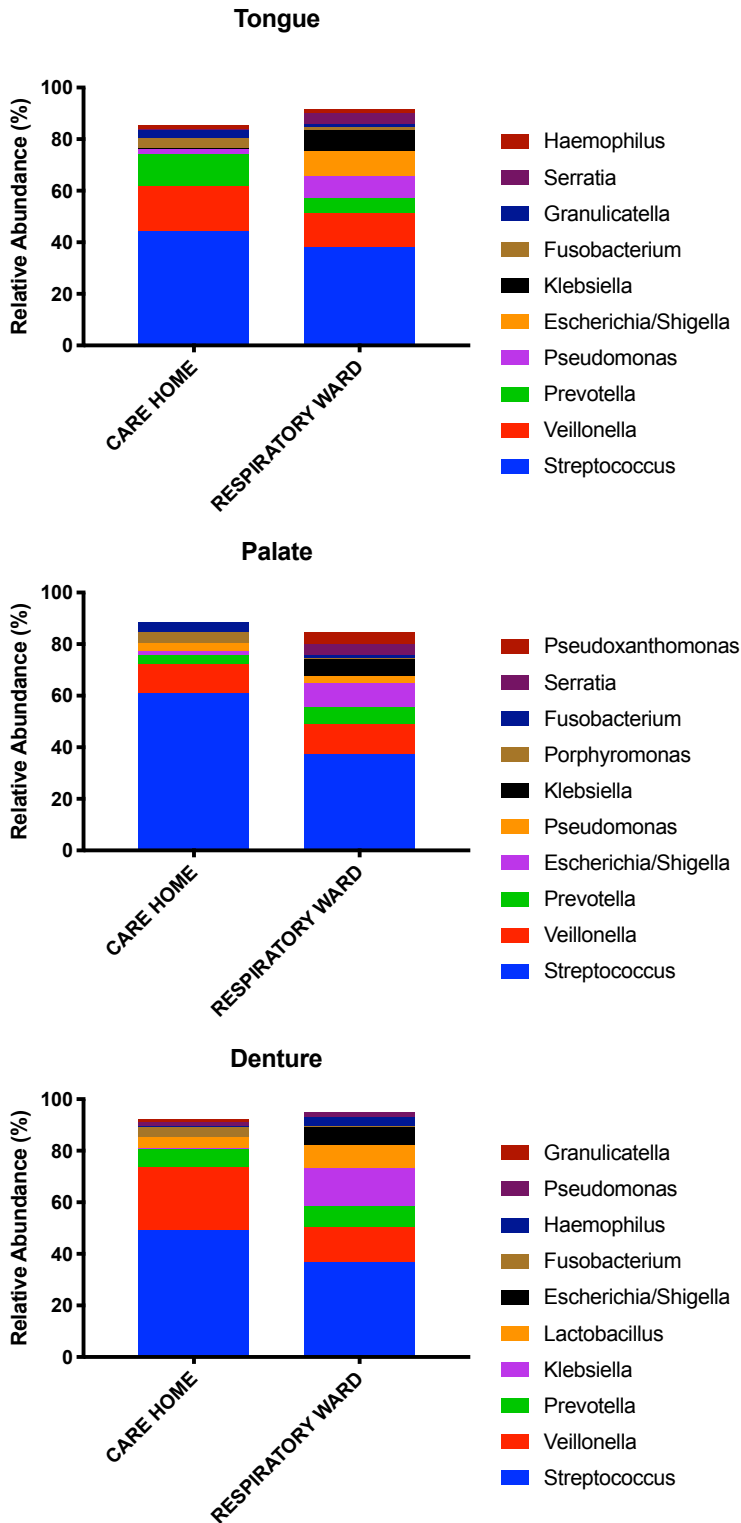
Figure 4.4.3C: Top 10 most abundant genera among oral samples from care home residents and respiratory ward patients

4.4.3.1: Relative abundance of putative respiratory pathogens

The relative abundance reflects PRP OTUs as a percentage of the total OTUs. There was a trend towards increased relative abundance of putative respiratory pathogens in pneumonia patients compared with care home residents (Figure 4.4.3.1). However, this only crossed the threshold of statistical significance for *Enterobacteriaceae*. PRP OTU proportions were similar in tongue and denture samples for both cohorts, while samples from the palate showed reduced PRP relative abundance, with the exception of *Enterobacteriaceae*, *Klebsiella* species, *Serratia* species and *E. coli.* Both *P. aeruginosa* and *S. aureus* – the target pathogens for isolation by conventional culture - were identified infrequently among the samples.

Tongue

Palate

Denture
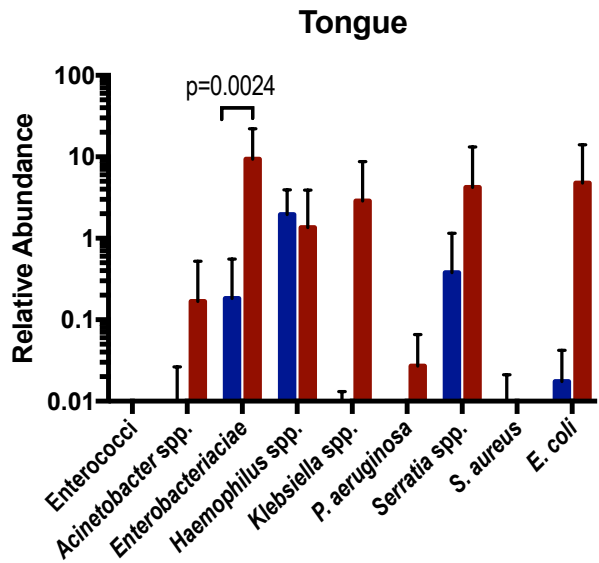
Figure 4.4.3.1:
Relative abundance (%) of putative respiratory pathogens from each oral site

OTUs are grouped at either genus or species level to collate bacteria associated with respiratory infection at the lowest discriminatory phylogenetic level. Note that the Y axis features a $\log_{10}$ scale. Mean values shown, error bars represent 95% confidence intervals.
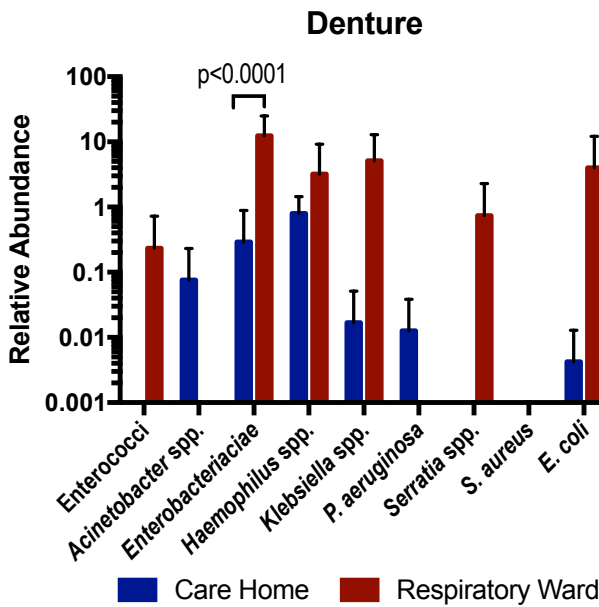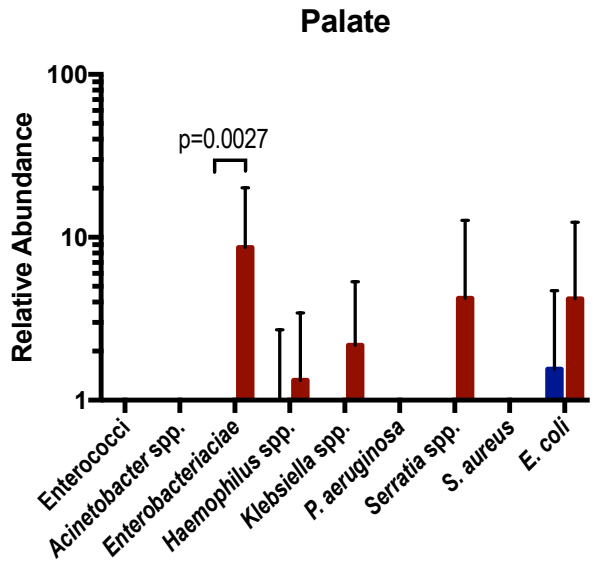
4.4.3.2: Association of Putative Respiratory Pathogens with pneumonia status

When the cumulative relative abundance of PRPs was compared between the two cohorts, a clear difference in pathogenic bioburden emerged (Figure 4.4.3.2a). This was especially pronounced in denture samples. There was a greater than 20-fold difference in PRP bioburden detected in denture samples of patients with pneumonia compared to respiratorily healthy care home residents (Figure 4.4.3.2b).
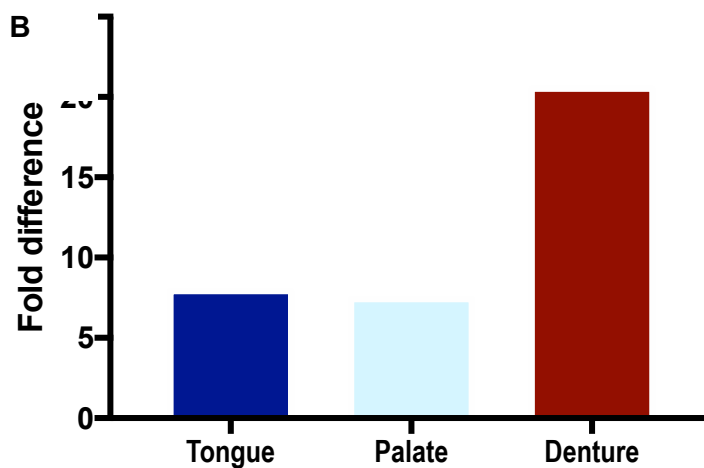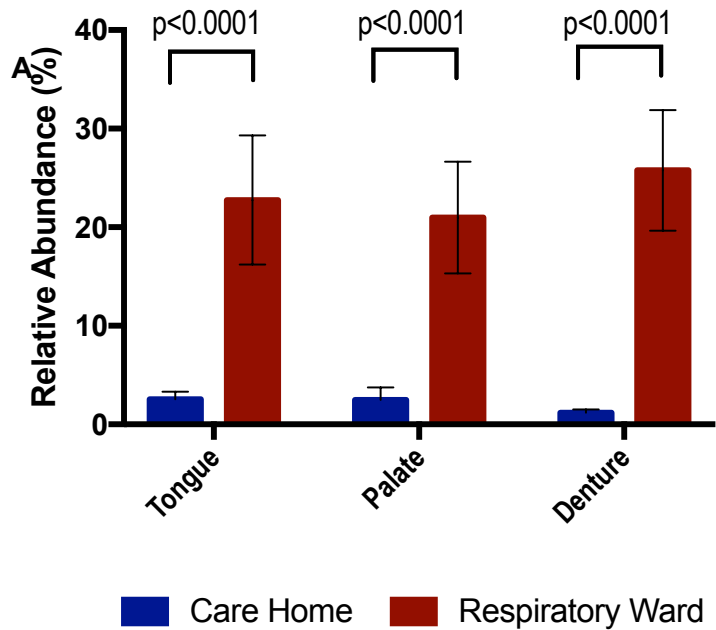
Figure 4.4.3.2:

A: Cumulative relative abundance (%) of PRPs identified in care home residents compared to respiratory ward patients. Mean values shown. Error bars represent 95% confidence intervals.

B: Fold difference of PRP cumulative relative abundance in respiratory ward patients, normalised to care home residents. Fold difference calculated using mean values reported in **a)**.
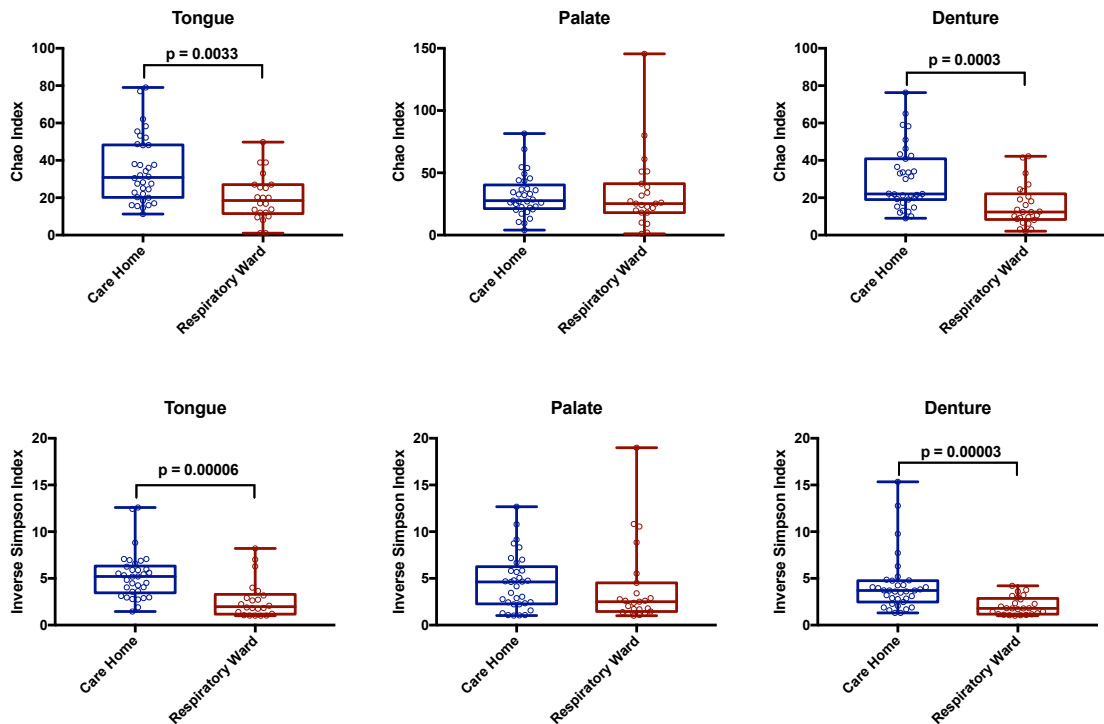
Figure 4.4.3.3 – Chao (upper panel) and Inverse Simpson (lower panel) indices for oral sites in each participant cohort
Individual data points with representative box plots shown.

4.4.3.3: Association of microbiome community diversity and species richness with pneumonia status

There was a statistically significant reduction in both community diversity and species (OTU) richness in pneumonia patients compared with care home residents, in tongue and denture samples only (Figure 4.4.3.3). There was no difference between palate samples for either richness or diversity indices.

Multivariate regression analysis using a forward stepwise approach revealed that only antibiotic status had a significant impact on microbial community diversity indices (using denture samples as the reference indices). As all patients in the pneumonia cohort and only 4 care home residents had received antibiotics, there was insufficient statistical power to justify subgroup analysis.

4.4.3.4: Principle component analysis of microbial communities

The non-metric multidimensional scaling (NMDS) ordination plots for each oral site, separated by participant cohorts are presented in Figure 4.4.3.4a-c. PERMANOVA revealed a statistically significant difference in beta diversity between participant cohorts for tongue (p = 0.02), palate (p = 0.032), and denture samples (p = 0.019),

although there was considerable overlap between samples overall. There was also a high level of dissimilarity between individual samples within each cohort.
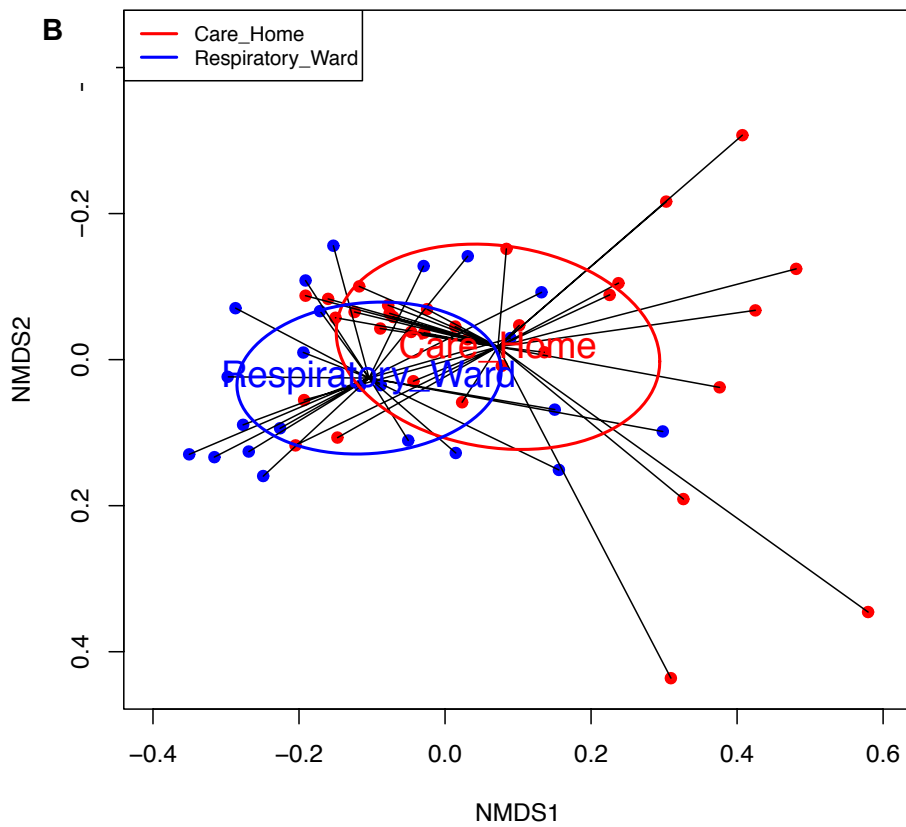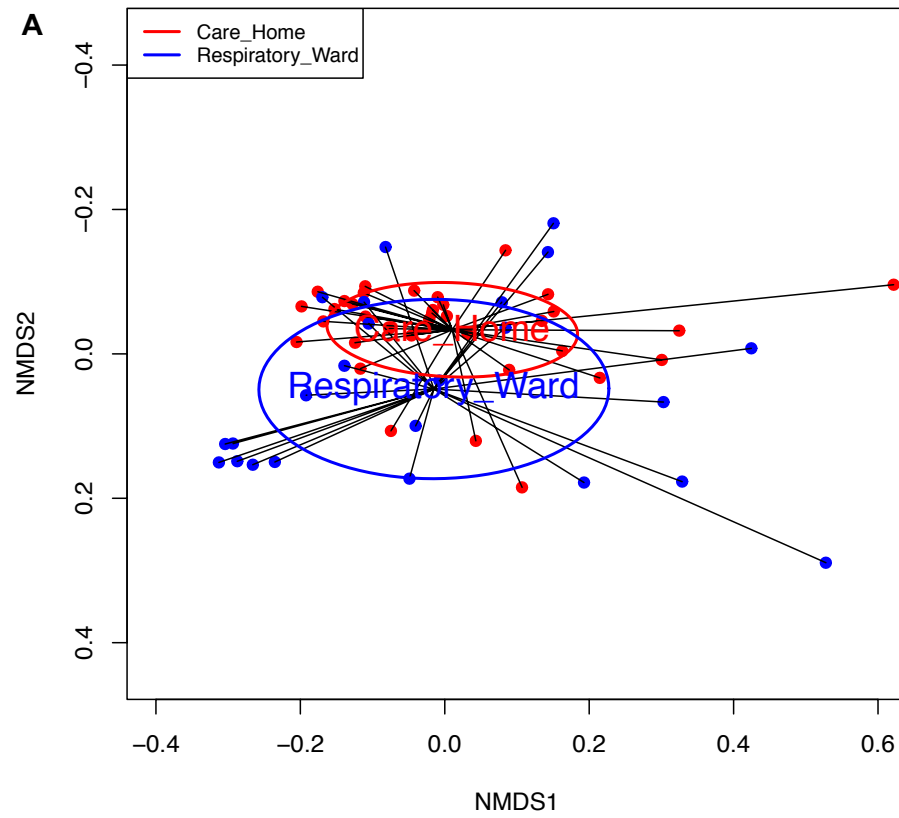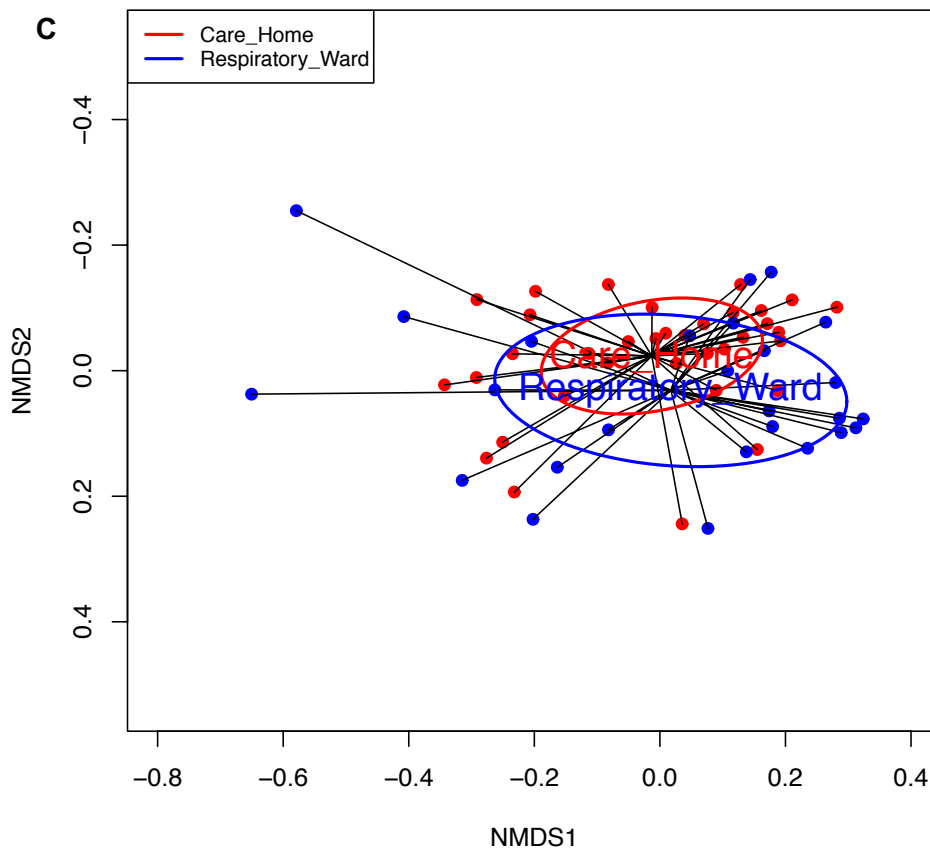
Figure 4.4.3.4: Non-metric multidimensional scaling ordination of oral samples

Scatter plot points represent individual sample ordination values coloured according to participant cohort. Groupings are encircled at 95% confidence interval boundaries.

a) Tongue samples
b) Palate samples
c) Denture samples

4.4.3.5: Heat map of microbial communities

Seven participants (3 care home residents, 4 pneumonia patients) were excluded from the analysis because metataxonomic data were not present for all 3 oral sites. Community diversity of the 30 most abundant genera and species in each sample set was analysed using the vegan package in R and the NMF package (Gaujoux and Seoighe 2010) was used to construct heatmaps for each oral site, comparing OTU clustering between participant cohorts. The ape package (Paradis et al. 2004) was used to construct comparative dendrograms overlaid onto heatmaps to highlight sample clusters with similar microbial phylogenetic profiles.

Heatmap data comparing OTUs between participants is presented in Figure 4.4.3.5 at genus (4.4.3.5a-c) and species (4.4.3.5d-f) level.

Compositional differences can be seen easily in these figures, with a tendency for respiratory ward patients' samples to cluster together, typified by increased abundance in PRP genera and species. However, the samples did not separate distinctly into participant cohorts, with clusters of samples from both participant cohorts showing considerable homogeneity in some cases.

A   **Top 30 genera in tongue samples**
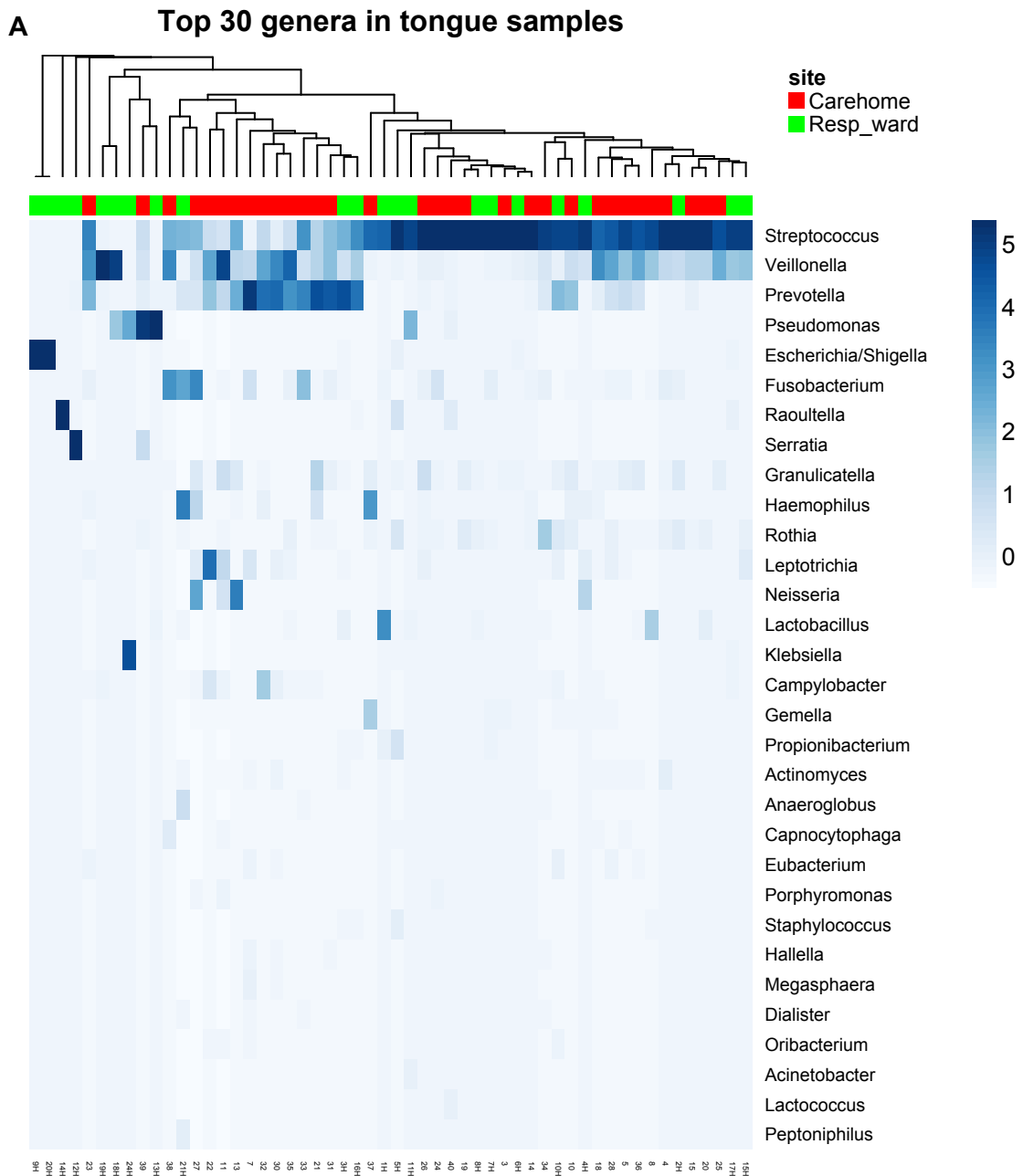
B   **Top 30 genera in palate samples**

site
Carehome
Resp_ward

Streptococcus
Veillonella
Prevotella
Escherichia/Shigella
Pseudomonas
Fusobacterium
Lactobacillus
Serratia
Raoultella
Haemophilus
Gemella
Leptotrichia
Neisseria
Granulicatella
Propionibacterium
Klebsiella
Campylobacter
Capnocytophaga
Atopobium
Rothia
Rubritepida
Anaeroglobus
Spirosoma
Staphylococcus
Dialister
Actinomyces
Enterococcus
Rubellimicrobium
Eubacterium
Friedmanniella
Porphyromonas

C   **Top 30 genera in denture samples**

D **Top 30 species in tongue samples**
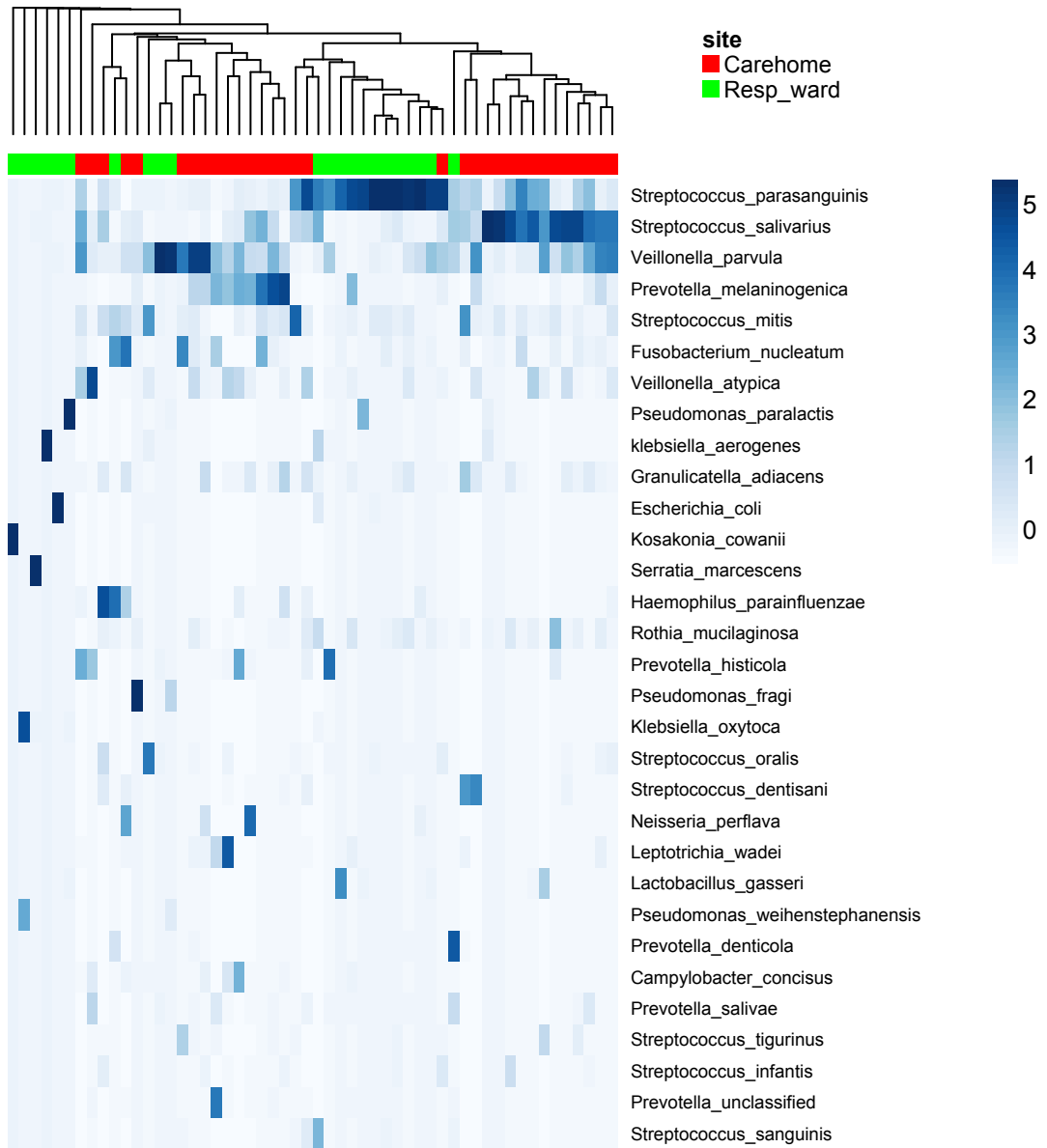
E   **Top 30 species in palate samples**
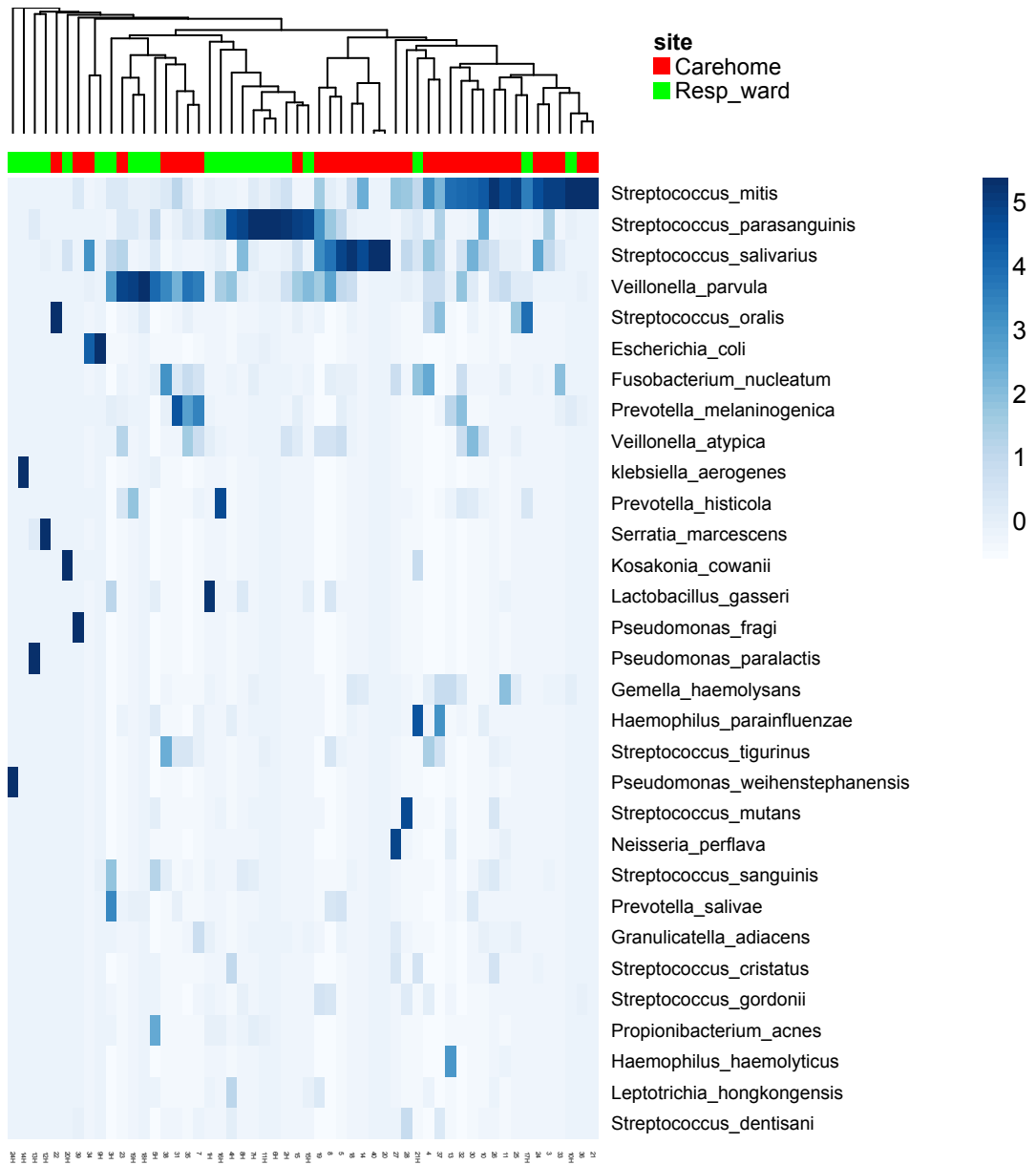
F Top 30 species in denture samples
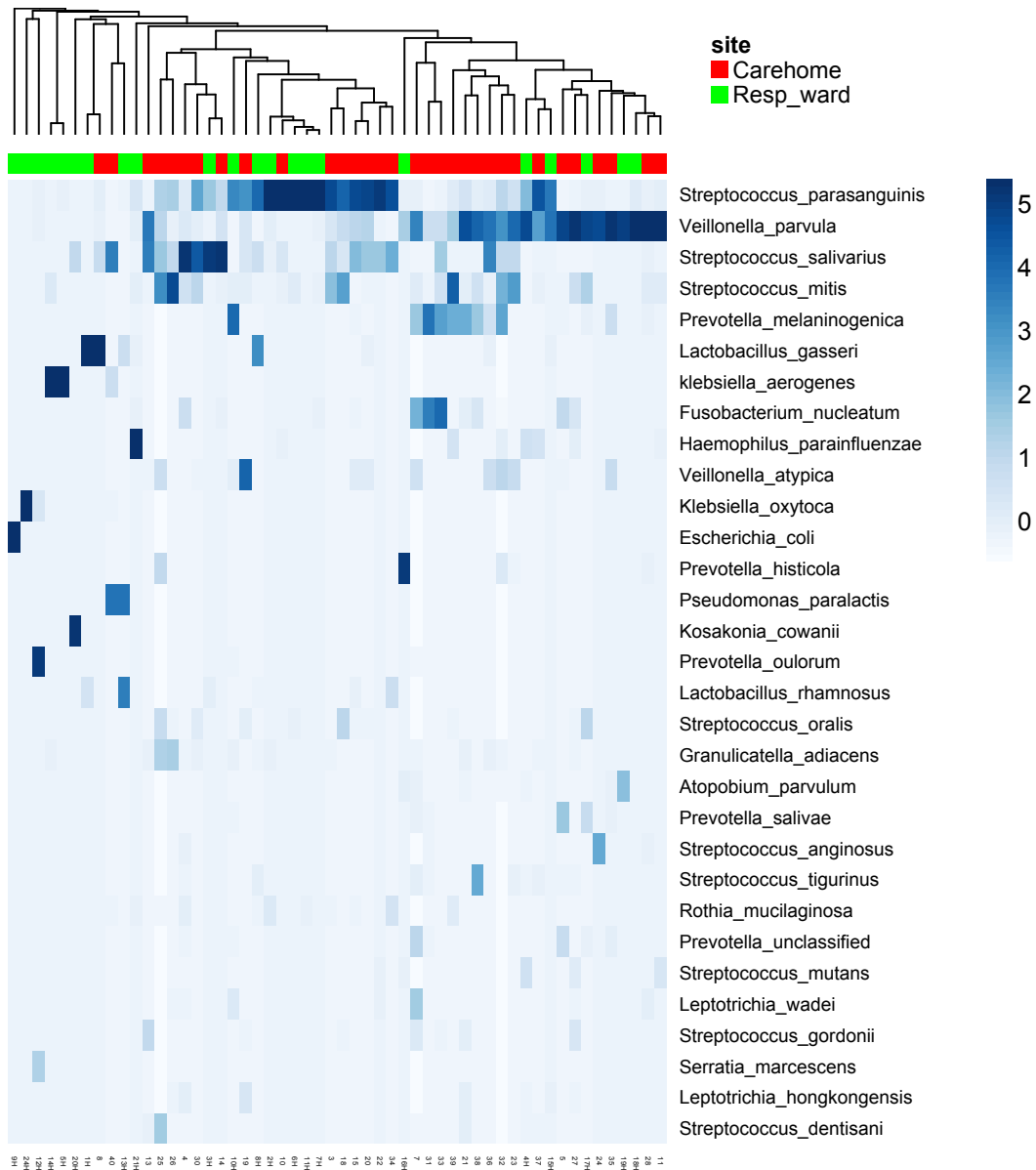
Figure 4.4.3.5: Heatmaps constructed from metataxonomic sequencing data demonstrating the most abundant 30 genera (A-C) and species (D-F) level OTUs.

Dendrogram indicates most closely related clusters of samples.

4.4.3.6: LeFSe analysis of microbial communities

There was a consistent increase in the relative abundance of the proteobacteria phylum in pneumonia patients for all oral sites. The PRP genera Klebsiella and *Enterobacteriaceae* were among the most elevated in abundance among pneumonia patients compared with care home residents in all oral sites. *Escherichia/Shigella*

genera were elevated in the tongue samples of pneumonia patients, while *Pseudomonas* species were also present in significantly increased abundance in all oral sites.

Respiratorily healthy care home residents had significantly increased relative abundance of bacterial phyla and genera associated with a 'normal' oral microbiome. There was an increase in species from the *Fusobacterium* and *Veillonella* genera and *Bacteroides* class in all oral samples compared to pneumonia patients. Additionally, palate and denture samples revealed an increase in species from the *Streptococcus* genus. The LDA scores demonstrating significant differences between participant cohorts for each oral site are presented in Figures 4.4.3.6a-c, and cladograms mapping the phylogenetic distribution of differentially abundant OTUs are shown in Figures 4.4.3.6d-f. It should be noted that LDA score magnitude indicates overabundance of the represented taxonomic level according to the class assignment (represent as red or green here), regardless of direction. This means that the LDA score of -4.7 for Bacteroidetes in Figure 4.4.3.6a indicates an overabundance of this phylum in care home residents (coded red), while the LDA score of +5.1 for Proteobacteria indicates an overabundance in pneumonia patients.

A

Tongue

B
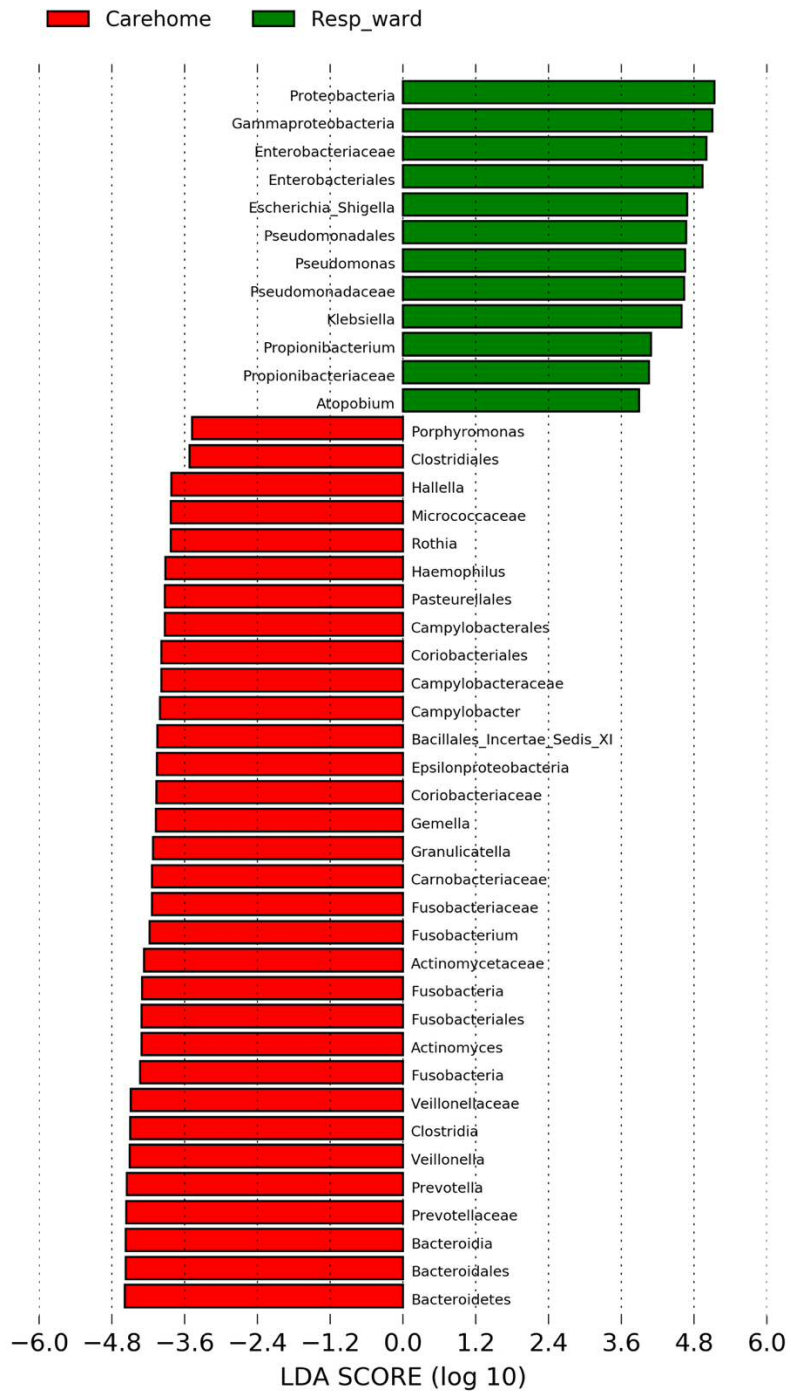
Palate
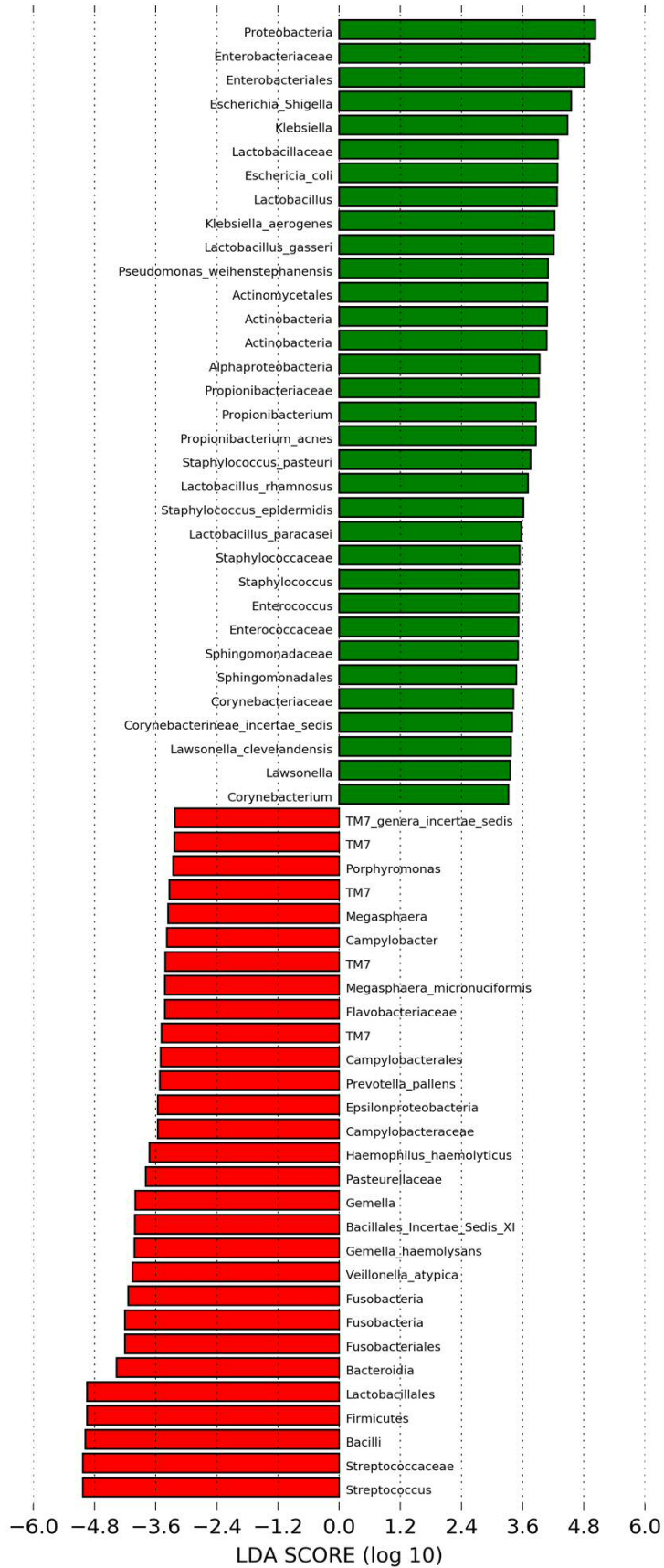
C
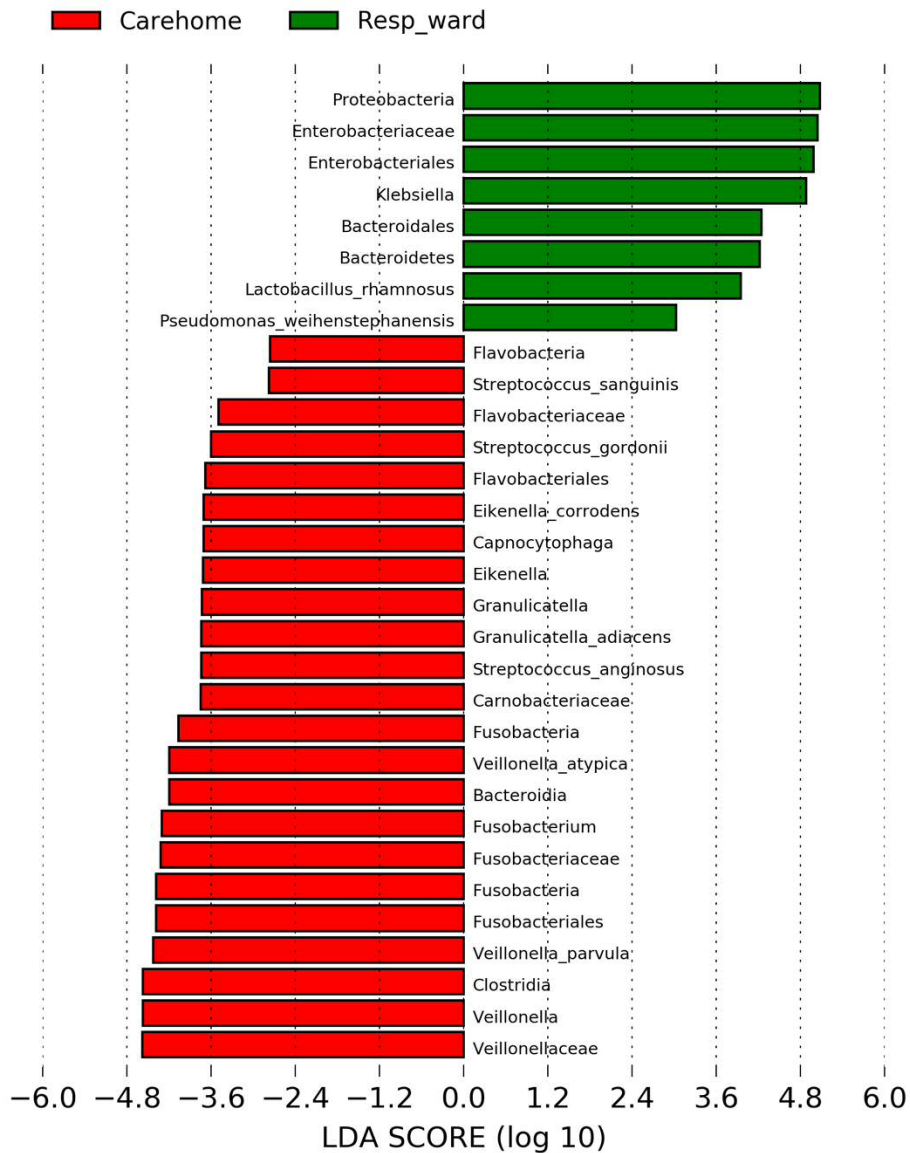
Denture

Figure 4.4.3.6: LeFSe analysis of differential OTU relative abundance between participant cohorts for each oral site

Above: Significantly different Linear Discriminant Analysis scores for samples from:
A. **Tongue**
B. **Palate**
C. **Denture**

Note all LDA scores are transformed to $\log_{10}$ scale
Below: Cladograms constructed from a-c mapping the phylogenetic relationships between differentially express genera:
A. **Tongue**
B. **Palate**
C. **Denture**

D

Tongue

Carehome
Resp_ward
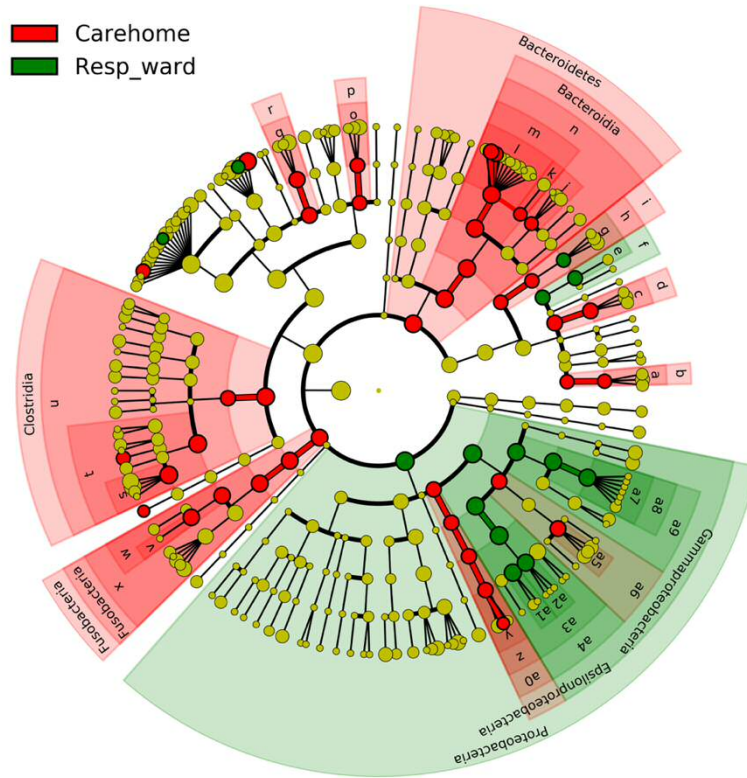
- a: Actinomyces
- b: Actinomycetaceae
- c: Rothia
- d: Micrococcaceae
- e: Propionibacterium
- f: Propionibacteriaceae
- g: Atopobium
- h: Coriobacteriaceae
- i: Coriobacteriales
- j: Porphyromonas
- k: Hallella
- l: Prevotella
- m: Prevotellaceae
- n: Bacteroidales
- o: Gemella
- p: Bacillales_Incertae_Sedis_XI
- q: Granulicatella
- r: Carnobacteriaceae
- s: Veillonella
- t: Veillonellaceae
- u: Clostridiales
- v: Fusobacterium
- w: Fusobacteriaceae
- x: Fusobacteriales
- y: Campylobacter
- z: Campylobacteraceae
- a0: Campylobacterales
- a1: Escherichia_Shigella
- a2: Klebsiella
- a3: Enterobacteriaceae
- a4: Enterobacteriales
- a5: Haemophilus
- a6: Pasteurellales
- a7: Pseudomonas
- a8: Pseudomonadaceae
- a9: Pseudomonadales

E

Palate

Carehome
Resp_ward
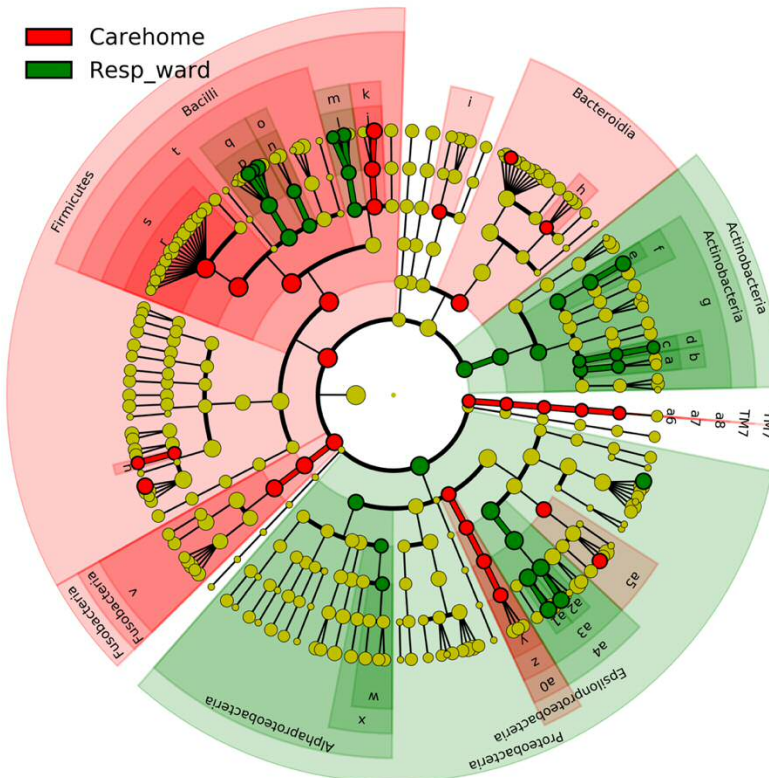
- a: Corynebacterium
- b: Corynebacteriaceae
- c: Lawsonella
- d: Corynebacterineae_incertae_sed
- e: Propionibacterium
- f: Propionibacteriaceae
- g: Actinomycetales
- h: Porphyromonas
- i: Flavobacteriaceae
- j: Gemella
- k: Bacillales_Incertae_Sedis_XI
- l: Staphylococcus
- m: Staphylococcaceae
- n: Enterococcus
- o: Enterococcaceae
- p: Lactobacillus
- q: Lactobacillaceae
- r: Streptococcus
- s: Streptococcaceae
- t: Lactobacillales
- u: Megasphaera
- v: Fusobacteriales
- w: Sphingomonadaceae
- x: Sphingomonadales
- y: Campylobacter
- z: Campylobacteraceae
- a0: Campylobacterales
- a1: Escherichia_Shigella
- a2: Klebsiella
- a3: Enterobacteriaceae
- a4: Enterobacteriales
- a5: Pasteurellaceae
- a6: TM7_genera_incertae_sedis
- a7: TM7
- a8: TM7

214

F Denture
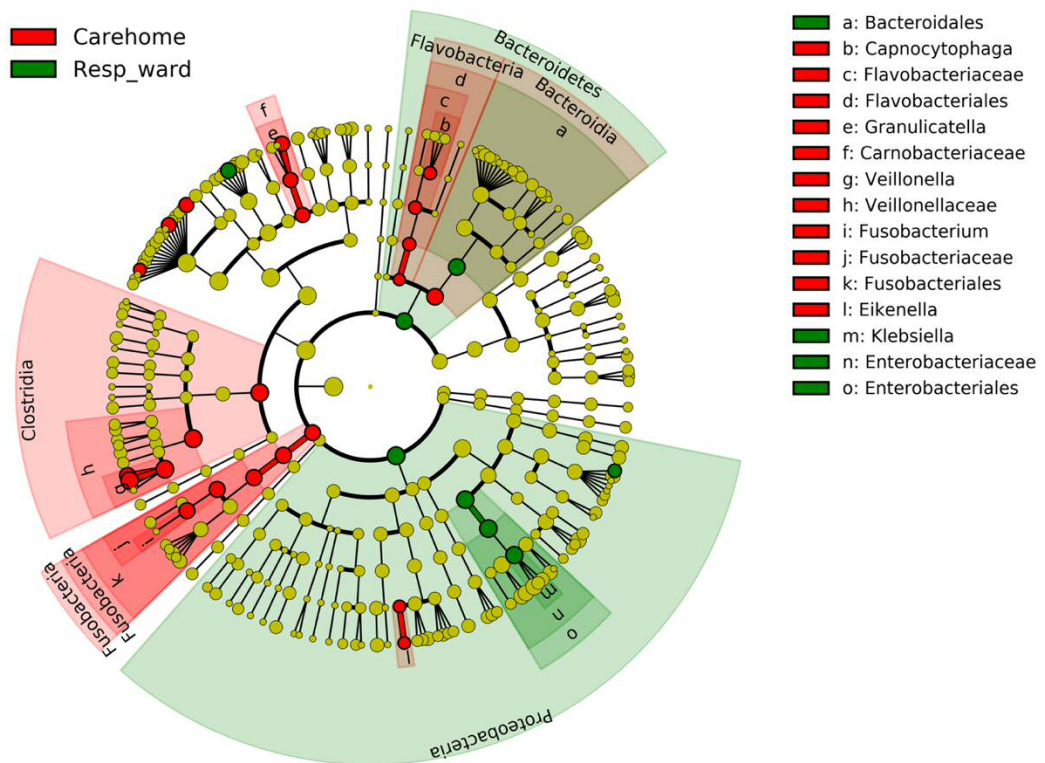
4.4.4: Analysis of salivary cytokine profiles

Unstimulated whole saliva was obtained from a total of 24 participants (16 care home residents and 8 respiratory ward patients). There was an inadequate volume of saliva for analysis in the remaining 37 participants. Indeed, many participants failed to express any detectable saliva after 2 min.

There was no significant difference in the expression of 6 pro-inflammatory cytokines analysed in participants, nor any discernible trend in expression levels (Figure 4.4.4). There was substantial variation in expression of salivary cytokines, with up to a 1000-fold difference in the expression of IL-8 within the respiratory ward patient cohort.
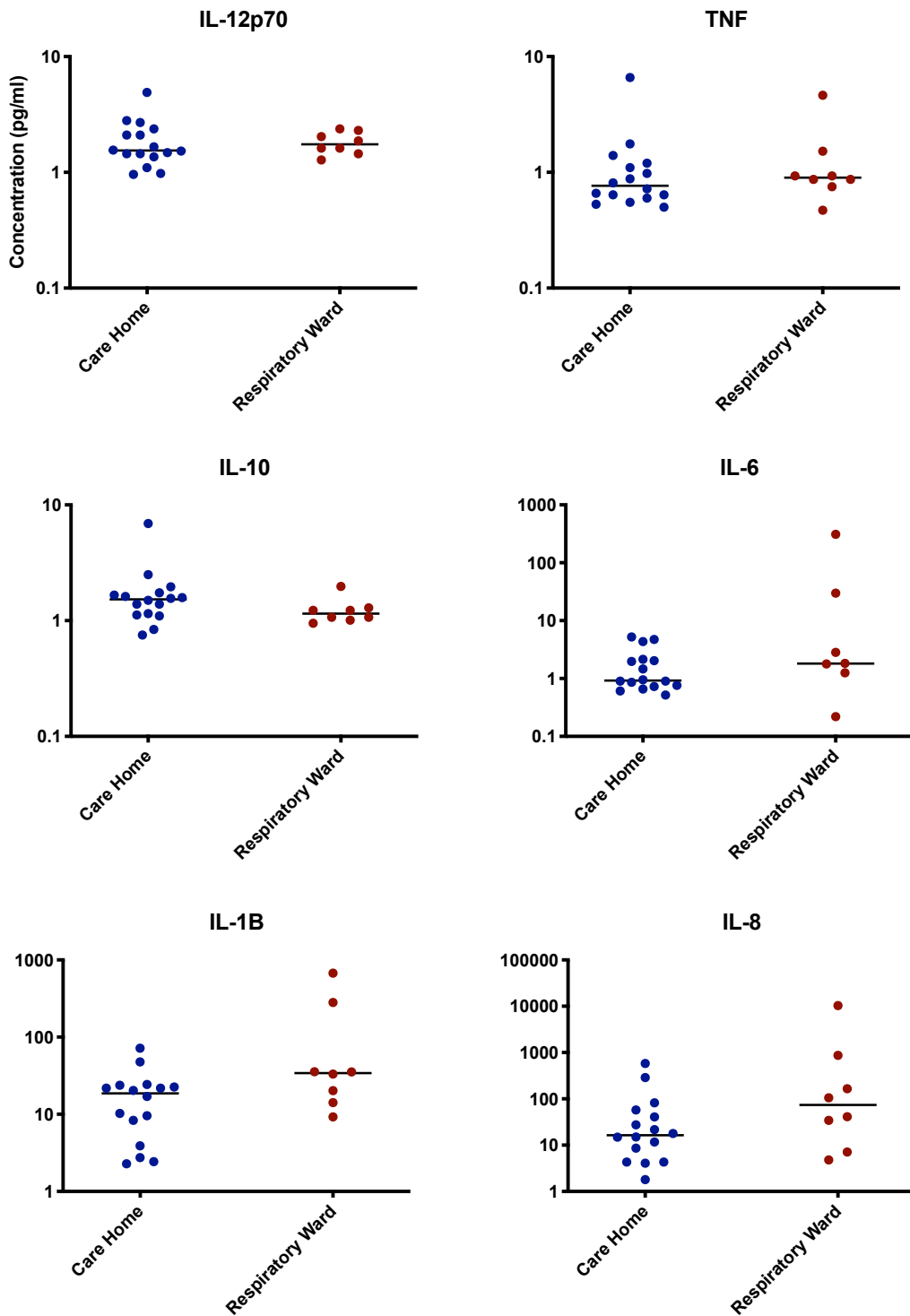
Figure 4.4.4: Salivary cytokine expression levels (pg/ml) measured in this study
Note that the Y axis uses a $\log_{10}$ scale.
Individual points represent each sample measured, horizontal black line indicates median expression level for each cohort.

4.5: Discussion

This was the first study to directly explore compositional shifts in the denture-associated oral microbiome correlated with pneumonia status; using contemporary molecular techniques to limit selectivity bias. A compositional shift in the denture-associated oral microbiota was evident, with a decrease in community diversity and species richness, and a concurrent increase in the relative abundance of putative respiratory pathogens.

In contrast to a number of prior studies conducted in elderly individuals, recovery of *S. aureus* and *P. aeruginosa* by culture was relatively low in both participant cohorts, suggesting that these species may have a diminished oral prevalence in severe community acquired pneumonia even following hospital admission. Instead, the findings presented indicate that bacteria of *Enterobacter* and *Klebsiella* genera may be more appropriate targets for investigation in such individuals.

More than half of all participants were unable to express adequate saliva for analysis of biomarkers. This alone is an important finding, as the multifaceted role of saliva in maintaining the normal oral microbiome, as well as tissue integrity and function is well documented (Lima et al. 2010). There was a high degree of inter-subject variability in salivary pro-inflammatory cytokine levels, suggesting that these may serve a limited role in identifying individuals with - or at risk of - pneumonia.

In order to reach, colonise and infect the lungs, bacteria must either pass from an external source through the oral cavity, or intrinsically from the gastrointestinal tract (Dickson and Huffnagle 2015). Thus, any relationship between the oral microbiome and pneumonia status may hold diagnostic promise, due to the close anatomical approximation of the oral cavity with the lungs and gastrointestinal tract, and the interface formed with the external environment.

It can be speculated that the findings of this study may support a potential sequential, if not necessarily causal process, with compositional shifts in the oral microbiome coinciding with pneumonia. While neither causality nor sequence can be said to have definitively been demonstrated in this research, it is difficult to envisage a series of events whereby bacteria (from either intrinsic or extrinsic sources) reach the lungs without previously or simultaneously colonising the oropharynx. Given the poor reliability of sampling the infected lung, which must be performed essentially 'blind', the ease of access to the oropharynx for microbial sampling could lead to rapid, reliable identification of potential causative microorganisms, and provide antimicrobial susceptibility profiles to aid diagnosis and treatment of pneumonia (Holter et al. 2015).

Limitations of the methods

This study has a number of limitations, with further work required to support a causal relationship between compositional shifts in the oral microbiome and pneumonia.

Due to the difficulties recruiting suitable patients within the ethical constraints of this study, a low sample size was achieved. Many care homes refused to enrol their residents, often without providing a reason. As private institutions, these sites were primarily concerned with the care of their residents, and likely wished to avoid any unnecessary disturbance of their residents that sample collection would require. Many care home residents suffered with cognitive impairment or frank dementia, and thus were excluded from the study. Similarly, pneumonia patients were frequently delirious due to fever, or had a background of cognitive impairment. Such age-related comorbidities are a major challenge to conducting research in this demographic (Chalmers 2000). There has been a recent move towards including primary care settings in research, which has typically been limited to academic centres historically (Palmer and Batchelor 2006). However, attempting to incentivise private organisations such as care homes to partake in studies presents a greater challenge still, as these institutions are not beholden to NHS clinical governance (Hall et al. 2009).

The cross-sectional design of this study was another major limitation. As recruited respiratory ward patients had received a diagnosis of pneumonia prior to recruitment, it was not possible to track changes in oral microbiome composition from respiratory health to disease. Similarly, there was no follow-up to examine shifts in microbial communities upon resolution of pneumonia. It is therefore not possible to determine if changes in the oral microbiome preceded pneumonia onset, a critical step in satisfying Bradford Hill criteria when attempting to establish a causal relationship (Hill 1965). The nasal microbiome was not considered, but could have exerted influence on the development of respiratory infection. Similarly, the role of the fungal and viral microbiome was not explored, both of which could have a major bearing on pneumonia development, particularly given the known role of influenza virus on subsequent pneumonia risk. Again, a longitudinal design would allow for elucidation of the relationships between oral (and nasal) carriage of microbes and pneumonia risk.

All patients with suspected pneumonia received empirical antibiotic therapy according to local policy, which reflects the British Thoracic Society guidelines on the management of severe community acquired pneumonia (Lim et al. 2009). As only a very low proportion of care home residents had received any antimicrobials in the preceding 30 days, and none had received a comparable regime to the first line

combination therapy of Amoxicillin/Clarithromycin used in suspected pneumonia patients; the importance of the potential role of antibiotic use as a confounder leading to altered oral microbial composition cannot be overstated. However, a number of factors suggest that while antibiotic use may have contributed to reduced community diversity and species richness, the differences cannot be entirely explained by antibiotic use alone. Firstly, it would be expected that denture-associated biofilms would be least affected by antibiotic use compared with other oral sites, due to both the protective effect of the biofilm in conferring antimicrobial tolerance to its constituents (Stewart 2015), and because antibiotics would have to cross the epithelial oral mucosal barrier, diffuse through the palatal microbial biofilm and then penetrate the denture-associated biofilm through the outer surface in sufficient concentration to perturb the microbial communities. It should be noted that *Enterobacteriaceae* are typically not susceptible to macrolide antibiotics such as Clarithromycin and are intrinsically resistant to Amoxicillin and other beta-lactamases. The aggressive use of these antibiotic regimes in pneumonia patients may act as a selective pressure to suppress growth and viability of normal oral microbes, particularly *Streptococcaceae*, leading to an increased relative abundance of more virulent microorganisms not normally associated with the oral microbiome, such as *Enterobacter* species. Nonetheless, the finding that the difference in relative abundance of PRPs was most pronounced in denture samples suggests that antibiotic use is unlikely to be the primary contributor to the changes in microbial community composition. Notably, no *S. aureus* isolates recovered from respiratory ward patients were resistant to amoxicillin, compared to over one quarter of those from care home residents. However, macrolide resistance was more than doubled in respiratory ward *S. aureus* isolates. There were much higher rates of resistance to the beta-lactam antibiotic piperacillin-tazobactam in care home residents' *P. aeruginosa* isolates compared with pneumonia patients, as was seen for the related cephalosporin ceftazidime. However, resistance of *P. aeruginosa* isolates to ciprofloxacin, a fluoroquinolone antibiotic, was found to be much higher among pneumonia patients than care home residents. While the low number of both *S. aureus* and *P. aeruginosa* isolates recovered precludes any reliable statistical evaluation, the differences in resistance patterns suggests that antibiotic treatment may not have exerted a major selective pressure upon the oral microbiota. This was particularly evident in the case of *S. aureus* isolates, where amoxicillin sensitive strains were isolated from respiratory ward patients' samples despite aggressive empiric therapy with this agent. Based on the sequencing data, it appears that *S. aureus* and *P. aeruginosa* are poor candidates for a targeted approach as was

employed in this study. Future studies should instead target *E. coli,* Enterobacteriacia, Klebsiella, Enterococcus and Serratia species.

Salivary cytokines did not reflect pneumonia status, nor oral PRP colonisation. A better alternative to evaluate the host-microbiome interface in the oral cavity would be collection of tissue fluid from the palatal mucosa underlying the denture. This may still offer useful predictive information, as local inflammatory responses might hint at both the immune status of an individual and the virulence of the microbial community. Equally, tissue fluid would be less prone to confounding from time of day, recent food or drink intake and medicines affecting salivary flow.

In future research it would be advantageous to follow participants from day of admission through to recovery and discharge (or death). However, this was not feasible within the limitations of this study due to the small clinical team, and barrier to participation that a longer-term study duration might create. By recruiting participants at admission, it would be possible to collect samples prior to antibiotic administration, allowing this confounder to be examined and accounted for. However, there are several practical challenges to achieving recruitment in this manner. Firstly, many patients may have already received empirical antibiotics from their GMP prior to, or at the time of, referral to hospital. A large number of patients are admitted with suspected respiratory infection, but most of the clinical signs and symptoms are non-specific. Therefore, only a small percentage of prospectively identified admissions will actually receive a diagnosis of bacterial pneumonia (Khalil et al. 2007). This means that a large number of samples would need to be collected, necessitating a large research team, logistical and financial resources, and disturbing patients who are already unwell; with many of the samples collected being discarded. Additionally, many patients screened were either unwilling or unable to consent to participate in this study. Emergency admissions are commonly pyrexic and delirious due to their acute illness. These patients would thus need to be excluded, increasing the duration of recruitment and the resources required, or consented retrospectively, as our group has previously done in ICU patients (Sands et al. 2016). At the present cost of sequencing, the number of samples needed to track patients serially over time and provide sufficient discriminatory information to allow diagnostic and prognostic evaluation would be prohibitively expensive.

An alternative route could be to evaluate an intervention aimed at improving oral health in patients at risk of bacterial pneumonia, such as care home residents, adequately powered to evaluate the impact of such care on pneumonia incidence. Secondary outcome measures including oral health measures such as plaque indices

and Newton's index, and compositional changes in the oral microbiome could be used to indirectly correlate the effect of enhanced oral care on pneumonia with modulation of the oral microbiome. Previous work in this area has been highly promising (Yoneyama et al. 2002, Bassim et al. 2008, Juthani-Mehta et al. 2014), but has typically evaluated nurse or carer led interventions rather than delivered by an oral health professional; and has not considered microbiological outcomes, measured compliance and oral hygiene effects in addition to respiratory disease incidence.

Despite the limitations highlighted above, this study was an important first step in evaluating the possible contribution of perturbations within the denture-associated oral microbiome to pneumonia. Having demonstrated an association between an altered microbiome and an increase in the bioburden of putative respiratory pathogens, the premise for a causal association is established. Future research should aim to further disentangle the relationship between oral health, the oral microbiome and pneumonia pathogenesis; as well as assessing the impact of effective oral and denture care on modulating the oral microbiome and decreasing pneumonia risk in susceptible individuals.

The results of this study can be used to inform a power calculation for both further observational or interventional studies. For instance, to give 80% power with an alpha of 5% in a study for an oral health intervention with an expected reduction of 20% in the mean relative abundance of PRPs on denture surfaces, a total sample size of 278 participants would be required. As the impact of oral care on PRP carriage has not been evaluated to date, it would be sensible to include an interim analysis at an early point in such a study to refine the estimate based on the effect size seen.

CHAPTER 5: DEVELOPMENT AND OPTIMISATION OF NOVEL ANTIMICROBIAL SILICONES TO PREVENT DENTURE-ASSOCIATED BIOFILMS

5.1: Introduction

With the growing threat of antimicrobial resistance, there is an urgent need for novel antimicrobial agents (Roca et al. 2015). Approximately 65% of healthcare associated infections are estimated to be biofilm-related (NIH, 2002). Biofilms offer a protective niche against both the host immune response and the action of many antimicrobials (Lindsay and Van Holy 2006). The 'biofilm lifestyle' entails a change in microbial cell behaviour, with altered metabolism due to nutrient privation and low oxygen tension in deep biofilm regions, which favours a reduction in many of the metabolic and proliferative pathways targeted by the majority of antimicrobials (Flemming et al. 2016). Further compounding the problem, the biofilm environment increases the rate of persister cell formation, wherein microbes can reside in a viable but dormant state and thus tolerate many antimicrobial agents regardless of resistance (Jolivet-Gougeon and Bonnaure-Mallet 2014).

As has been outlined in previous sections, removable dentures offer a route for the acquisition of environmental pathogens, which can subsequently be transferred into the oral cavity in a protected, stagnant environment that encourages biofilm formation. With age, progressive atrophy of the alveolar ridges may lead to dentures becoming ill-fitting and uncomfortable, limiting function and oral health related quality of life (Polzer et al. 2010). In many functionally limited, frail elderly individuals in institutional care settings, regular provision of new dentures is challenging due to expense, availability of dental care and a decreased capacity to tolerate new dentures with declining oral muscular control (Holm-Pedersen et al. 2005). A potential means of mitigating this problem is to reline dentures, which can be achieved using either the addition of acrylic, or polyvinyl-siloxane (PVS) rubber materials.

Silicone reline materials offer the advantage of cushioning the denture to increase comfort, particularly in the case of atrophic ridges which result in high concentration of masticatory pressures on tissues (Palla et al. 2015). Additionally, silicone materials allow areas of undercut to be engaged which can enhance retention and stability of removable dental prostheses. However, these materials degrade and become porous over time (Hashem 2015), which encourages microbial colonisation (Bulad et al. 2004; Taylor et al. 2008). Candidal species in particular are able to infiltrate the silicone rubbers, generating biofilms which are highly recalcitrant to removal (Hahnel et al. 2012). In order to combat this problem, a number of researchers have attempted to incorporate antimicrobial agents into silicone rubbers (Lefebvre et al. 2001; Geerts et al. 2008; Salim et al. 2012; Bueno et al. 2015).

5.1.1: Silver as an antimicrobial agent

Silver and silver metal salts have a storied history of application as antimicrobial agents, with the use of such compounds in vessels for transporting water and in a variety of medical applications in ancient Egyptian, Greek and Roman civilisation documented (Alexander 2009). The first application of silver in modern medicine was the use of silver nitrate to treat wounds and ulcers, followed by the use of silver in suture thread. In the early 20th Century, colloidal silver (a suspension of silver particles in liquid, usually water) was popularised for the treatment of ophthalmic and other infections including bacterial sepsis (Politano et al. 2013). In modern medicine, silver is used in a number of applications, including dressings for chronic wounds (Lo et al. 2009), linings for catheters (Beattie and Taylor 2011) and endotracheal tubes (Li et al. 2012). More recently, interest has grown in the use of silver nanoparticles in the medical setting (Burdușel et al. 2018). Silver nanoparticles possess antimicrobial properties which are independent to silver metals. Instead, these may relate to the shape and size of the nanoparticles leading to penetration of microbial membranes, release of silver (Ag^+) ions and induction of oxidative stress or interference with intracellular signalling (Rai et al. 2009). Despite growing use of silver nanoparticles in a wide range of applications, there are a number of toxicity and safety concerns (AshaRani et al. 2008). The mechanisms of toxicity of silver nanoparticles are poorly understood. Furthermore, nanoparticles are readily dispersed both within tissues (Lankfeld et al. 2010) and across the environment (Panyala et al. 2008), meaning the effects of such compounds could be impactive on a global scale. Caution, therefore, is warranted in the use of nanoparticles over metallic silver or its ionic salts.

A particularly attractive feature of silver as an antimicrobial is the broad range of microbial targets it affects. There are at least 7 distinct cidal mechanisms of ionic silver which have been characterised in the literature:

1) The Ag^+ cation can bind nucleic acids to form a number of complexes which alter the molecular structure of DNA and RNA, increase base pair melting temperatures and consequently inhibiting replicative and protein synthetic pathways. (Arawaka et al. 2001; Kozasa et al. 2008)

2) Inactivation of enzymes through binding to thiol groups in amino acids such as cysteine (Feng et al. 2000).

3) Generation of reactive oxygen species (ROS) secondary to inhibition of respiratory chain enzymes in an oxygen-dependent manner. This is an effect that appears to be related to interaction with thiol groups in amino acids, although other mechanisms of direct ROS generation are plausible (Park et al. 2009).

4) Inhibition of ATP production through inactivation of key respiratory enzymes and ribosomal proteins, including cytochrome proteins. Again, this is secondary to interactions with thiol groups in key amino acids and proteins (Bragg and Rainnie 1974, Yamanaka et al. 2005).

5) Proton leakage at cell membranes through interaction with either a broad range of membrane-associated proteins or the membrane phospholipid bilayer (Dibrov et al. 2002; Jung et al. 2008).

6) Disruption of microbial adhesion through ionic interactions which interfere with hydrogen bonds, electrostatic and hydrophobic interactions required for attachment to biofilm matrix and surfaces. Although silver ions at low concentration demonstrated no biocidal effect against established biofilms, they did increase permeability of the biofilm matrix, which could enhance the effectiveness of concurrent antimicrobial treatments. When used as a lining material for a sterile surface, it could be expected that silver ions would contribute to anti-adhesive properties (Chaw et al. 2005).

7) Inhibition of glycolytic pathways through irreversible inactivation of phosphomannose isomerases (Wells et al. 1995).

The combined anti-adhesive and broad range of non-specific microbiocidal properties of ionic silver make its therapeutic use, particularly in medical devices attractive, as resistance is relatively low (Percival et al. 2008). However, overexpression of efflux pumps is one mechanism known to confer resistance to silver in Gram negative pathogens such as *P. aeruginosa* (Mijnendonckx et al. 2013). Additionally, the benefit of ionic silver against established biofilm is to increase permeability of the EPS matrix to other antimicrobial agents, rather than to directly kill cells (Morones-Ramirez et al. 2013). The synthesis of silver complexes with other antimicrobial compounds has therefore been an area of exploration for drug development research recently. A particularly promising prospect is the combination of silver salts with nucleophilic heterocyclic carbene (NHC) compounds (Gordon et al. 2010).

5.1.2: Functionalisation of nucleophilic heterocyclic carbenes (NHCs) and conjugation with transition metals

NHCs consist of pentane rings with a strong affinity for transition metals such as silver, gold, mercury and palladium (Figure 5.1.2). They are typically derived from imidazolium salts and are a popular compound due to their stability and the relative ease of synthesis (Anderson and Long 2010). A wide range of complexes can be formed, with 1 or multiple carbene groups complexed with a metal core. Additionally, considerable variation of the functional groups attached to the nitrogen atoms is readily achievable. This affords a huge array of potential compounds, which can be

tailored to achieve specific biochemical effects. The use of metal-NHC complexes as antimicrobial agents was first investigated by Çetinkaya et al. (1996), using complexes with ruthenium and rhodium. Silver-carbene complexes rapidly grew in popularity however, due to the inherent antimicrobial activity of ionic silver and widespread use of silver in medicine. The compounds also offer good antifungal activity against Candida species (Gök et al. 2014). The Ag(I) ionic state is stabilised through the addition of carbene complexes, enhancing the effectiveness of silver (Gordon et al. 2010).
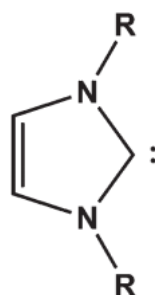
Figure 5.1.2: Typical structure of an imidazolium-derived nucleophilic heterocyclic carbene.
R = Functional groups which may vary.
(Image from Majhi et al. 2014)

Silver NHCs have been found to exhibit antimicrobial activity against a range of PRPs including *S. aureus* and *P. aeruginosa* (Melaiye et al. 2004). The addition of carbenes appears to result in slow, sustained delivery of silver ions, which enhanced antimicrobial activity compared to the silver nitrate control. A murine *P. aeruginosa* respiratory infection study showed up to 100% survival following treatment with silver NHC complexes, which suggests that such compounds can be used safely at least in acute infective episodes (Cannon et al. 2009). However, extensive toxicity testing on cell lines have been contradictory, with some studies showing detrimental effects on epithelial cells, hepatocytes and lymphocytes, likely due to the impacts of ionic silver on the respiratory chain; while a number of studies have found no cytotoxicity of ionic silver (Hollinger 1996; Hidalgo and Domınguez 1998; Ghosh and Banthia 2004; Pellei et al. 2012). The toxicity of NHCs is likely to vary with the specific molecular structure and functional groups present. Therefore, each compound likely requires testing on a relevant range of cells and tissues.

5.1.3: Antimicrobial properties of triclosan
Triclosan is a chlorinated phenol (Figure 5.1.3) which is widely used for its broad-spectrum antimicrobial properties. Triclosan has been shown to be effective at

inhibiting *S.* aureus, *E. coli, C. albicans,* and *S. pneumoniae* (Regös et al. 1979), although it is generally ineffective against *P. aeruginosa* due to multi-drug efflux pumps and the presence of a specifc enoyl-acyl carrier protein reductase FabV (Fang et al. 2010). It can be found in disinfectants, soaps, dentifrices among other household, personal care and medical products (Suller and Russell 2000). Triclosan has been found to have low toxicity at the concentrations used in many of the above applications, which has led to its widespread use in such a broad range of settings (Jones et al. 2000). However, a potential consequence of the ubiquitous use of triclosan in products marketed directly to the general public is the dissemination of triclosan throughout the environment in waste water. A particular concern is the potential hormone and development disrupting effects on fish and marine wildlife (reviewed in Dann and Hontela 2011), who are first exposed to triclosan via effluence. However, it should be noted that at present there is no evidence to support any meaningful detrimental effects in ecologically relevant exposure levels.

Triclosan has biocidal activity at high concentrations, while it is bacteriostatic at lower concentrations through different mechanisms (Maillard 2002; Russell 2004). Bactericidal mechanisms involve multiple targets; triclosan can diffuse through bacterial cell walls to disrupt RNA, lipid and protein synthesis through interactions with biosynthetic enzymes, as well as membrane disruption leading to cell lysis. At sublethal concentrations, triclosan appears to interact with nucleic acids in the cytoplasm. It is thought that in both circumstances outlined above, the primary effector target is the enoyl-acyl carrier protein (ACP) reductase enzyme in bacteria, responsible for fatty acid synthesis. A similar mechanism is likely present in fungi, although this has yet to be properly defined. It is unlikely that triclosan targets the ergosterol pathway however, as synergistic effects have been found when combining triclosan with Amphotericin B or Fluconazole, both of which act primarily upon ergosterol biosynthesis (Movahed et al. 2016).

Triclosan resistance has been reported in *S. aureus* strains (Suller and Russell 2000), although this has yet to impact clinical care of individuals and appears to occur at a low level overall. More worryingly, reports have suggested that triclosan may induce cross-resistance to antibiotics (Yazdankhah et al. 2006). This may occur through an alteration of the ACP biosynthetic pathway in instances where this pathway is targeted by antibiotics, particularly pertinent to the development of isoniazid resistance in *Mycobacterium tuberculosis* (Russell 2004). In Gram negative microorganisms such as Acinetobacter or Pseudomonas species, resistance is likely related to high constitutive expression of efflux pumps. Worryingly, evidence suggests that triclosan exposure among such microorganisms may drive clones with

higher rates of efflux pump expression (Chuanchuen et al. 2003). The impact of triclosan on *S. aureus* is controversial, as an early study demonstrated plasmid-mediated mupirocin resistance to also confer triclosan resistance (Cookson et al. 1991). However, subsequent research has not found this mechanism (Suller and Russell 2004). Triclosan exposure may also drive selection of small-colony variants, which exhibit a reduced growth rate and altered metabolic behaviour due to defects in the electron transport chain (Seaman et al. 2006). These changes in cell biochemistry drive antimicrobial resistance in a manner comparable the (although distinct from) the antimicrobial tolerance conferred to persister cells in biofilms outlined in Section 1.1.3.6.

Despite such potential concerns, triclosan remains a popular antimicrobial agent with an excellent record of clinical safety.
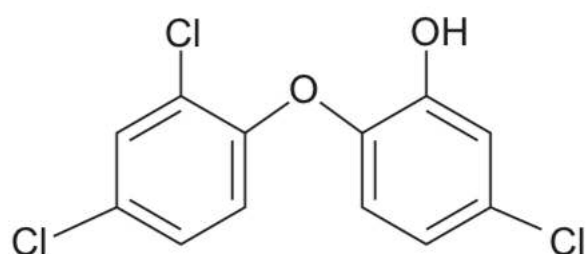
Figure 5.1.3: Chemical structure of triclosan.

Despite being halogenated, triclosan is considered to be essentially non-ionic, which aids biofilm matrix penetration.
(Image from Hontela and Habibi 2013)

5.1.4: Silicone as a denture biomaterial - key features and chemical properties

Silicone is used across a wide range of biomedical applications due to its favourable properties: stability after autoclaving; maintenance of physicochemical properties over time; anti-adhesive properties, biocompatibility and imperviousness to degradation by the host immune cells or microbes (Quinn and Courtney 1988). Silicone monomers can be polymerised into rubber by a variety of catalysts and chemistries. The resultant polymer is a soft, flexible and elastomeric material which can be polymerised in moulds to permit highly customisable topographies. This makes them ideal materials for use as denture soft-liners, their soft, viscoelastic nature facilitating the distribution of occlusal forces across a wide surface area of oral tissue to improve comfort during masticatory function (Dootz et al. 1992). Additionally, their high dimensional stability and surface conformity allows an intimate association with the oral mucosa, include engagement of areas of undercut which can improve denture retention and stability (Goiato et al. 2008).

However, such soft denture lining materials have several disadvantages of biological consequence. Long-term resiliency of silicones is limited, and the materials tend to degrade over time, becoming permeable to microbes (Herman et al. 2008; Taylor et al. 2008). *Candida albicans* in particular has been found to readily invade silicone rubbers in denture liners, with penetrating hyphae proving recalcitrant to removal (Nevzatoğlu et al. 2007). Silicones also offer relatively poor resistance to abrasion, with degradation of the surface upon aggressive brushing, particularly if abrasive dentifrices or unsuitable denture cleansers are used (Jin et al. 2003). This can increase both surface area available for colonisation by microbes and surface roughness, which may generate sheltered niches for microbes to evade removal by chemo-mechanical cleansing. The bond between silicone and PMMA can also break down (Gundogdu et al. 2014). This may lead to partial detachment of the silicone liner to the acrylic surface which generates a nidus not only sheltered from chemo-mechanical removal but also host immune cells and saliva. These limitations have reduced the effectiveness of silicones for long term use, necessitating frequent replacement which is costly and resource intensive. One approach to minimise microbial colonisation of these materials has been to incorporate antimicrobial agents into the silicone polymers.

5.1.5: Antimicrobial silicones - current concepts and modalities

Biomaterials may exhibit antimicrobial activity by one, or a combination of, two primary features (Francolini et al. 2017). Materials which inhibit microbial attachment or perturb biofilm formation without directly killing microbes or inhibiting growth can be termed anti-adhesive or anti-fouling. Alternatively, biomaterials may either kill microbes in a contact dependent manner (non-releasing materials) or by release of antimicrobial substances from their surface into the surrounding environment (releasing materials). An important consideration for releasing materials is the 'rechargeability' of the antimicrobial contents if long-term activity is important. Either release and reattachment of the antimicrobial component must occur continuously by dynamic equilibrium, or it must be possible to recharge the antimicrobial reservoir without necessitating replacement of the entire material (Sun et al. 2012; Lv et al. 2013).

Much attention regarding antimicrobial silicones has fallen upon the prevention of infections associated with indwelling urinary catheters. These devices offer an attractive testing ground for antimicrobial coatings, as antimicrobial activity is typically required for up to 12 weeks before the catheter is changed (Crouzet et al. 2007; Wilde et al. 2013). Symptomatic urinary tract infections associated with long-term catheters

affect up to 12.6% of catheterised patients (Nicolle 2014). A number of coatings have been trialled to reduce the incidence of infections in catheterised patients. One large, multicentre UK randomised control trial (Pickard et al. 2012) compared 3 different catheters in normal NHS use: a PTFE-coated device (control); a silver alloy-coated catheter, and a nitrofurazone antibiotic-impregnated catheter. Only the nitrofurazone-impregnated devices demonstrated a statistically significant difference in catheter-associated urinary tract infections (CAUTIs), although these catheters resulted in greater discomfort during use and removal. A recent systematic review (Drekonja et al. 2008) examined a total of 13 randomised control trials examining the impact of antimicrobial catheters on the development of catheter-associated bacteriuria or fungiuria (CABF). None of the included studies directly examined the development of symptomatic infections. Interestingly, the authors noted that studies of silver-coated catheters conducted prior to 1995 demonstrated substantial reductions in CABF rates (Median relative risk 0.32; range: 0.24-0.44), while studies conducted subsequently failed to show a meaningful reduction in CABF rates by such devices. The reasons for more recent studies failing to replicate such promising earlier results is unclear. However, the authors speculate that one factor may be the use of latex catheters in the earlier studies, while later studies employed silicone or silicone-coated devices. This demonstrates the importance of the enhanced anti-adhesive properties of silicone over latex and supports the strategy of combining anti-fouling and antimicrobial components in such devices. In contrast, epidemiological research offers support for the effectiveness of silver-alloy catheters in reducing CAUTI rates. A report from the National Healthcare Safety Network found a 58% relative reduction in infection in silver-coated catheters compared to standard catheters (Lederer et al. 2014).

A number of alternative antibiotics and antimicrobial agents have been tested *in vitro*, but their use remains unproven in a clinical setting. Unsurprisingly, most research pertaining to denture lining materials has focussed on antifungal activity against *C. albicans*. To date, no clinical research has been conducted on antimicrobial silicones in dentistry.

5.2: Aims and Objectives

The principle aims of this work package were to:

1. Screen a range of novel imidazolium-derived NHC compounds and their silver complexes against relevant planktonic PRP cultures and biofilms
2. Develop a method for incorporating such compounds into silicone biomaterials
3. Test the antimicrobial activity and toxicity of silicones incorporating silver-NHC compounds

Hypotheses

- Imidazolium-derived NHC compounds will demonstrate antimicrobial activity against planktonic PRPs and biofilms
- Antimicrobial activity will be enhanced by complexing with ionic silver, with a reduction in the concentration of antimicrobial required and/or antimicrobial activity against species where previously no activity was found
- Incorporation of antimicrobial complexes into silicone materials will not adversely effect the physicochemical properties of the materials
- Silicones with antimicrobial complexes incorporated will demonstrate antimicrobial activity against the same microorganisms as the compounds in suspension
- Silicones with antimicrobial complexes incorporated will demonstrate low toxicity

5.3: Materials and Methods

5.3.1: Preparation of experimental compounds

A number of NHCs were prepared by collaborators in the School of Chemistry, Cardiff University by deprotonation of imidazolium salts, with functional groups consisting of saturated or unsaturated hydrocarbon chains which varied in length, terminating in an alkyl group. Compound structure and purity was confirmed by the presence of characteristic peaks through mass spectroscopy nuclear magnetic resonance (MS-NMR). The chemical structure of each test compound is presented in Table 5.3.1. Synthesised compounds were dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich, Gillingham, UK) at 10 mg/ml, and silver-complexes shielded from light due to photo-reactivity in solution. Compounds were stored for up to 30 days at room temperature prior to use.

Table 5.3.1: Chemical structure of test imidazolium-derived NHC compounds

Compound	Chemical Formula	Chemical Structure
1	$C_{14}H_{26}N_2O_3S$ Mol. Wt.: 302.43	
2	$C_{18}H_{34}N_2O_3S$ Mol. Wt.: 358.54	
3	$C_{15}H_{20}N_2O_3S$ Mol. Wt.: 308.4	
4	$C_9H_{15}NO_4S_2$ Mol. Wt.: 265.35	
5	$C_{14}H_{26}BrNOS$ Mol. Wt.: 336.33	
6	$C_{16}H_{31}BrN_2$ Mr 331.34	
7	$C_{14}H_{10}F_{17}IN_2$ Mol. Wt.: 656.12	
8	$C_{15}H_{27}BrN_2$ Mol. Wt.: 315.29	

5.3.2: Selection and culture of microbial reference strains for antimicrobial testing

A panel of microbial reference strains representative of pathogenic species frequently isolated in healthcare-associated infections was selected for evaluation (Table 5.3.2). This included yeast, gram-negative and gram-positive microbes to account for possible differences in activity of prospective antimicrobial compounds.

Table 5.3.2: Reference strains used for *in vitro* biofilm model

Microorganism	Strain	Isolated from
Candida albicans	ATCC 90028	Blood
Staphylococcus epidermidis	RP62A	Intravascular catheter-associated infection
Staphylococcus epidermidis	ATCC 14990	Nose
Staphylococcus aureus	NCTC 6571	Not specified
Staphylococcus aureus	NCIMB 9518	"human lesion"
Escherichia coli	NCTC 12923	Faeces
Pseudomonas aeruginosa	ATCC 15692	Infected wound

5.3.3: Screening of experimental compounds for inhibition of microbial growth using the broth microdilution method

Initial screening of antimicrobial activity of test compounds was conducted using a minimum inhibitory concentration (MIC) assay. Test compounds were dissolved in dimethylsulfoxide (DMSO) at 10 mg/ ml concentration to produce stock solution. This stock solution was subsequently diluted 2-fold to produce a range of concentrations to 78 µg/ ml. DMSO dilutions were then further diluted 1 in 50 in either Mueller-Hinton broth (Sigma-Aldrich, Gillingham, UK) for bacterial strains or RPMI 1640 medium (Sigma-Aldrich, Gillingham, UK) supplemented with 2% glucose and 0.165 M 3-(N-morpholino)propanesulfonic acid (MOPS), to produce concentrations ranging from 200 µg/ ml to 1.56 µg/ ml in 1% DMSO solution.

100 µl of each compound in broth was added in triplicate to a 96 well microtiter plate (Starstedt, Leicester, UK). DMSO was added to each respective growth medium at 2% v/v concentration and added to two further triplicate columns in the microtiter plate

for positive and negative controls. Subsequently, an inoculum of the test microorganism was prepared. The test microbe was cultured overnight in appropriate growth medium aerobically at 35 ˚C. Overnight bacterial suspensions were diluted to a 0.5 MacFarland standard by measuring absorbance at 620 nm using a spectrophotometer, to give an optical density of 0.8-0.1. *Candida albicans* was diluted similarly to an inoculum optical density of 0.9-1.1.

Standardised inocula were then diluted 1:1000, giving an approximate inoculum concentration of 1×10^5 bacterial CFU/ ml, and 1×10^4 Candida CFU/ ml. 100 µl of test microbial suspension was added to each well containing antimicrobial solution, and the positive control wells. 100ul of blank growth medium was added to the negative control wells. This resulted in a final test concentration of 100 µg/ ml to 0.78 µg/ ml of each compound.

After inoculation, microtiter plates were incubated aerobically at 35 ˚C for 16-20 h. Absorbance was then measured at 620 nm using a plate reader (OMEGA, BMG Labtech, Aylesbury, UK). The negative control absorbance value was subtracted from all wells to correct for background absorbance. The MIC value for each compound was determined as 80% reduction in absorbance relative to positive control wells. If a compound was inhibitory against the test microbial species, the assay was retained for further use in testing the minimum biocidal concentration. Each experimental was subsequently repeated twice.

5.3.4: Determining minimum biocidal concentrations (MBCs) of antimicrobial compounds

Microtiter plates from MIC assays in which compounds had demonstrated inhibition of growth in the test microbial species were used for MBC testing. The liquid medium was removed from each well ranging from 1 concentration below the MIC value, to the maximum concentration, after mechanically agitating the contents with the pipette tip to remove adherent microorganisms from the plastic surface. The microbial suspension was then added to plain growth medium at a concentration of 1:20, to ensure that any antimicrobial compound was diluted to well below the MIC. Suspensions were incubated aerobically at 35 ˚C for a further 24 h, then centrifuged at 4000 RPM for 5 min to pellet the cells. The supernatant was discarded, and the cell pellet resuspended in 50 µl of PBS. The entire volume of resuspended cells was inoculated onto Mueller-Hinton agar using a spiral plater (Don Whitley Scientific, Bingley, UK). Both positive and negative control wells were processed identically. Plates were incubated for a further 24-48 h, until colony growth was evident in positive controls. The MBC value was determined as the concentration at which less than 50

colonies of test microorganisms were present. MBC testing followed every MIC repeat, and so was repeated twice.

5.3.5: Assessing biofilm eradication potential of experimental antimicrobial compounds using a microtiter plate assay

An overnight microbial suspension was prepared under the conditions described in section 5.3.3. The suspension was standardised to a MacFarland standard of 0.1 for bacteria or 1 for Candida as described previously. 100 µl of this inoculum was then added to a 96 well microtiter plate, in 9 triplicate wells, with blank growth medium added in triplicate to further wells to act as a negative control. Microtiter plates were incubated without shaking for 24 hours to allow biofilm formation. Following this, growth medium and loose, non-adherent cells were removed by pipetting, and each well rinsed with 100 µl PBS.

Test compounds prepared as described in section 5.3.3 were then added to each well to provide a concentration ranging from 200 µg/ ml to 1.56 µg/ ml. To the positive and negative control wells, growth medium containing 2% v/v DMSO was added. The test biofilms were incubated for a further 24 h, then the antimicrobial solutions removed by pipetting. Wells were washed twice with 100 µl PBS, then biofilms were resuspended in 100 µl growth medium and mechanical agitated to ensure adequate resuspension. The same procedure was completed for positive and negative control wells to ensure equivalent treatment.

Turbidity was measured by optical density at 620 nm using a spectrophotometer (BMG Labtech, Aylesbury, UK). The optical density reading from the negative control wells was subtracted from all test wells to compensate for absorbance of blank growth medium. The minimum biofilm eradication concentration (MBEC) for each compound was defined as the lowest concentration at which turbidity was reduced by 80% relative to positive controls. MBEC tests were subsequently repeated twice.

5.3.6: Incorporation of experimental antimicrobial compounds into silicone materials by bulk-loading

Silicones were mixed using a proprietary two-part mixture of base polymer Part A) and a catalyst containing polymer (Part B) in a 10:1 v/v ratio. Following initial hand mixing to eliminate air bubbles, the silicone mixture was loaded into a rotary mixer and mixed under vacuum at high speed for 3 min. Silicones were then decanted into metal moulds and cured by heating at approximately 80ºC for 2 h. This produced 5 mm thick sheets of silicone rubber with a uniform, macroscopically smooth surface, which were sectioned using a scalpel into 10 x 10 mm coupons.

Triclosan and triclosan acetate were dissolved in an excess volume of acetone and added to the silicone mixture during hand mixing. After rotary mixing under vacuum, excess acetone was evaporated using a rotary evaporator (Büchi Rotavap R300, Büchi Labortechnik AG, Flawil, Switzerland) at 50 °C and the silicones heat-cured as previously described.

To dissolve test compounds while ensuring minimal solvent residue in the final silicone material, 100 mg of each test NHC compound and silver salt was combined with a 1% DMSO: 99% acetone mixture (v/v). Initial attempts to incorporate the test materials into silicone polymers resulted in a failure of the silicone to cure. It was speculated that this was likely due to interaction of NHC compounds and ionic silver with the platinum catalyst responsible for cross-linking of the silicone polymer and therefore alternative materials were explored.

5.3.7: Incorporation of experimental antimicrobial compounds into acetoxy-resin material

An acetoxy resin (A-100 Type A Medical Adhesive, Technovent, Bridgend, UK) was trialled as an alternative material for loading of the test materials. This product has a history of application in the biomedical field, and bonds effectively to silicone rubbers and acrylic biomaterials. However, unlike PVS materials, this product cures by condensation upon contact with atmospheric moisture which is independent of a metal catalyst.

Again, 100 mg of test NHC compounds were dissolved in 1% DMSO: 99% acetone v/v to a total volume of 10 ml and thoroughly hand mixed into 9.8 g of acetoxy resin material. Excess acetone was removed using a rotary evaporator (Büchi Rotavap R300, Büchi Labortechnik AG, Flawil, Switzerland) at 50 °C. Acetoxy resin materials were subsequently applied to silicone rubber prepared as per section 5.3.6 and cured over night at room temperature, protected from light. However, the viscosity of the material made the resultant surface finish macroscopically irregular and was approximately 1 mm thick, which was felt to offer unacceptable dimensional control for prospective clinical applications. Consequently, an alternative method of generating a thin, even coating of material was sought, using a 'dip-coating' method.

5.3.8: Formulation of acetoxy-resin silicone oil dispersion containing experimental antimicrobial compounds

Acetoxy resins were combined with test compounds as described above, then immediately added to a silicone-based fluid (G608 Pro Bond Remover, Technovent, Bridgend, UK) in a 1:1 ratio. The resultant mixture was mixed vigorously by hand

using stainless steel mixing balls (8 mm diameter) to ensure an even dispersion was generated. Silicone rubber sheets were dipped into the mixture and the excess drained. The coated sheets were then cured on a flat, level surface overnight at room temperature, protected from light. A total of 3 'dip coats' were applied. Test materials were sectioned into 10 x 10 mm coupons for subsequent testing.

All test materials were disinfected by immersion in 100% ethanol for 60 s prior to use.

5.3.9: Screening of antimicrobial activity of materials using zone of inhibition assays

Overnight suspensions of test microbial species were prepared as described previously and standardised to an optical density of 0.1 and 1 for bacterial and Candida respectively in 0.9% PBS, using absorbance measured by spectrophotometer at 620 nm. Prepared inocula were then added to Mueller-Hinton agar plates using a sterile cotton swab and spread evenly across each surface to produce a microbial "lawn". Within 15 min of inoculation, the test material was aseptically added to each plate using sterile tweezers, and gently pressed against the surface to ensure adequate contact. Plates were incubated aerobically at 35 °C for 16-20 h, until even microbial growth was observed across the surface. Circular areas in which microbial growth was inhibited were measured in full diameter using digital callipers, reading from the underside of each plate.

5.3.10: Evaluating antimicrobial activity of materials using Live/Dead staining and quantitative analysis of CLSM imaging

Standardised inocula in 0.9% PBS were prepared from overnight suspensions of test microorganisms as described in Section 5.3.9. Representative microorganisms selected were *C. albicans*, *E. coli*, and an *S. aureus* strain to represent yeasts, Gram-negative and Gram-positive bacteria respectively. Sterilised test materials were placed in triplicate in 12 well microtiter plates, and 100 µl of microbial inoculum added by pipetting to the centre of each material. Well plates were sealed using Parafilm (Sigma Aldrich, Gillingham, UK) and incubated aerobically for 24 h at 37 ºC.

After incubation, samples were labelled with the BacLight Live/Dead stain and imaged using a Leica SP5 confocal laser-scanning microscope as described in Section 2.3.8.2.

Microscopic images were analysed using the COMSTAT 2.1 plugin in ImageJ, as described in Section 2.3.8.2.1, to provide surface area coverage data (μm^2).

5.3.11: Characterisation of physico-chemical properties of antimicrobial materials

A range of tests of physico-chemical properties silicone and acetoxy resin materials were undertaken to ensure that no adverse effects were generated through the incorporation of experimental compounds. The tensile strength of acetoxy resins was not investigated as this was applied as a coating to silicones and not intended for independent use. Otherwise all tests were undertaken on the selected experimental materials from Section 5.3.10.

5.3.11.1: Evaluation of tensile strength and tear resistance of materials

Unmodified silicone specimens were used as controls. A total of 10 'dumbbell' shaped specimens (10 mm x 45 mm x 2 mm) of each test material were fabricated. At the midpoint length of each sample, a scalpel was used to make a cut 4 mm across the sample, to initiate a tear. Samples were loaded into a Mecmesin MultiTest-d machine (Mecmesin Ltd, Slinfold, UK) and loaded at a speed of 300 mm/min. The proprietary software was used to calculate the percentage elongation and maximum load for each material until tearing.

5.3.11.2: Evaluation of surface roughness of materials by optical profilometry

Test materials formulated as described in sections 5.3.6 and 5.3.8 were tested according to ISO 4287:1997 (Geometrical product specifications (gps) -- surface texture: profile method) using a white light interferometer (Micro XAM-100-HR, KLA-Tencor, Wokingham, UK) with the assistance of Dr. E Brousseau, School of Engineering, Cardiff University. A total of 6 samples for each material were analysed, with mean Ra values (the arithmetic average of the roughness profile) calculated by the proprietary software.

5.3.11.3: Evaluation of hydrophobicity of materials by dynamic contact angle analyser

Test materials were prepared in 10 mm x 10 mm x 1 mm coupons, with 6 replicates of each material tested. Following 30 s immersion in ethanol to remove any surface contaminants, specimens were transferred into a Dynamic Contact Angle analyser (DCA-312, Thermo Cahn Instruments, Madison, USA), being careful to avoid contact of samples with skin. Samples were immersed in water and the advancing contact angle recorded.

5.3.12: Exploration of cytotoxicity of compounds and materials

The following experiment was conducting in the Institute of Life Sciences 1 (ILS1) building, Swansea University, working in collaboration with Dr. Tom Wilkinson.

Test materials were prepared using the experimental compounds which demonstrated greatest antimicrobial activity by MIC, MBC, MBEC and zone of inhibition tests. Materials were sterilised by immersion in ethanol, which was allowed to evaporate in a fume cabinet. Test materials were then aseptically transferred to 12-well plates. Whole blood from clinically healthy donors was collected by the clinical research facility affiliated with Dr Wilkinson's research group and 500 μl of blood added to the surface of each material, taking care to ensure that the entire material surface area was covered. Additionally, blood was added to duplicate blank wells, to act as negative controls (background); plain silicone and a plain acetoxy resin-coated silicone were used as internal negative controls for triclosan-loaded silicones and silver-complex modified acetoxy resins, respectively. Lipopolysaccharide (LPS) derived from *E. coli* (Sigma Aldrich, Gillingham, UK) at a concentration of 1 μg/ml as a positive control.

Incubation of blood with the test materials was conducted for 0, 2 and 4 h. For 2 and 4 h incubations, samples were incubated aerobically at 37 °C with shaking (120 RPM) to prevent sedimentation of blood.

Following incubation with test materials, blood was transferred to sterile 1.5 ml microcentrifuge tubes by gentle pipetting. Samples were subsequently centrifuged at 3000 RPM for 5 min, and the platelet-free plasma supernatant transferred to new sterile microcentrifuge tubes. Samples were frozen at -80 °C prior to analysis of cytokines. Cytokine analysis was conducted using the BD Biosciences cytometric bead array for human inflammatory cytokines (BD Biosciences, Wokingham, UK), as described in Section 4.3.5.6. An outline of the screening process and subsequent evaluations for test compounds is displayed in Figure 5.3.12.

Figure 5.3.12: Screening process and evaluations for test compounds and materials

Initial screening of NHC compounds (n=8) and silver complexes (n=5, 3 chemically unstable), silver nitrate, triclosan (control) and triclosan acetate by MIC testing

No inhibition in:
5 NHC compounds
2 silver complexes

Second stage screening of NHC compounds (n=3) and silver complexes (n=3), silver nitrate, triclosan (control) and triclosan acetate by MBC testing

No biocidal effect in:
1 silver complex

Third stage screening of NHC compounds (n=3) and silver complexes (n=2), silver nitrate, triclosan (control) and triclosan acetate by MBEC testing

No biofilm eradication in:
Any compound for *P. aeruginosa*
NHC compounds (n=3) excluded due to narrow spectrum of activity

Incorporation of silver complexes (n=2), triclosan and triclosan acetate into acetoxy resins

Zone of inhibition testing to evaluate release of materials from silicones

'Cytotoxicity' blood assay for pro-inflammatory cytokines by FACS

Direct inoculation of materials for L/D staining and CLSM evaluation

Surface roughness , tear resistance and contact angle evaluation

5.4: Results

5.4.1: Minimum Inhibitory Concentrations (MICs) for test compounds

The majority of tested compounds demonstrated some inhibitory activity against at least one of the pathogens (Table 5.4.1). There was no clear trend of modulation of antimicrobial activity by length of hydrocarbon chains. However, halogenated compounds appeared to inhibit microbial growth at lower concentrations overall. The addition of silver to the NHC compounds led to a greater spectrum of antimicrobial activity (fewer uninhibited microorganisms) but did not appear to result in a reduced MIC value in microorganisms against whom the NHC ligand was already effective. This may be due to a net reduction in the quantity of ligand present. Only one compound, Ag $C_{16}H_{31}BrN_2$, was effective against all microorganisms at the concentrations tested. Several of the NHCs could not be complexed with ionic silver; silver particles precipitated from these solutions and sedimented at the base of the containers. These compounds were thus excluded from further testing. Similarly, compounds which all microorganisms were able tolerate at the tested concentrations were also excluded. Triclosan demonstrated activity against all microorganisms with the exception of *P. aeruginosa*.

Table 5.4.1: Minimum Inhibitory Concentrations (MICs) for test compounds

	C. albicans ATCC 90028	E. coli NCTC 12923	P. aeruginosa ATCC 15692	S. aureus NCIMB 9518	S. aureus NCTC 6571	S. epidermidis ATCC 14990	S. epidermidis RP62A
$C_{14}H_{26}N_2O_3S$	R	R	R	R	R	R	R
Ag $C_{14}H_{26}N_2O_3S$	25	R	100	R	R	100	100
$C_{15}H_{20}N_2O_3S$	R	R	R	R	R	R	R
Ag $C_{15}H_{20}N_2O_3S$				Unstable			
$C_9H_{15}NO_4S_2$	100	R	R	R	R	R	R
Ag $C_9H_{15}NO_4S_2$	50	R	R	50	25	25	25
$C_{14}H_{26}BrNOS$	50	R	R	50	25	25	25
Ag $C_{14}H_{26}BrNOS$				Unstable			
$C_{18}H_{34}N_2O_3S$	R	R	R	100	100	50	50
Ag $C_{18}H_{34}N_2O_3S$	12.5	100	100	R	R	50	50
$C_{16}H_{31}BrN_2$	6.25	50	R	3.125	3.125	1.5625	1.5625
Ag $C_{16}H_{31}BrN_2$	1.5625	50	100	3.125	3.125	3.125	3.125
$C_{14}H_{10}F_{17}IN_2$	6.25	25	R	6.25	6.25	1.5625	1.5625
Ag $C_{14}H_{10}F_{17}IN_2$	25	100	R	12.5	12.5	12.5	12.5
$C_{15}H_{27}BrN_2$	25	50	R	6.25	6.25	6.25	6.25
Ag $C_{15}H_{27}BrN_2$				Unstable			
$AgNO_3$	<0.78125	50	12.5	25	25	12.5	12.5
Triclosan	6.25	1.5625	R	<0.78125	<0.78125	<0.78125	<0.78125
Triclosan Acetate	R	<0.78125	R	<0.78125	<0.78125	<0.78125	<0.78125

MICs for test compounds are expressed in μg/ml
R = no inhibition observed at all concentrations tested

5.4.2: Minimum Biocidal Concentrations (MBCs) for test compounds

Only those compounds which demonstrated effective inhibition of at least one microbial species were tested for biocidal activity (Table 5.4.2). As was observed in MIC tests, the complexing of the NHC ligands with ionic silver resulted in a broader spectrum of biocidal activity but did not alter the concentration at which this occurred in most cases. With the exception of *S. epidermidis* strains, triclosan compounds had minimal biocidal effects. Again, one compound, Ag $C_{16}H_{31}BrN_2$, demonstrated biocidal activity against all strains tested.

Table 5.4.2: Minimum Biocidal Concentrations (MBCs) for test compounds

	Species						
	C. albicans ATCC 90028	*E. coli* NCTC 12923	*P. aeruginosa* ATCC 15692	*S. aureus* NCIMB 9518	*S. aureus* NCTC 6571	*S. epidermidis* ATCC 14990	*S. epidermidis* RP62A
Ag $C_{18}H_{34}N_2O_3S$	R	R	R	X	X	R	R
$C_{16}H_{31}BrN_2$	R	R	X	50	50	50	50
Ag $C_{16}H_{31}BrN_2$	25	50	100	50	100	12.5	25
$C_{14}H_{10}F_{17}IN_2$	25	R	X	100	25	25	25
Ag $C_{14}H_{10}F_{17}IN_2$	25	R	X	100	50	25	25
$C_{15}H_{27}BrN_2$	R	R	X	50	50	25	25
AgNO$_3$	50	R	100	R	100	100	100
Triclosan	R	R	X	R	R	50	100
Triclosan Acetate	R	R	X	R	R	50	50

MBCs for test compounds are expressed in μg/ml
R = no biocidal effect noted at all concentrations tested
X = not tested due to lack of inhibition noted in preceding MIC tests

5.4.3: Minimum Biofilm Eradication Concentrations (MBECs) for test compounds

No compound was effective against *P. aeruginosa* biofilms (Table 5.4.3). However, most compounds, most notably the triclosan compounds, demonstrated anti-biofilm activity against the Gram-positive Staphylococci. Silver nitrate was ineffective against all bacterial biofilms. Again, Ag $C_{16}H_{31}BrN_2$ demonstrated the broadest range of activity, with anti-fungal and activity against *E. coli*, although only at the highest concentration tested were *E. coli* biofilms affected. By comparison, the triclosan compounds demonstrated much greater effectiveness against Staphylococci (MBEC 6.25 μg/ml for *S. aureus* strains, 3.125 μg/ml for *S. epidermidis* strains vs. 100 μg/ml and 12.5 μg/ml respectively for Ag $C_{16}H_{31}BrN_2$), but no effectiveness against *E. coli* or *C. albicans* at the concentrations evaluated.

Table 5.4.3: Minimum Biofilm Eradication Concentrations (MBECs) for test compounds

	C. albicans ATCC 90028	E. coli NCTC 12923	P. aeruginosa ATCC 15692	S. aureus NCIMB 9518	S. aureus NCTC 6571	S. epidermidis ATCC 14990	S. epidermidis RP62A
				Species			
$C_{16}H_{31}BrN_2$	100	200	X	100	100	100	100
Ag $C_{16}H_{31}BrN_2$	50	200	R	100	100	12.5	12.5
$C_{14}H_{10}F_{17}IN_2$	200	R	X	100	50	50	50
Ag $C_{14}H_{10}F_{17}IN_2$	100	R	X	100	100	50	50
$C_{15}H_{27}BrN_2$	R	R	X	200	200	50	50
$AgNO_3$	100	R	R	R	R	R	R
Triclosan	R	R	X	6.25	6.25	3.125	3.125
Triclosan Acetate	R	R	X	6.25	6.25	3.125	3.125

MBECs for test compounds are expressed in μg/ml

R = no biofilm eradication noted at all concentrations tested

X = not tested due to lack of biocidal activity noted in preceding MBC tests

5.4.4.1: Bulk-loading of antimicrobial compounds into silicones

Attempts to incorporate both NHCs and silver-NHC complexes into silicones resulted in an inhibition of curing of the silicone, due to a failure in polymerisation. This inhibition remained regardless of whether room temperature or heat-cured silicones were used. There was also a strong, brown discolouration of the silicone materials.

5.4.4.2: Incorporation of silver-NHC compounds into an acetoxy-resin silicone oil dispersion

The incorporation of both NHCs and silver-NHC complexes into an acetoxy-resin material proved to be successful, with no effect on the normal curing time of this material. Subsequent formation of a dispersion by combining the test compounds and acetoxy-resin in a silicone oil also proved successful, with the formation of a smooth, thin layer of material on silicone surfaces following 'dip-coating'. There was a brown-red discolouration observed in these materials, but this was markedly reduced compared to that seen in silicones.

5.4.5: Antimicrobial activity in silicones and acetoxy resin materials assessed by zone of inhibition (ZOI) assays

Initial screening of antimicrobial activity by zone of inhibition assays demonstrated limited activity for silver-NHC complexes, but a strong inhibitory effect of triclosan compounds against Staphylococci (Table 5.4.5). The silver-NHC complexes were hypothesised to exert antimicrobial activity in a contact-dependent manner rather than by release and diffusion from materials, and so further testing by direct inoculation of surfaces was undertaken.

Table 5.4.5: Zones of Inhibition for test silicone and acetoxy-resin materials

	Species						
	C. albicans ATCC 90028	*E. coli* NCTC 12923	*P. aeruginosa* ATCC 15692	*S. aureus* NCIMB 9518	*S. aureus* NCTC 6571	*S. epidermidis* ATCC 14990	*S. epidermidis* RP62A
Triclosan	0	15	0	45	40	45	45
Triclosan Acetate	0	16	0	44	36	45	46
Ag $C_{16}H_{31}BrN_2$	0	0	0	0	0	15	7
Ag $C_{14}H_{10}F_{17}IN_2$	0	0	0	0	0	0	11

ZOIs expressed in mm corresponding to the diameter of the zone of no growth observed

5.4.6: Assessment of antimicrobial activity of silicones and acetoxy-resins

The tested materials had different effects on each of the tested species. *Candida albicans* was able to adhere to and remain viable upon all materials (Figure 5.4.6 A & B). While there was an approximate 50% reduction in total viable adherent microbes (Figure 5.4.6 B) on the Ag $C_{16}H_{31}BrN_2$-modified acetoxy resin material compared to the control, this was neither statistically significant nor clinically relevant. Surprisingly, *E. coli* adhered very poorly to the plain acetoxy resin control, despite high adherence to the silver-complex modified materials and silicones (Figure 5.4.6 D). Despite this reduced adherence, the overall viability of adherent *E. coli* to all test materials was similar, at around 40-60% (Figure 5.4.6 C). Triclosan acetate caused an almost 97% reduction in the proportion of viable *S. aureus* compared to plain silicone controls (Figure 5.4.6 E), with a decrease in absolute quantity of viable microbes of 98.8% and a decrease in the total quantity of adherent microbes (live or dead) of 97.8% (Figure 5.4.6 F). As was seen with *E. coli*, there was no impact of incorporation of silver-complexes in acetoxy resins on the adherence or viability of *S. aureus*. In fact, incorporation of Ag $C_{16}H_{31}BrN_2$; the most promising antimicrobial in earlier screening, appeared to result in both greater adherence and a greater proportion of viable *S. aureus.*

A *C. albicans* ATCC 90028

B *C. albicans* ATCC 90028

C

E. coli NCTC 12923

D

E. coli NCTC 12923

S. aureus NCIMB 9518

S. aureus NCIMB 9518
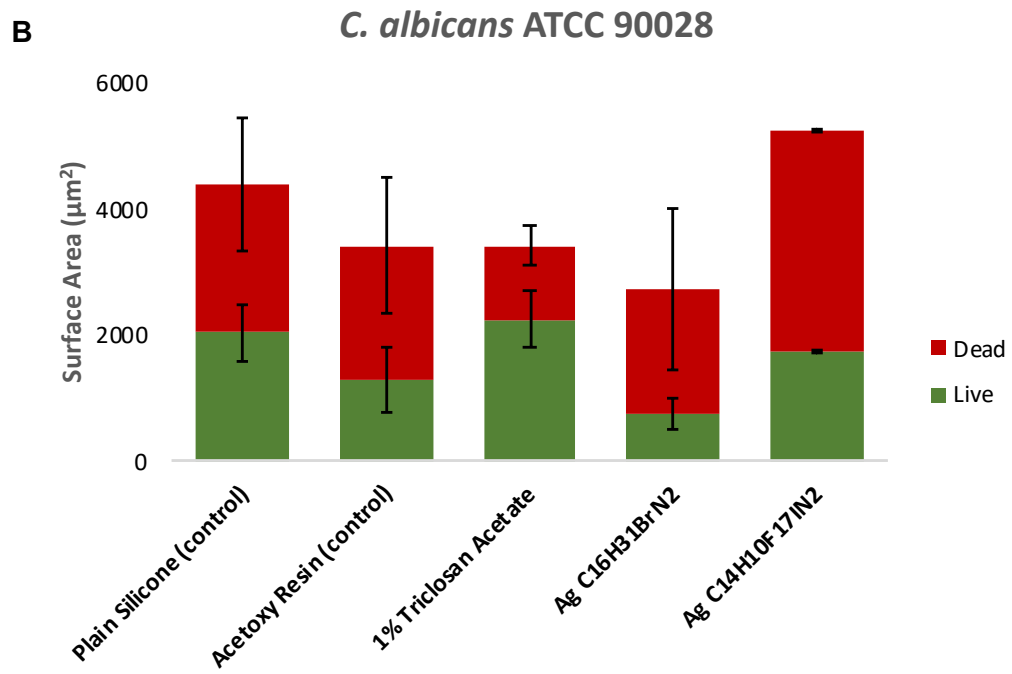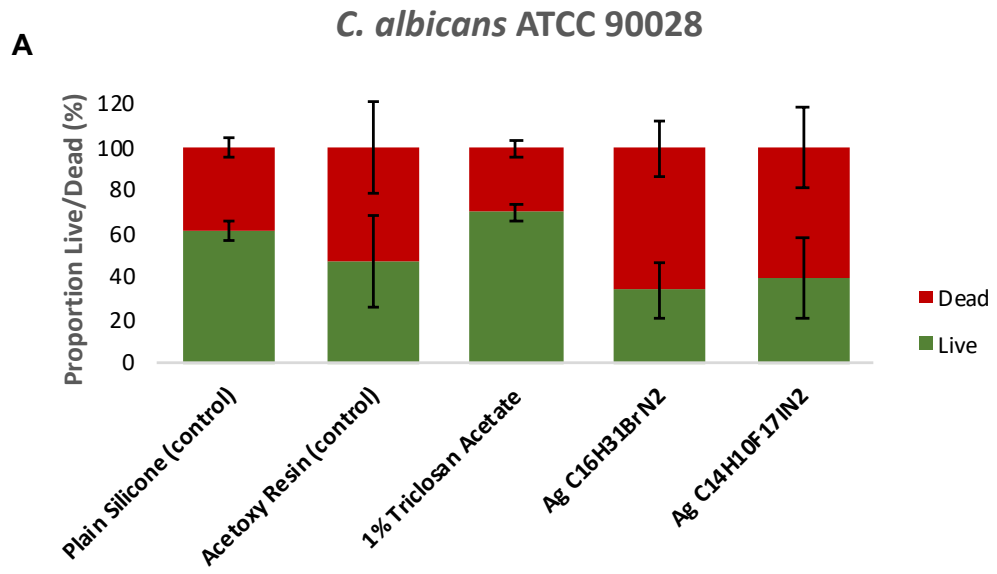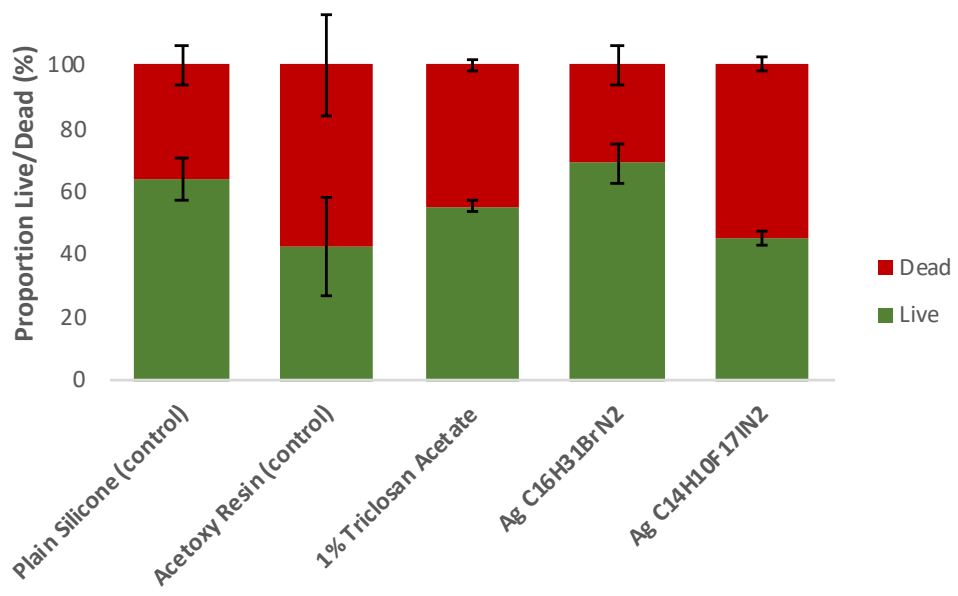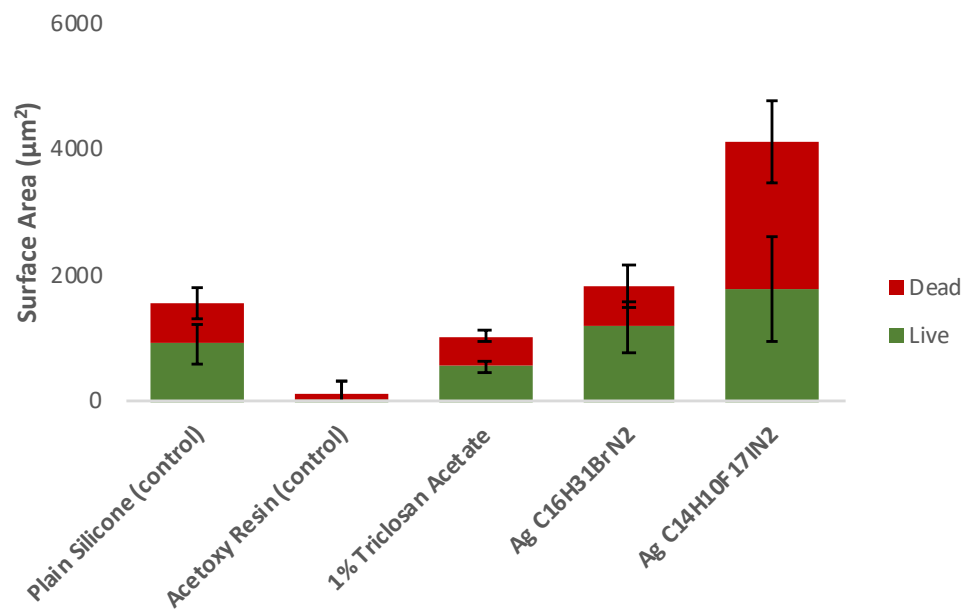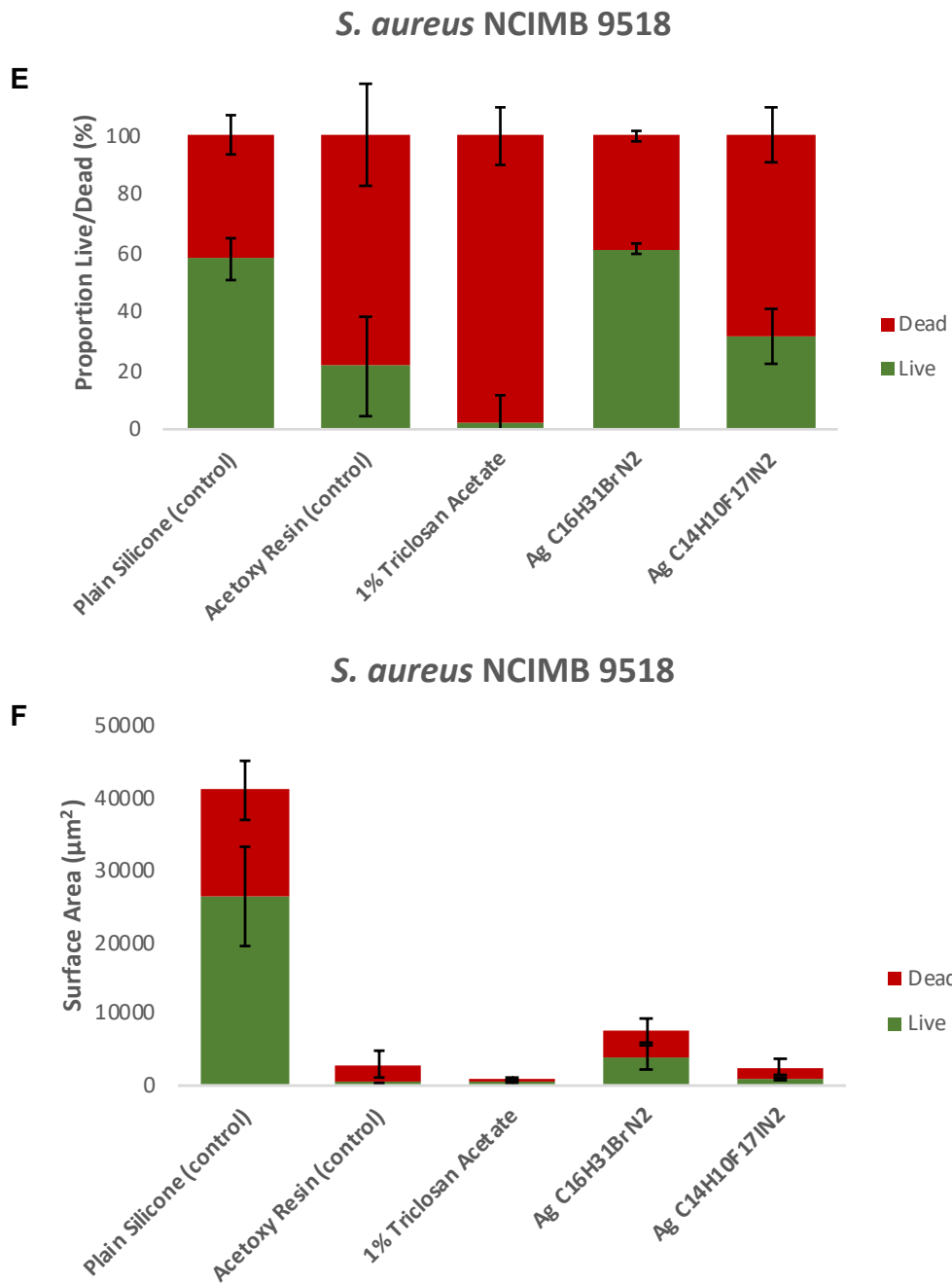
Figure 5.4.6: Antimicrobial activity of test materials as assessed by quantitative evaluation of Live/Dead stain of microbial inocula on material surfaces

A) Proportion of live/dead *C. albicans*
B) Surface area occupied by live/dead *C. albicans*
C) Proportion of live/dead *E. coli*
D) Surface area occupied by live/dead *E. coli*
E) Proportion of live/dead *S. aureus*
F) Surface area occupied by live/dead *E. coli*

Mean values shown in columns. Error bars represent SEMs. All samples were tested in triplicate; N=3.

5.4.7: Impact of triclosan and triclosan acetate bulk-loading on physico-chemical properties of silicone

A notable visible effect of incorporation of triclosan materials in silicones was a white colouration to the material (data not shown).

5.4.7.1: Tensile strength and tear resistance

There was no significant effect of the addition of triclosan compounds on the percentage elongation of silicone materials (Table 5.4.7.1). However, both triclosan and triclosan acetate significantly reduced the tear resistance of materials. Despite strong statistical significance however, the magnitude of the difference was relatively small (an approximately 25% reduction). The significance of such changes in a clinical setting are questionable.

Table 5.4.7.1: Tensile strength and tear resistance of silicones

Material	Mean % elongation (S.D.)	Mean maximum load (N) before tear (S.D.)
Plain Silicone (control)	435.86 (57.54)	32.20 (4.28)
1% Triclosan	438.46 (28.29)	23.62**** (3.17)
1% Triclosan Acetate	451.98 (44.19)	25.84** (3.97)

** $P=0.0019$, **** $P<0.0001$ (One-way ANOVA with Dunnett's test vs. plain silicone control group). N = 10.

5.4.7.2: Contact angle

The addition of triclosan compounds to silicone led to statistically significant increases the hydrophilicity of the materials (Table 5.4.7.2). This may be due to residual acetone within the material.

Table 5.4.7.2: Contact angles for silicones measured in water

Material	Contact Angle (°) (S.D.)	Interpretation
Plain Silicone (control)	96.3 (6.37)	Hydrophobic
1% Triclosan	71.2**** (6.02)	Weakly hydrophilic
1% Triclosan Acetate	69.7**** (5.64)	Weakly hydrophilic

**** $P<0.0001$ (One-way ANOVA with Dunnett's test vs. plain silicone control group) N = 10.

5.4.7.3: Surface roughness

The addition of triclosan compounds to silicones resulted in an approximately 3-fold increase in the Mean R_a values, which was statistically significant (Table 5.4.7.3).

Table 5.4.7.3: R_a Values for silicone materials

Material	Mean R_a (S.D.)
Plain Silicone (control)	0.0682 (0.018)
1% Triclosan	0.178**** (0.008)
1% Triclosan Acetate	0.17**** (0.012)

**** $P < 0.0001$ (One-way ANOVA with Dunnett's test vs. plain silicone control group)
N = 6

5.4.8: Impact of incorporation of silver-NHC compounds on physico-chemical properties of acetoxy-resin materials

The silver-NHC compounds imparted a slight silver-brown colouration to the acetoxy resin materials, although this was less strong than had been seen during attempted bulk-loading of silicone materials.

5.4.8.1: Contact angle

There was no significant impact on the hydrophobicity of acetoxy resins following the addition of silver-NHC complexes (Table 5.4.8.1). The materials were very weakly hydrophilic overall.

Table 5.4.8.1: Contact angles for acetoxy resins measured in water

Material	Contact Angle (°) (S.D.)	Interpretation
Acetoxy Resin (control)	87.2 (4.72)	Very weakly hydrophilic
Ag $C_{16}H_{31}BrN_2$	85.0 (7.14)	Very weakly hydrophilic
Ag $C_{14}H_{10}F_{17}IN_2$	88.5 (4.37)	Very weakly hydrophilic

N = 10.

5.4.8.2: Surface roughness

The addition of silver-NHC complexes had no significant effect on the mean R_a value of the acetoxy resins (Table 5.4.8.2). The roughness of the acetoxy resin materials was intermediate to plain silicone and triclosan-loaded silicones.

Table 5.4.8.2: R_a Values for acetoxy resin materials

Material	Mean R_a (S.D.)
Acetoxy Resin (control)	0.117 (0.012)
Ag $C_{16}H_{31}BrN_2$	0.132 (0.033)
Ag $C_{14}H_{10}F_{17}IN_2$	0.120 (0.017)

N=6

5.4.9: Cytotoxicity of antimicrobial materials - Evaluation by a patient-sourced blood toxicity model

IL-12p70

IL-1B

IL-6
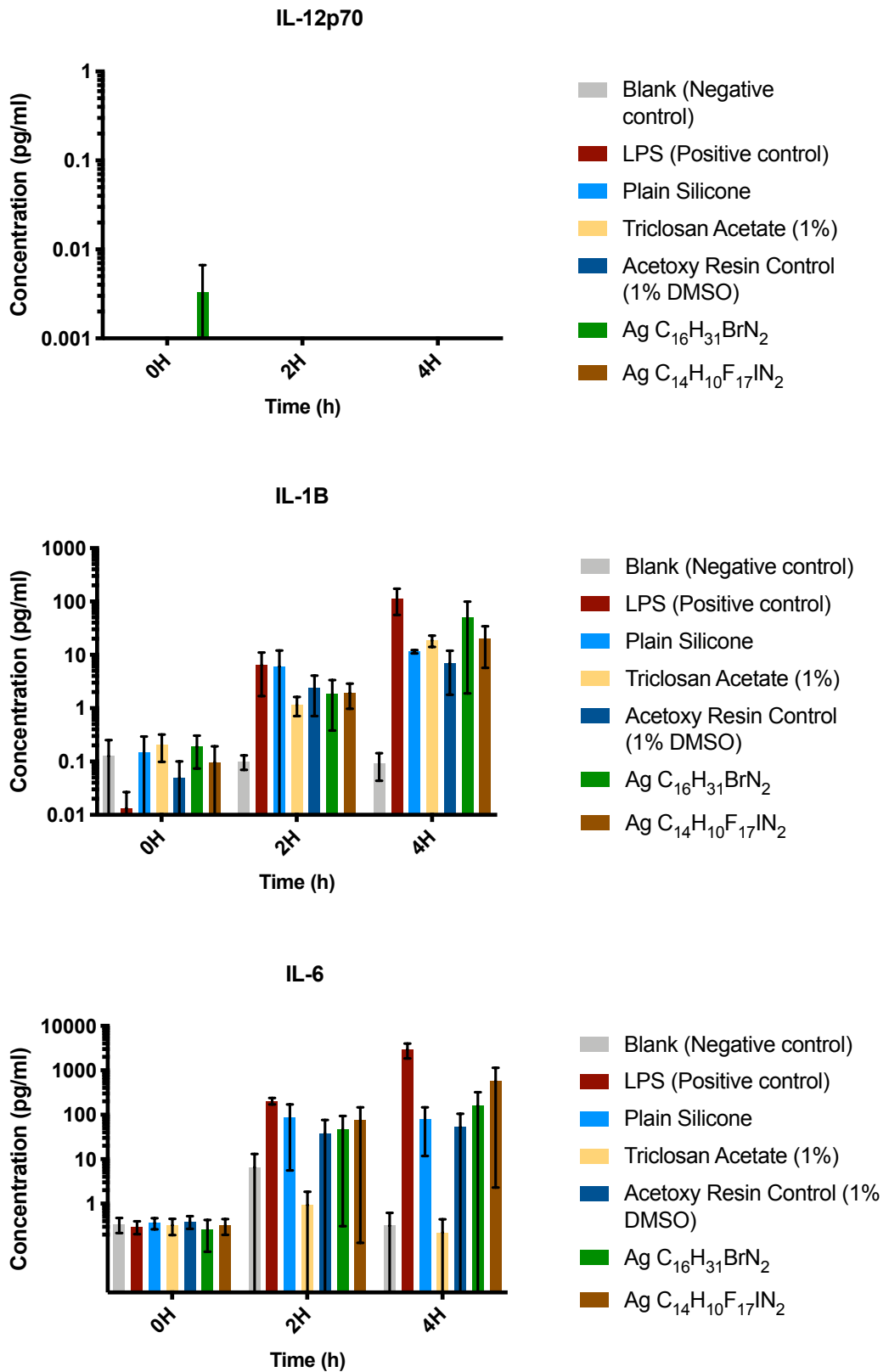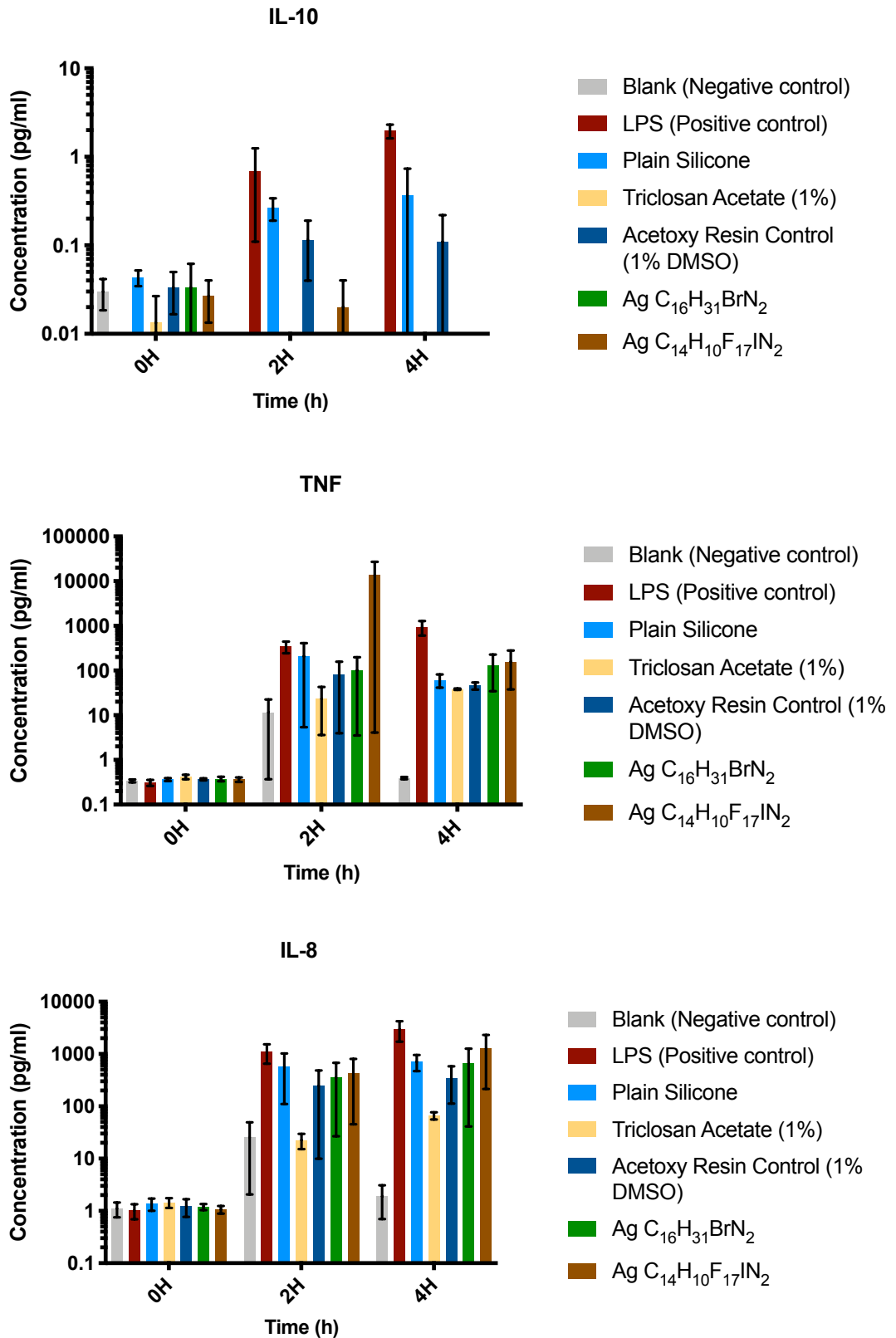
Figure 5.4.9: Stimulated pro-inflammatory cytokines in donor-derived whole blood inoculated onto material surfaces

N = 3
Mean values shown; error bars represent standard error of the mean (SEM).

None of the tested compounds demonstrated a statistically significant difference in eliciting pro-inflammatory cytokines from inoculated blood compared to plain silicone or acetoxy resin controls at any time point (Figure 5.4.9). However, most materials (including plain silicone) generated an increase in IL-1B from 0.1 to between 1.2-6.035 pg/ml; IL-6 from 6.6 to between 38.4-88.0 pg/ml; TNF from 11.5 to between 23.3-13533.1 pg/ml, and IL-8 from 25.8 to 247.9-566.3 pg/ml compared to blank controls at 2 h. At 4 h further differences were seen approximately 1 order of magnitude higher. Of note, triclosan acetate appeared to be the least inflammation-inducing compound. For both IL-12p70 and IL-10, levels were below the threshold of detection for several samples, rendering meaningful comparison unfeasible. There was a high degree of heterogeneity in the responses, indicated by the broad error bars. This variability was between samples from different donors rather than within samples (data not shown), with over a 1000-fold difference in concentrations of cytokines between donors.

5.5: Discussion

In addition to the clinical problem of denture-associated infections; there is growing concern about the possibility of denture biomaterials acting as a reservoir for exogenous pathogens or promoting promulgation of potential pathobionts which may subsequently seed infection at distant anatomical sites such as the respiratory tissues (Coulthwaite and Verran 2007). As tooth loss is associated with a number of systemic comorbidities (Felton 2016), and edentulism primarily affects the elderly, the risk of infection and associated morbidity and mortality is increased. This is particularly true for frail, elderly individuals such as those in long-term institutional care, for whom access to regular dentistry and the ability to tolerate extensive treatment may be limited (Fox 2010). A number of such individuals have decreased motor learning capacity, which may be due to age-related neuromuscular changes, or as a consequence of disease, such as neurological deficit secondary to cerebrovascular accident (stroke). This population also frequently suffers from xerostomia, which may be related to medications, systemic illnesses such as diabetes, or aging-associated reduction in salivary output (Nederfors 2000). As a result of the confluence of poor access, ill-health, xerostomia, and reduced ability to tolerate treatment, provision of new dentures may be highly challenging. Relining existing dentures is therefore an attractive alternative, as this is relatively simple to undertake, can be achieved in 1 – 2 visits, and in the case of soft denture-liners, is reversible in the event of poor tolerance of alterations (Kimoto et al. 2004; Pisani et al. 2013). Such materials also offer a cushioning effect which can increase comfort in individuals with atrophied ridges.

Unfortunately, the biological impact of such materials, which are usually silicone-based, is less favourable. Silicones are vulnerable to degradation due to friction or abrasion compared with PMMA resins (Hermann et al. 2008). While much of the literature has focussed on the permeability of silicone denture-liners to *C. albicans* hyphae (Verran and Maryan 1997; Bulad et al. 2004; Skupien et al. 2013), it seems likely that surface defects might be equally plausibly exploited by pathogens frequently encountered in institutional care settings, particularly given such species' ability to form biofilms on other indwelling devices like catheters or endotracheal tubes (Percival et al. 2015).

The most widely-explored approach to overcome the issue of denture-associated biofilms and infections has been to employ antimicrobials; typically, antifungal agents with activity against Candida species (Geerts et al. 2008; Rathore et al. 2009; Salim et al. 2012). It is therefore a natural extension of such an approach to employ

antimicrobials with broad-spectrum activity against a range of relevant pathogens to reduce the risk of colonisation by putative respiratory pathogens.

In this chapter, a range of novel, nucleophilic-heterocyclic carbenes and their silver complexes were screened for inhibitory and biocidal activity against a range of relevant pathogens frequently encountered on medical devices and dentures. Additionally, triclosan and a novel triclosan derivative triclosan acetate, hypothesised to have better incorporation into silicones, were trialled against the same panel of microorganisms. Compounds demonstrating promising biocidal activity were subsequently applied to established biofilms. A method for incorporation of the most promising silver complexes and triclosan compounds into silicone-compatible (for the silver complexes) materials, or directly into silicone (for the triclosan compounds) was formulated. These novel materials were screened for antimicrobial activity using a zone of inhibition test and for anti-adherent effects by direct inoculation and examination using CLSM. Furthermore, the impact of addition of antimicrobial compounds on some basic physicochemical properties of the materials was examined. Finally, the cytotoxicity of materials was explored using a novel donor-derived whole-blood assay to assess stimulation of pro-inflammatory cytokine release.

The antimicrobial compounds in free solution offered considerable promise, with NHCs demonstrating some antimicrobial activity alone, with complementary broad-spectrum activity provided by the complexation with ionic silver. One compound in particular, Ag $C_{16}H_{31}BrN_2$, was effective against biofilms of all test species with the exception of *P. aeruginosa* (Table 5.4.3). However, while the triclosan compounds were able to be incorporated directly into silicones, attempts at bulk-loading of silver complexes or NHC ligands alone into silicone materials resulted in a failure of the silicones to polymerise. This is likely due to interaction of the NHC ligands with the platinum catalyst, blocking or reducing its ability to coordinate cross-linking of silicones. In the case of the silver complexes, this occurs by substitution of complexes formed with silver to platinum; a process termed transmetalation (Lalonde et al. 2013). This phenomenon is due to the slightly increased electronegativity of platinum compared to silver. A potential solution could be to employ an alternative catalyst with reduced electronegativity to facilitate silicone cross-linking. Tin is widely used as a silicone catalyst for a number of applications, but has some disadvantages compared to platinum-cured silicones (Fawcett et al. 2013). Firstly, the degree of polymerisation shrinkage is increased during curing of the material, with a resultant reduction in dimensional accuracy (Colas and Curtis 2004). There are concerns about the use of tin as a catalyst due to toxic products generated, both from an environmental

perspective and the possibility of direct cytotoxicity from residual tin products in biomedical applications (Tanzi et al. 1994; Burnell et al. 2000). One such example of silicones which employ tin catalysts are the acetoxy resins, which cure by condensation following incorporation of water from environmental moisture. In this work package, it was found that curing of acetoxy resins was not inhibited by the presence of NHC or silver-complexes, supporting the transmetalation hypothesis. A notable by-product of such a curing reaction is the formation of acetic acid (Colas 2005). This was observed during curing of test materials by the generation of a vinegar-like smell. Acetic acid is itself antimicrobial (Ryssel et al. 2009) and incomplete removal of this material from the acetoxy resin control material during antimicrobial testing may explain the apparent antimicrobial activity of this material observed (Figure 5.4.6 A-F). The silver-NHC complexes failed to demonstrate any significant antimicrobial activity against the microbial strains tested, nor demonstrated an anti-adhesive effect. Previous studies have employed metals (Salvarci et al. 2016) or antimicrobials such as chlorhexidine to achieve antimicrobial silicone surfaces (Darouiche et al. 2006; Garner et al. 2015; Phuengkham and Nasongkla 2015). However, in the majority of cases, silicones are coated with the test compounds, rather than direct incorporation of the compounds into the silicones themselves. While some authors have found successful retention of antimicrobial activity directly incorporating zinc-oxide (Ozkan et al. 2015) and titanium compounds (Depan and Misra 2014) into silicone polymers, the metals were used in nanoparticle form. Direct coating of silicones by the test silver-NHC complexes was not practical in this work due to the photo-reactivity of the complexes rendering their application to clinical scenarios unrealistic. Additionally, studies employing such methods rely upon controlled release of antimicrobial coatings, while the present work aimed to achieve *in situ* antimicrobial activity without significant release and systemic uptake of the compounds. Although metallic nanoparticles offer promising antimicrobial activity, the effects of their biological and environmental accumulation are a growing concern among the scientific community (Zuzana et al. 2011).

In contrast, triclosan and triclosan acetate both retained antimicrobial activity against Staphylococci. The large zones of inhibition generated by such materials suggests that this results at least in part by diffusion of the triclosan compounds from the silicone surface (Table 5.4.5). There was both a reduction in the absolute number of adherent microorganisms and the proportion of viable Staphylococci present on the surface of silicones containing 1% triclosan acetate (Figure 5.4.6 E and F). While concerns exist regarding the environmental effects of triclosan (Dann and Hontela 2011), it is likely that the quantities produced in this relatively niche application would

have limited significance in the context of the widespread use of this compound. Nevertheless, such concerns may constrain the use of triclosan compounds in biomedical applications in future.

All test materials showed no significant increase in stimulation of pro-inflammatory cytokines in a novel donor-derived whole blood assay (Figure 5.4.9). However, while this model is excellent for obtaining more representative data with greater biological validity, the high degree of variability in cytokine responses from each donor may mask underlying patterns. Because blood from just 3 donors was used, this variability results in a strong noise: signal ratio. Ideally, a larger sample size would have been used, but this was not possible due to financial and time constraints. An ideal approach would be to pair such testing with basic cytotoxicity assessments on relevant cell lines such as oral fibroblasts and epithelial cells, and possibly subsequent testing on 3D organotypic tissue models. It is possible that the low level of cytotoxicity exhibited by the test compounds is due to their entrapment in silicone cross-linked polymer chains, which may also be responsible for the lack of antimicrobial activity seen.

The metal-NHC complexes had little effect on the physicochemical properties of the acetoxy resins. The triclosan compounds appeared to lead to a reduction in the contact angle of silicones, leading to more hydrophilic surfaces. This result might appear surprising, as triclosan, like silicone, is highly hydrophobic. The addition of triclosan also increased the surface roughness of silicone materials however, and the interaction of surface roughness and hydrophobicity may explain the observed effect (Teughels et al. 2006). In addition, there was a significant decrease in tear resistance, although the magnitude of this effect was not especially large, and so its clinical relevance is open to interpretation. However, given the role of surface degradation in facilitating infiltration of microbial biofilms in silicone surfaces, this effect should not be disregarded when considering the application of triclosan-modification of silicone for clinical applications.

Limitations of the methods

This work had several important limitations. Only 1-2 strains of each representative pathogen were used to test the antimicrobials. Some (*C. albicans* ATCC 90028, *S. epidermidis* RP62A, *S. aureus* NCIMB 9518 and *P. aeruginosa* ATCC 15692) were originally isolated from human infections, although none were associated with respiratory infection or the oral cavity. Furthermore, virulence and microbial behaviour following multiple generations of microorganisms cultured in the laboratory is likely to be highly different to *in vivo* characteristics of the original isolates. It may

be that the results of antimicrobial testing are therefore unapplicable to pathogens in the medical setting. However, as an initial screening method, this is a necessary pragmatic trade-off. There is no strong reason to assume that orally-derived strains of the same microorganisms would behave in a strongly dissimilar manner.

Only a limited range of compounds were tested, and due to the complexity of their synthesis only limited quantities were available. The silver coordination complexes were all comprised of a 1:1 ratio of silver to NHC. For a more comprehensive evaluation, each basic structure would be tested with a range of hydrocarbon tail lengths and incorporating different positions of saturated hydrocarbons. The most effective compounds Ag $C_{16}H_{31}BrN_2$ and Ag $C_{14}H_{10}F_{17}IN_2$ had the two longest hydrocarbon tail lengths. However, it is not clear from the current research if tail length is an important factor, or if some other aspect of the molecular structure is responsible for their better antimicrobial performance.

The zone of inhibition testing and direct inoculation evaluation demonstrated minimal antimicrobial effectiveness of the silver complexes. It was assumed that this was due to a lack of release of the compounds from the material surface. However, the concentration gradient in the agar used for zone of inhibition testing could have been measured by liquid chromatography, to quantify any release and the diffusion properties of the compounds. Similarly, a blank inoculum with growth medium could have been undertaken alongside the inoculation experiments for subsequent removal and characterisation. This would elucidate if any compound is released from the surface, and if any chemical changes occur from incorporation into silicone materials. Future work should seek to include such testing, regardless of the effectiveness of materials evaluated.

The tested compounds Ag $C_{16}H_{31}BrN_2$ and Ag $C_{14}H_{10}F_{17}IN_2$ showed considerable promise as antimicrobial agents against a range of relevant pathogenic microorganisms. However, further work is required to ensure this antimicrobial activity is retained when such compounds are incorporated into biomaterials such as silicones. A method of surface-coating by which the compounds are reliably tethered to the biomaterial surface may be one avenue to explore in future. Triclosan and triclosan acetate can be successfully incorporated into silicone biomaterials, with strong antimicrobial activity against Staphylococci and no apparent provocation of a host pro-inflammatory cytokine response. However, the impact of these materials on the mechanical properties of silicones requires further characterisation to ensure these do not impart adverse clinical effects.

CHAPTER 6: EXPLORATION OF THE ANTI-BIOFILM POTENTIAL OF HIGHLY CONTROLLED DELIVERY OF MICROWAVE ENERGY USING A CAVITY RESONATOR OR COAXIAL PROBE

6.1: Introduction

6.1.1: Principles of microwave energy - generation and properties

The term "microwaves" refers to electromagnetic radiation with a frequency of between 300 MHz and 30 GHz, corresponding to a wavelength of between 1cm and 1m (Collin RE, 2001). While microwaves have many applications, summarised in Figure 6.1.1, the primary focus of this section will be their use in heating and disinfection.
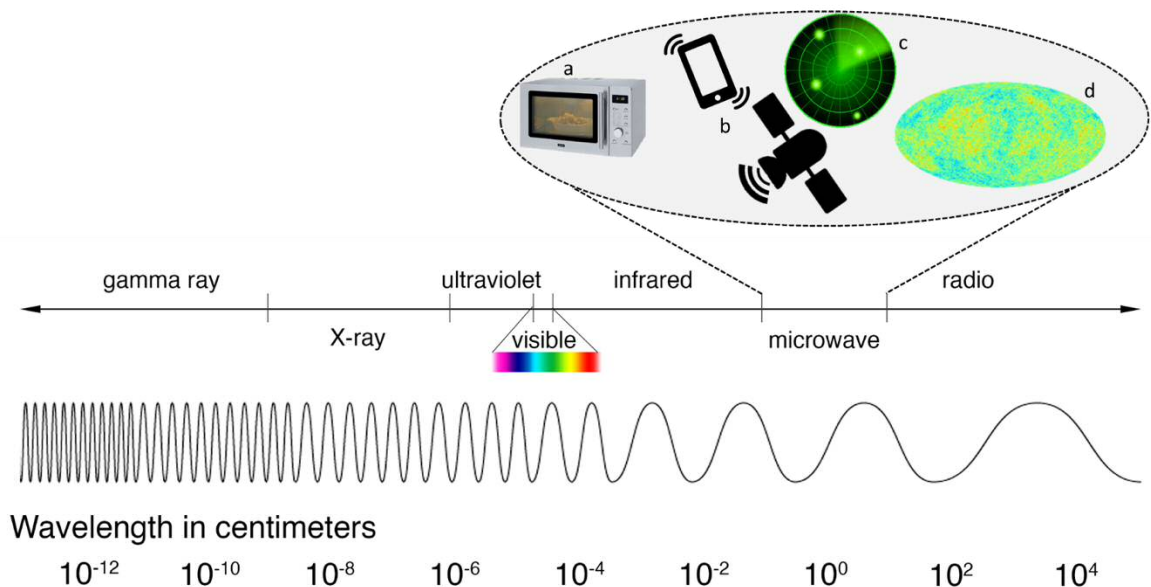
Figure 9: Microwave electromagnetic frequency and selected applications.
a) Domestic microwave oven (Magnetron)
b) Communications technology
c) Radar
d) Radio astronomy
    (Image adapted from NASA "Imagine the universe" URL: http://imagine.gsfc.nasa.gov/science/toolbox/spectra1.html)

The application most commonly associated in the lay public with microwave energy is use of magnetron ovens for cooking in domestic settings. The basic theory of microwave generation within a magnetron oven is reviewed in Vollmer (2004). The generated electromagnetic waves (which are standardised to 2.45 GHz in Europe) cause oscillation in dipolar molecules, most notably water, due to the alternating electric field. The net result of this increased molecular vibration is heat generation, termed dielectric heating (Grant and Halstead, 1998). This form of heating contrasts conventional heating methods whereby heat is generated in the surrounding environment, with the target being heated from outside to inside.

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The ability of microwave energy to cause heating in a material is linked to the propensity of the target material molecules to polarise in alignment with the electric field. This is determined by the dielectric properties of the material. PMMA materials have a low dielectric constant (the term relative permittivity is preferred), indicating a declivity of the constituent molecules to polarisation by application of an electric field. This has two important consequences: relatively low heating of the PMMA biomaterial in the presence of microwave electric field; and minimal interference of the PMMA resin with the electric field. The relevance of these properties will be discussed in later sections.

The widespread use of magnetron ovens, their low cost and ease of use make them attractive targets for sterilisation of acrylic dentures, particularly in institutional settings, where chemo-mechanical methods of denture hygiene are typically under-utilised (Gornitsky et al. 2002). However, it is important to note that any prostheses with metal components may not be used in such a device, as reflected microwaves may lead to high charge density at metal vertices and edges, leading to electrical arcing. Such phenomena can lead to damage to the microwave oven or denture components.

6.1.2: Use of microwave energy for disinfection

While more commonly associated with cooking in a domestic setting, the potential for microwave energy to be used for disinfection and sterilisation in a wide range of industrial settings has been explored (Chandrasekaran et al. 2015). In dentistry, microwave disinfection has been considered as a possible replacement for conventional measures such as autoclaving for processing of some waste materials (Cardoso et al. 2007), dental instruments (Tarantino et al. 1997), impression materials (Abdelaziz et al. 2004), dental casts (Berg et al. 2005) as well as dentures. In all such cases, the primary mechanism of microbial inactivation or killing has been linked to heating effects. However, it has been postulated that there may be a separate biological impact of microwave energy that is independent of heat generation.

6.1.3: State of the evidence - disinfection of dentures by microwave ovens

A growing body of research has been accumulated over the last two decades examining the potential use of domestic microwave ovens in denture sterilisation. However, conclusive evidence is lacking due to a failure to establish standard protocols and a lack of integration between microbiological outcomes and material properties. Studies often differ in terms of number, duration and power of microwave

exposures, use of dentures or smaller acrylic samples, range of microbes examined, methods of assessing disinfection or sterilisation, and assessments of material properties.

Several studies have demonstrated the effective disinfection of a range of microbes on dentures using microwave exposures. For instance, Webb et al. (2005) used 350 W for 10 min on dentures of patients with denture stomatitis as part of an effective treatment for this condition. Studies aimed at *Candida* biofilm eradication due to this species being implicated in denture stomatitis have also been conducted with variable methods, exposure times and results (Campanha et al. 2007, Neppelenbroek, 2008). A limitation of this method is that while microorganisms may be inactivated, antigenic components are not removed entirely, thus can still elicit an inflammatory response. Ribeiro et al. (2009) demonstrated the effectiveness of microwaves in sterilising dentures containing *S. aureus*, amongst other pathogens. Importantly, most of the studies use culture methods to determine effectiveness of sterilisation, and none use direct measures of vitality by microscopy.

The minimum effective time and exposure able to adequately disinfect denture surfaces appears to be 3 min at a power of 650 W (Neppelenbroek et al. 2008, Ribeiro et al. 2009, Sanita et al. 2009). However, further clarification of optimal power and time configurations is required to optimise this approach.

Studies investigating changes in denture materials due to microwave exposure have considered a range of important properties. Most authors found an improvement in hardness of acrylics after microwave treatment (Ribeiro et al. 2008, Konchada et al. 2013). This may be a result of increased polymerisation of residual monomer due to heat generated during treatment (Klironomos et al. 2015). Impact resistance was largely unaffected as a result of microwave treatment also (Senna et al. 2011).

However, detrimental effects have been noted in bond strength of denture teeth to the base resin (Consani et al. 2010), as well as hardness of some brands of denture teeth (Campanha et al. 2005, Vasconelos et al. 2013). Most importantly, microwave treatment was found to affect dimensional stability of denture acrylic, with 900 W delivered for as short as 3 min leading to significant alterations with repeated exposure (Senna et al. 2011).

While insufficient evidence exists regarding the effect of microwave energy on dentures, it is clear that there is potential detriment to the materials, induced by heating, which is difficult to predict. An alternative method of delivering microwave energy which could avoid generation of heat and offer a more standardised approach to denture disinfection might mitigate these drawbacks.

While PMMA resin has a low relative permittivity which limits the degree of heating during microwave exposure, the use of microwave ovens to sterilise acrylic dentures appears to generate significant heating. Microwave ovens are designed to impart heat energy to their contents. This is achieved primarily by essentially random distribution of microwave energy which fluctuates over time. Not only does this result in difficulty achieving predictable sterilisation of denture surfaces, as different areas of the PMMA biomaterial surface will receive different exposures to microwaves, but it also necessitates high powers and prolonged exposures to ensure sufficient energy is delivered. Furthermore, several researchers have placed dentures in a volume of water prior to microwave sterilisation, with subsequent deliberate heating of the water to achieve microbiocidal effects.

6.1.4: The cavity resonator - advantages in denture disinfection

The cavity resonator (Figure 6.1.4A & B) offers a simple method of delivering microwave energy in a predictable and consistent manner. The cavity itself is bounded by metallic walls, and often contains air (although other substances may be used). An external power source is coupled to the cavity through a port and provides an electron source for excitation of electromagnetic waves. The wavelength, and thus frequency is determined largely by the size of the cavity. To ensure maximum efficiency and power delivery, it is important to match the input signal to this frequency. In this way, microwaves behave similarly to sound waves, with the cavity acting as an acoustic chamber. The various possible frequencies at which electromagnetic waves are able to propagate within the cavity are termed harmonic resonances.

An important option when considering the application of cavity resonators for disinfection of dental prostheses is the ability to alter the field to accommodate the added impedance of the denture within the cavity. Because microwave resonance is determined by the geometry of the cavity, any object placed into it will modify the resonant frequency, which must be matched by the input signal to ensure high operating efficiency. By adding a directional coupler to the input port of the cavity, Imtiaz et al. (2015) were able to rapidly measure perturbations of the electric field due to different volumes of water placed in the cavity via a novel matching network approach. As well as improving accuracy and speed of feedback, this approach reduced the size and weight of the cavity resonator system, which makes it highly portable. For this reason, this system will be used to optimise experimental methods within the project.
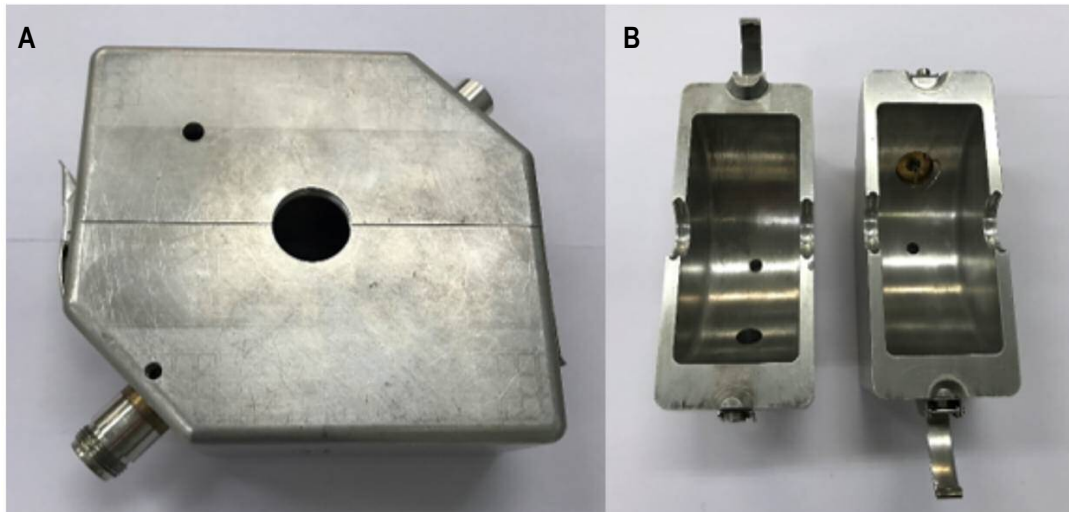
Figure 6.1.4: The cavity resonator used in the experiments presented in this chapter

A) External view of the assembled cavity – the central opening is a viewing port
B) The disassembled cavity showing the cylindrical internal cavity volume

6.1.5: Strategies to reduce heat generation by microwave energy

The possibility of athermal effects due to microwave energy is a contentious topic in the literature. The difficulty of accurately measuring temperature changes at a sufficiently small scale means there is little evidence to support or deny such effects. Several mechanisms have been proposed for athermal mechanisms in killing microbes (reviewed in Knorr et al. 1994).

Kozempel et al. (2000) demonstrated of microwave exposure at between 5 and 6 kW of power at 2.45 GHz when temperature was controlled below 45 °C. This is the only study to date which has successfully applied high power microwave energy to biological samples with a concomitant mitigation of heating effects.

In contrast, it was found that microwave irradiation demonstrated reduction of viable *Staphylococcus aureus* cell counts compared with temperature-matched controls (Khalil and Villota, 1988). The effects seemed to be primarily due to membrane damage, although altered protein expression was also noted. This follows from findings by Dreyfuss et al. (1980) who found that levels of certain metabolic enzymes were elevated in microwave treated compared with heat treated controls. However, these results seem to support a growth-promoting effect of low-level microwave power, rather than cell damage, as lactate dehydrogenase levels were unaltered. SEM analysis of *Mycobacterium bovis* following either microwave treatment for 90 s or immersion in boiling water for the same time revealed membrane discontinuities in the former which were not present in the latter (Rosaspina et al. 1994). However, no convincing evidence has emerged since this publication to support such findings.

It is important to note that while the presence of non-thermal effects of microwave energy have important implications, for the purpose of denture sterilisation they are a secondary concern. The primary motivation in the sterilisation of dental prostheses is to achieve biofilm eradication without causing detrimental heating of the materials.

6.2: Aims

This work package aimed to:

1. Develop a simple simulation model to evaluate the interaction of microwave energy, PMMA material, biofilm and temperatures generated in each material

2. Develop a method to reliably test disinfection of biofilms grown on acrylic surfaces using a cavity resonator

3. Explore methods to reduce heat generation by microwave energy and evaluate the athermal effects of microwave energy on biofilms

Hypotheses

- Microwave electric field energy delivered in a cavity resonator will effectively and reliably kill microorganisms in biofilms on acrylic surfaces at low levels of power (≤43 dBm)

- There will be a difference in the exposure time required to kill biofilms composed of different species, due to water content, cell wall rigidity and surface area: volume ratios

- Biocidal effectiveness of microwave energy delivery will differ with different orientations of biofilms in the electric field. Biofilms positioned parallel to the field will undergo a higher degree of polar interactions and therefore microorganisms will be killed at lower exposure times compared with biofilms positioned perpendicular to the electric field

- Reduction of microwave power or exposure time will reduce heat generation, but will also reduce biocidal activity

- Delivery of microwave energy in duty cycles, controlled for total exposure time, will reduce heat generation without impacting biocidal activity

6.3: Materials and Methods

6.3.1: Simulation of experimental parameters using COMSOL multiphysics software

All simulation work was undertaken by a Final Year Student (Mohammed Khan) at the School of Engineering, Cardiff University, who was co-supervised by Joshua Twigg and Dr Jonathan Lees. Simulations of planned experimental exposures were undertaken using COMSOL Multiphysics software. A PMMA cylinder and cylindrical cavity resonator were modelled using the default values for physical and dielectric properties in the software material library. For all experiments, air was input as the only medium within the cavity. The 'biofilm' was substituted with a 20 μm thick film of water for simplicity.

Initial simulations were undertaken to ensure that no significant disturbance of the electric field (the component of microwave energy responsible for biological effects and heating) occurred during placement of the acrylic sample. This simulation was undertaken in two orientations – with the acrylic sample and biofilm perpendicular to the electric field axis; and with the acrylic sample and biofilm parallel to the electric field axis. Subsequently, the impact of heating on the biofilm sample was modelled over time in both orientations.

6.3.2: Calibration of cavity resonator

A portable microwave applicator was assembled and kindly provided by the School of Engineering. This applicator was capable of providing up to 43 dBm of output power (equivalent to 20 W). The input power was measured continuously during exposure through a directional coupler which directed a small proportion of the signal to a power meter. The remaining microwave signal terminated in a simple loop antenna inside the cavity resonator. Signal generation was controlled from a laptop connected to the microwave applicator, with simultaneous detection of output power, using the locally developed 'Micromagic' software (Professor Adrian Porch, School of Engineering, Cardiff University). Once appropriate output power had been confirmed, the empty cavity was calibrated to ensure optimal coupling of the microwave signal. This was undertaken using a coaxial cable to measure the minimum reflected power (termed an S11 plot). A threshold for coupling was selected at -20 dB, indicative of delivery of 99% of microwave energy to the cavity. This process was then repeated following loading of the sample into the cavity. Coupling was adjusted by careful positioning and rotation of the antenna within the cavity.

6.3.4: Calibration of thermal imaging camera

A thermal imaging camera (thermoIMAGER TIM QVGA, Micro-Epsilon UK Ltd, Birkenhead, UK) was used to provide continuous temperature measurements of the samples, by focussing on the sample through the access port in the cavity resonator. To ensure adequate focus of the camera on the sample, a disc of dry ice was loaded into the sample position to provide an easily demarcated point for focus of the camera.

To ensure accurate measurement was being achieved using the thermal imaging camera, calibration was undertaken using a block heater, with simultaneous measurement of temperatures in 10 °C from room temperature to 80 °C recorded by the thermal imaging camera, a fibreoptic temperature probe and a mercury thermometer.

6.3.5: Preparation of experimental acrylic biofilms for microwave exposure

Acrylic coupons were prepared and preconditioned with artificial saliva as described in Sections 2.3.1 and 2.3.2. Subsequently 72 h microbial biofilms were prepared using the species used in Chapter 2, with the methods outlined in Section 2.3.7. Microbial biofilms were removed from the aerobic incubator at least 1 h prior to use to ensure equilibration with room temperature, then aseptically transferred to the cavity . Each sample was supported on a polystyrene cylinder to ensure location in the centre of the cavity. This was cleaned with 100% ethanol between uses.

6.3.6: Exposure parameters and measurement of bulk temperature

Initial exposures were undertaken at 43 dBm for durations of 0 (negative control), 2, 5, 10 and 20 s, with samples oriented both perpendicular and parallel to the axis of the electric field. Subsequent exposures were undertaken with samples oriented parallel to the electric field as this demonstrated greater microbiocidal effects. A range of powers were evaluated for a duration of 10 s (the minimum exposure time which reliably demonstrated eradication of viable microorganisms in denture acrylic associated biofilms): 100% (positive control), 50%, 25%, 10%, 1% and 0% (negative control) of full power (43 dBm). The impact of pulsatile microwave energy delivery was also evaluated, with duty cycles of 100% (positive control), 50%, 25%, 10%, 1% and 0% (negative control) at full power (43 dBm). The total exposure time was controlled. For example, a 1% duty cycle was delivered over a total time of 1,000 seconds, ensuring the same total microwave energy was imparted to the cavity for

each experiment. Bulk temperature changes were measured in real time during exposure of samples and subsequently until samples cooled below 35 °C.

6.3.7: Evaluation of biofilm killing by swab sampling

After cooling following microwave exposure, samples were transferred aseptically to new 12 well-plates using forceps and the exposed surface swabbed with a sterile cotton swab. Swab tips were transferred to a 1.5 ml microcentrifuge tube containing 1 ml PBS and vortex mixed at high speed for 1 min. Subsequently, serial 1:10 dilutions were undertaken, and suspensions inoculated onto selective agar plates for each species, as outlined in Table 2.3.3B, using a spiral plater (WASP – Don Whitley, Sheffield, UK). Agar plates were then incubated for 24-48 h aerobically at 37 °C and microbial colonies counted. A summary of experimental input and analyses is displayed in Figure 6.3.7.

Figure 6.3.7: Flow chart of experimental input and analyses

Single species biofilms (P. aeruginosa, S. aureus and C. albicans). 3 replicates, 2 subsequent repeats.

5 species 'mixed' biofilm. 3 replicates, 2 subsequent repeats.

Continuous exposure to microwave electric field in parallel and perpendicular orientations for different times.

Perpendicular sample orientation exluded from further analysis.

Continuous exposure to microwave electric field at different output powers.

Exposure to microwave electric field at different duty cycles.

Continuous temperature monitoring (Thermal imaging camera)

Culture analysis (spiral plating after recovery from swabs)

6.4: Results
6.4.1: COMSOL simulation models for microwave exposures
6.4.1.1: Interaction of electric field and model biofilms in cavity resonator

A simple model of the internal cavity resonator was generated (Figure 6.4.1.1A), with aluminium walls and air as the internal medium. A polystyrene cylinder with an acrylic coupon was added, with a 20 μm thick film of water simulating the biofilm. Simulations of microwave energy delivery at approximately 2.45 GHz demonstrated geometric separation of magnetic and electric field (Figure 6.4.1.1B), with strong electric field intensity in the cavity centre with polarisation occurring perpendicular to the cavity diameter. The magnetic field circulated circumferentially around the cavity perimeter. High electric field intensity was homogeneously distributed in the central portion of the cavity in a sufficient volume to ensure uniform exposure of the biofilm whether the surface was perpendicular (Figure 6.4.1.1C) or parallel (Figure 6.4.1.1D) to the electric field.
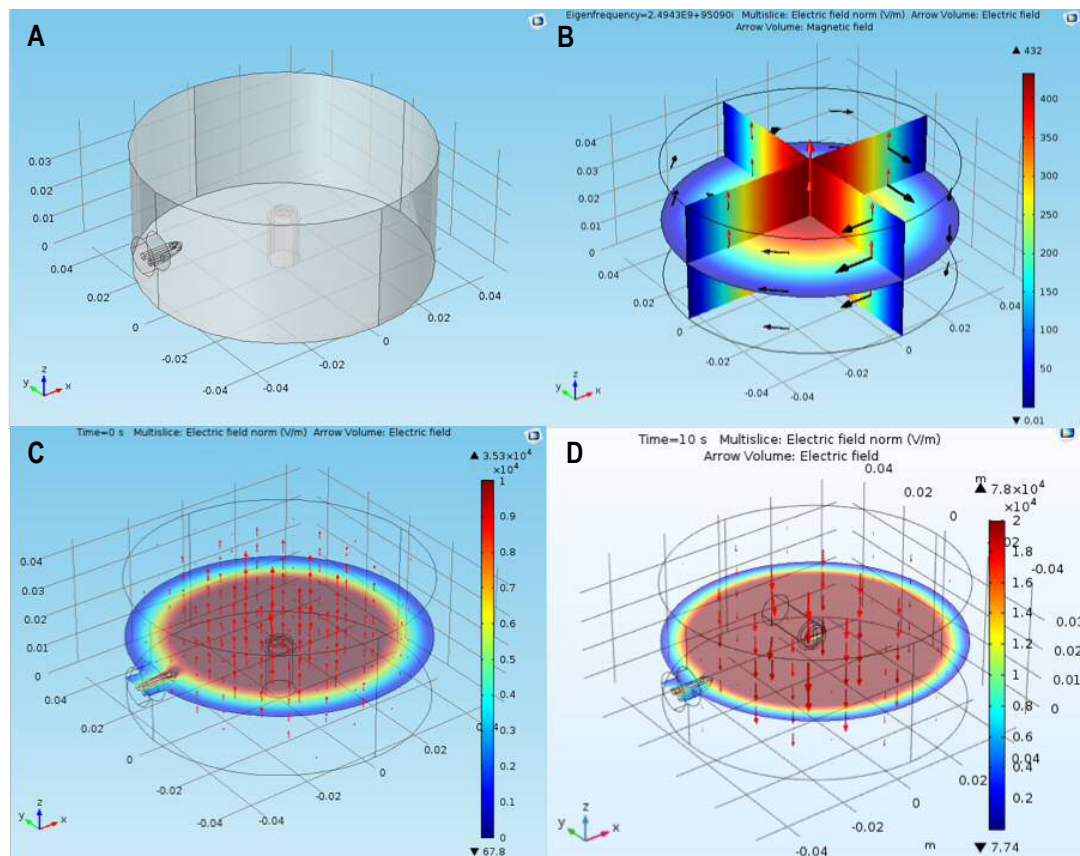
Figure 6.4.1.1: COMSOL Multiphysics simulations of experimental setup

A) Geometric parameters of cavity resonator showing 'biofilm' sample oriented to the volumetric centre of the cavity, with the planar surface of the biofilm perpendicular to the axis of electric field polarisation.
B) Electromagnetic field distribution in empty cavity at approximately 2.45 GHz frequency. Red arrows indicated direction of electric field; black arrows indicate direction of magnetic field.
C) Electric field intensity with sample oriented perpendicular to the axis of depolarisation.
D) Electric field intensity with sample oriented parallel to the axis of depolarisation.

6.4.1.2: Impact of exposure time on heat generation

Simulations produced at sections axial to the electric field corresponding to the planar 'biofilm' surface revealed that in perpendicular orientation (Figure 6.4.1.2A), samples might be expected to heat from the outer circumference (Figure 6.4.1.2B) initially, with progressive heating towards the centre (Figure 6.4.1.2C). A maximum temperature of 72.6°C was seen after 10 s continuous exposure at 43 dBm (Figure 6.4.1.2D). Importantly, a central portion of the sample remained at least 20°C below this temperature, however.
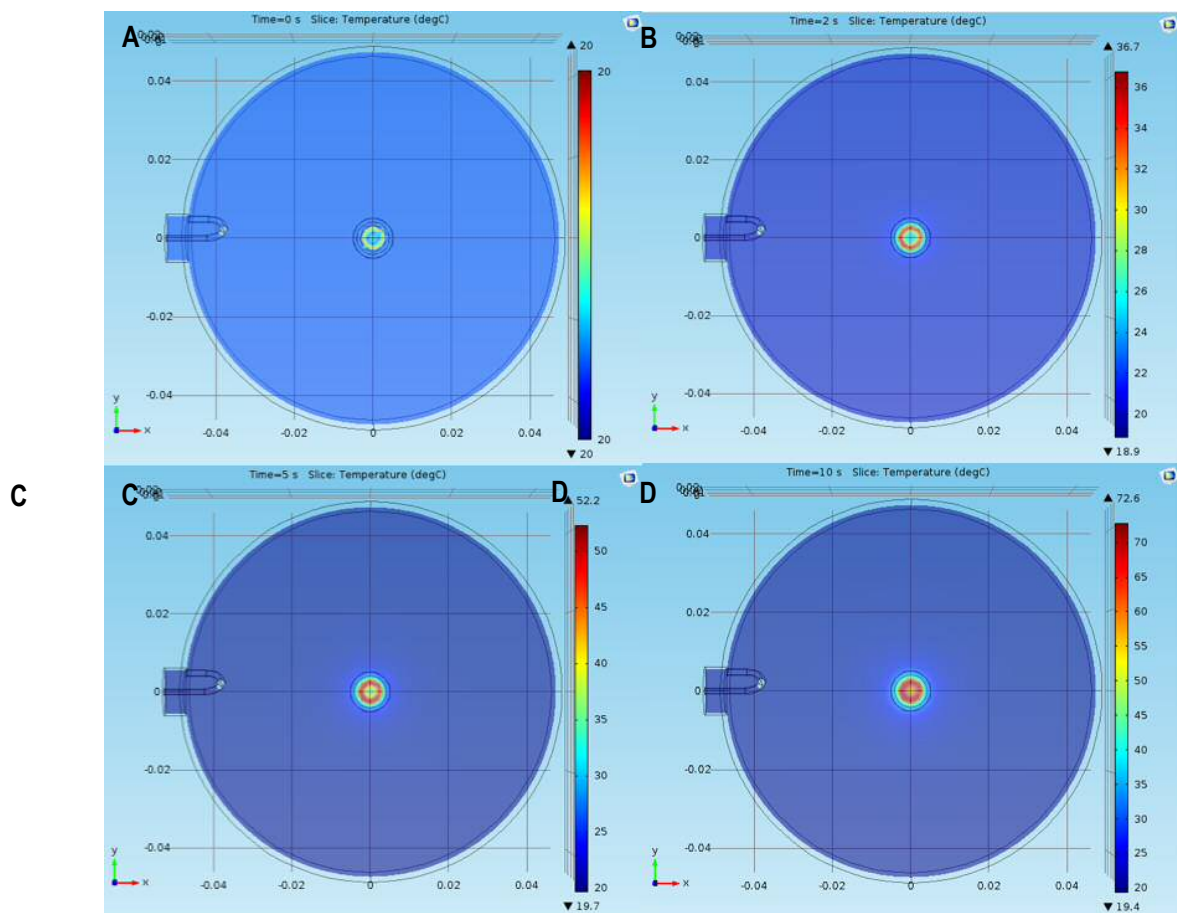
Figure 6.4.1.1: COMSOL Multiphysics simulations of experimental setup

A) Cross-sectional view of simulated 'biofilm' planar surface in cavity resonator, with surface temperature of 20°C.

B) After 2 s of exposure to the electric field, the sample shows peripheral heating, with minimal temperature change of the central portion.

C) The volume of heated area increases by 5 s, but the annular pattern of heating remains. The central portion of the sample has not exceeded 35°C, despite temperatures exceeding 50°C peripherally.

D) At 10 s, significant heating has occurred across almost the entire sample surface to at least 70°C, but a small central volume remains below 50°C.

When the sample was oriented parallel to the electric field (Figure 6.4.1.2E), the pattern and extent of heat generation differed substantially. Initial heating occurred at diametrically opposing edges corresponding to the electric field poles (Figure 6.4.1.2F). At 5 s, the distribution of heat was largely homogeneous across the sample surface (Figure 6.4.1.2G). After 10 s exposure to microwave electric field (Figure 6.4.1.2H), the 'biofilm' surface was seen to heat to over 400°C across the entire surface, with a thin band of greater heat intensity crossing the surface diametrically corresponding to the centre of the electric field.
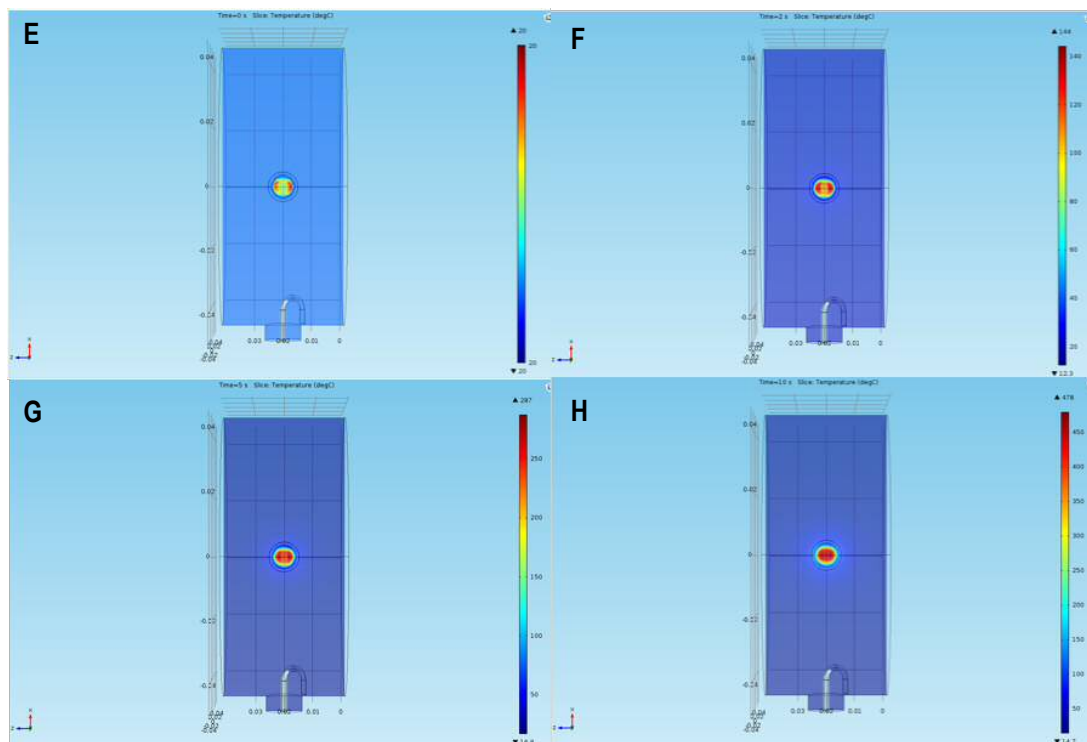
E) Cross-sectional view of simulated 'biofilm' planar surface in cavity resonator oriented parallel to the electric field, with surface temperature of 20°C.

F) After 2 s of exposure to the electric field, the sample shows heating at the poles, with some heating of the central portion.

C) By 5 s the entire sample volume has heated to over than 200°C. Heating is concentrated to the poles, with a thin diametric band seen from pole to pole.

D) At 10 s, the surface temperature of the sample exceeds 400°C, with maximum intensity now across a diametric band oriented along the central portion of the electric field.

6.4.2: Continuous exposure of acrylic biofilms to microwave energy in a cavity resonator – biocidal and thermal effects

Initial experiments undertaken with continuous exposure of samples to 43 dBm microwave energy examined the effects of sample orientation on the microbiocidal effects of the electric field. Attempts to analyse samples using the Live/Dead assay with CLSM were unsuccessful, possibly due to deformation of the acrylic surface, or

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debris generated during exposure of samples (data not shown). Consequently, only culture analysis could be undertaken. In single species biofilms, samples oriented parallel to the electric field showed elimination of microorganisms recoverable by culture after 10 s exposure (Figure 6.4.2A). When samples were oriented perpendicular to the electric field however (Figure 6.4.2B), both *P. aeruginosa* and *C. albicans* were cultured after 10 s exposure, while *S. aureus* was cultured in some samples after 20 s exposure. As was seen in previous analysis of mixed species biofilms, recovery of *C. albicans* by culture was not possible due to overgrowth of *P. aeruginosa*. Again, there was an elimination of *S. aureus* in samples oriented parallel to the electric field, while *P. aeruginosa* was reduced by approximately 8-log (Figure 6.4.2.C). In mixed biofilm samples oriented perpendicular to the electric field, both *S. aureus* and *P. aeruginosa* were cultured after 10 s exposure, albeit substantially reduced (Figure 6.4.2D). After 20 s exposure, both microorganisms were unable to be recovered by culture.
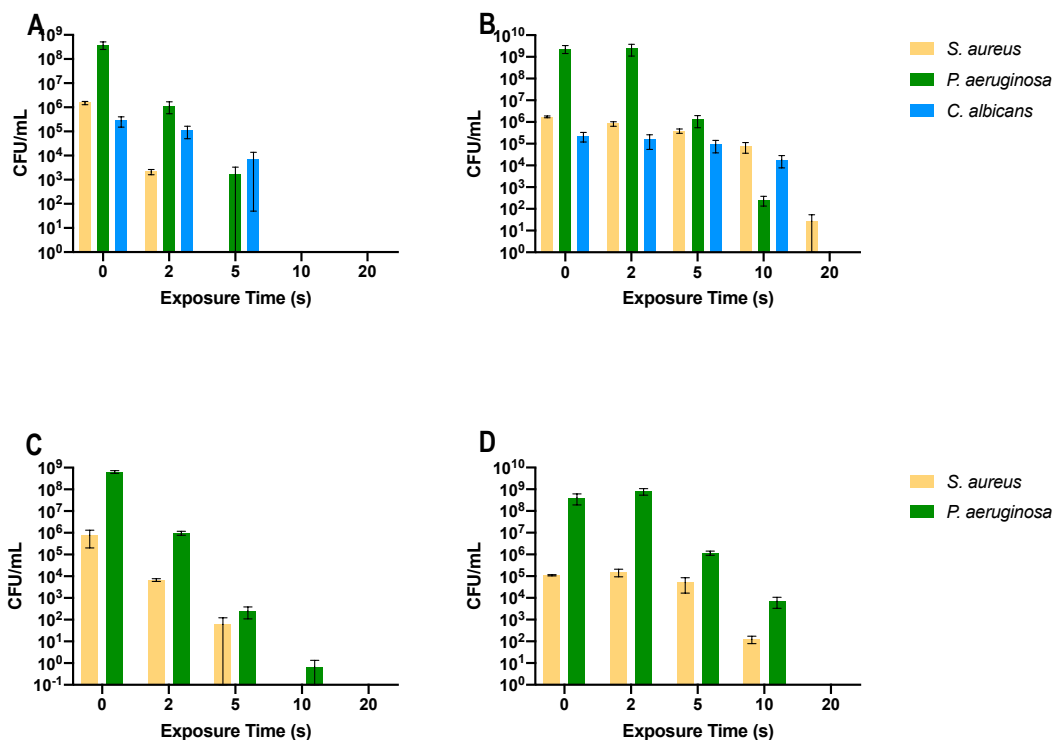
Figure 6.4.2: Recovery of microorganisms by culture after exposure to microwave energy
A) Single species biofilms oriented with the planar biofilm surface parallel to the electric field
B) Single species biofilms oriented with the planar biofilm surface perpendicular to the electric field
C) Mixed species biofilms oriented with the planar biofilm surface parallel to the electric field
D) Mixed species biofilms oriented with the planar biofilm surface perpendicular to the electric field
Experiments performed in triplicate with 2 experimental repeats. Mean values shown. Error bars represent standard error of the mean. 0 s exposure time = negative control.

The impact of sample orientation and biofilm species composition on sample temperature was also analysed. Heating was consistently higher with increased exposure times (Figure 6.4.2E) regardless of sample orientation or species composition. The highest temperatures were seen in *P. aeruginosa* biofilms, with similar heating in both *S. aureus* and *C. albicans* biofilms. The lowest temperatures occurred in mixed species biofilms. Temperatures in perpendicular sample orientations were similar to those predicted by COMSOL modelling, while parallel samples showed maximum temperatures of approximately 1/6th of the predicted temperatures in simulations.

The data from figure 6.4.2E was converted to show the change in temperature by subtracting the starting room temperature for each experimental run from the maximum temperature. This revealed statistically significant increases in temperature change in *S. aureus* and *P. aeruginosa* for samples oriented perpendicular to the electric field (Figure 6.4.2F). Despite failing to reach the threshold of statistical significance, a trend for increased temperatures in the perpendicular orientation was seen for *C. albicans*, and to a lesser extent in mixed species biofilms. There was considerable variability in the magnitude of temperature change for each exposure.

Analysis of stills from thermal imaging records (Figure 6.4.2G) revealed that the distribution of temperature across the sample surface was similar to that predicted in COMSOL simulations. In parallel orientation of the sample, temperatures initially increased at the poles, the rapidly spread across the surface, with almost uniform heating by 5 s exposure. In samples oriented perpendicular to the electric field, heating followed an annular pattern, with initial heating occurring at the sample periphery, before spreading inwards towards the sample centre. As was seen in the COMSOL simulations, the central portion of the sample reached a lower maximum temperature compared to the periphery.

Following the results from section 6.4.2 demonstrating the increased biocidal effectiveness of sample orientation parallel to the electric field, all subsequent experiments were undertaken in this orientation only.
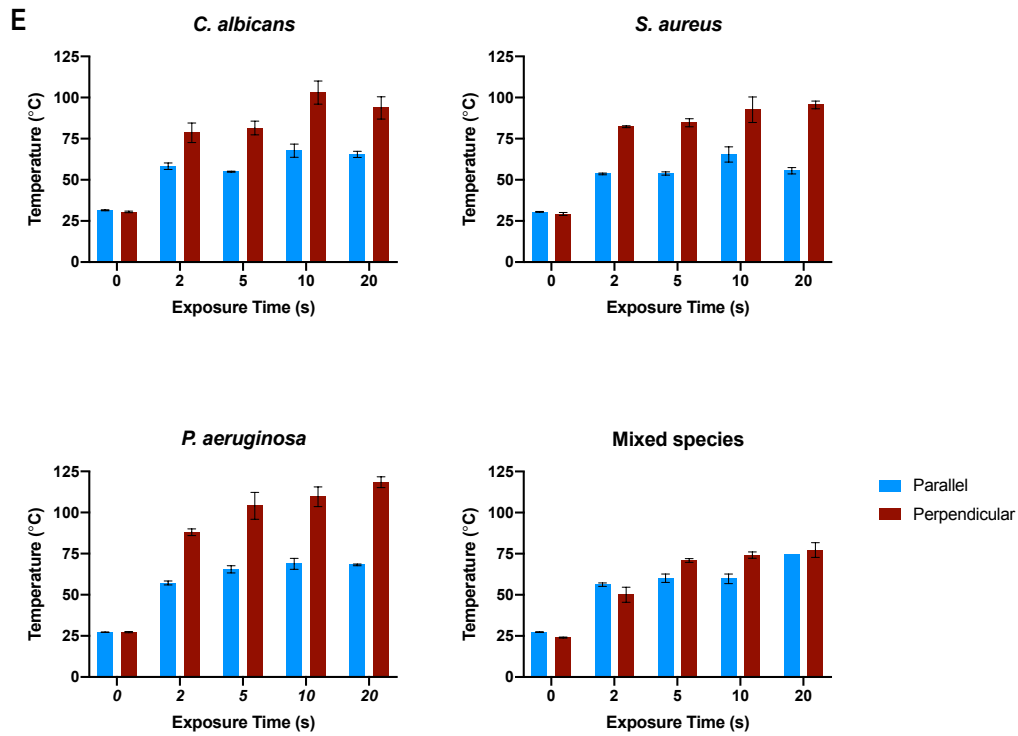
Figure 6.4.2E: Maximum temperature of biofilms on acrylic surfaces following exposure to microwave energy
Experiments performed in triplicate with 2 experimental repeats. Mean values shown. Error bars represent standard error of the mean. 0 s = negative control.
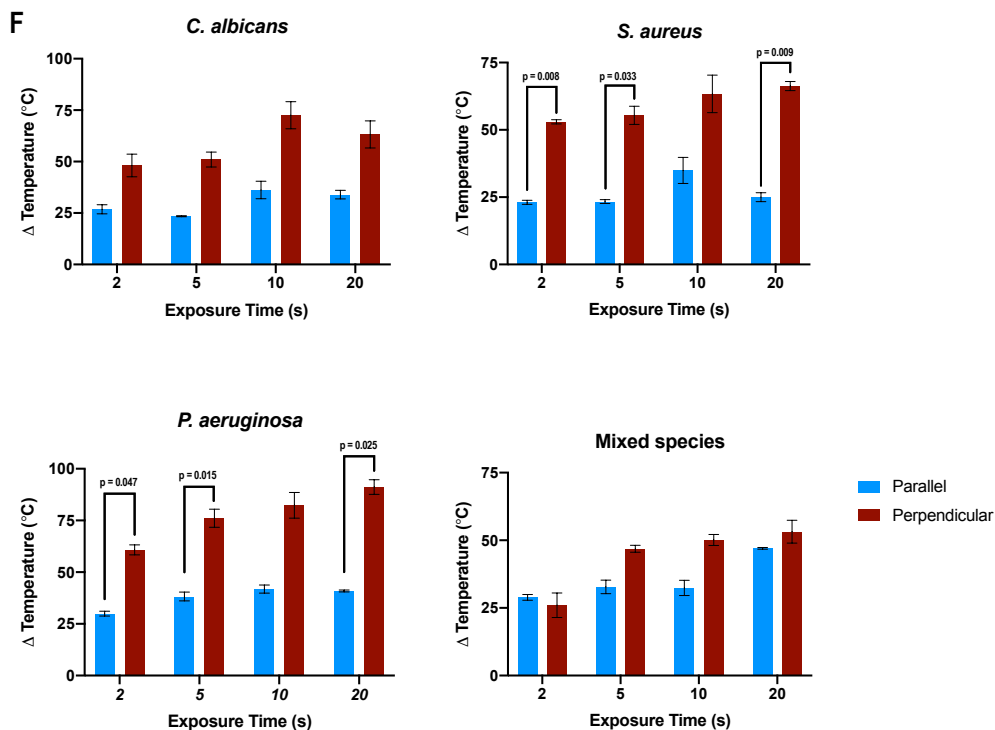
Figure 6.4.2F: Change in temperature of biofilms on acrylic surfaces following exposure to microwave energy
Experiments performed in triplicate with 2 experimental repeats. Mean values shown. Error bars represent standard error of the mean. P-values demonstrate adjusted significance after 2-Way ANOVA with Bonferroni correction.
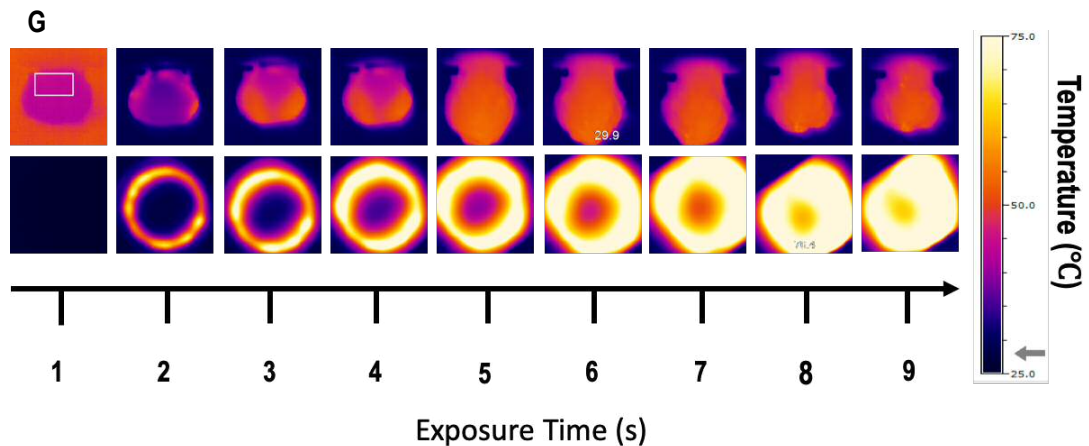
Figure 6.4.2G: Distribution of temperature of biofilms on acrylic surfaces following exposure to microwave energy
Upper panel shows biofilm samples oriented parallel to electric field.
Lower panel shows biofilm samples oriented perpendicular to electric field.
Stills captured from representative thermal imaging videos at 1 s intervals during exposure.

6.4.3: Impact of altering power or pulsing of microwave energy delivered to acrylic biofilms in a cavity resonator on thermal and biocidal effects

Both reductions in power and pulsed delivery (pulsed over a total time of 1 s in duty cycles; *e.g.* 1% duty cycle = 10 ms microwave energy, 990 ms recovery) led to a reduction of biocidal effects of microwave energy (Figure 6.4.3 A-D). Both methods of microwave energy delivery failed to eliminate all viable microbes, with an inverse correlation seen between either percentage power or duty cycle and biocidal effects. There was a very high degree of variability in microbiocidal effects seen at higher power settings and duty cycles, as indicated by the broad error bars.

The impact of either modulating power of microwave energy or pulsed delivery on temperature was similar (Figure 6.4.3E & G). Minimal rise in temperature was seen at 1% power, or using 1% duty cycles, with a sharp rise to approximately 45-55 °C noted at 10% power or duty cycles. There was subsequently a small, proportionately linear increase in temperature with increasing power or pulse duration.

As had been noted during initial experiments, there appeared to be differential effects of microwave energy on temperature generation, depending on the species composition of biofilms (Figure 6.4.3F & H). This effect only reached the threshold of significance in pulsed delivery experiments, although the same trend was seen in power modulation experiments. At lower duty cycles, mixed biofilms demonstrated significantly less heating than *P. aeruginosa* and *C. albicans* biofilms. This does not appear to have affected the biocidal effects of microwave energy however, as recovery rates of *P. aeruginosa* is equivalent in single or mixed species biofilms (Figure 6.4.3B & D).

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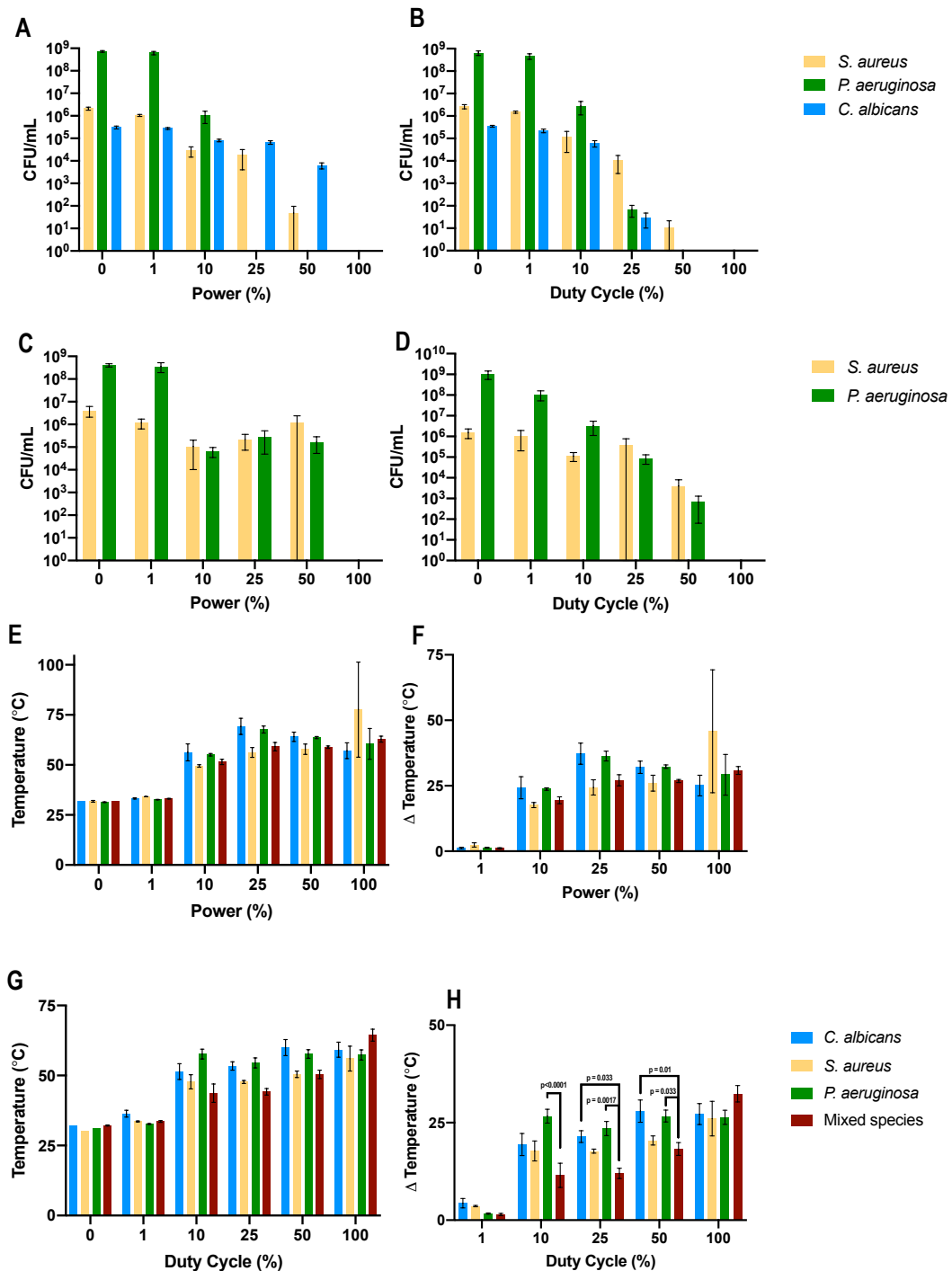
Figure 6.4.3: Impact of modulating power and pulsed delivery of microwave energy on recovery of microorganisms by culture

A) Modulation of power delivered in single species biofilms

B) Pulsed delivery in single species biofilms

C) Modulation of power delivered in mixed species biofilms

D) Pulsed delivery in mixed species biofilms

E) Impact of modulating microwave power on temperature generation

F) Change in temperature seen during microwave power modulation

G) Impact of pulsed delivery of microwave energy on temperature generation

H) Change in temperature seen during pulsed microwave energy delivery

N=3. Mean values shown. Error bars represent standard error of the mean.

P values demonstrate adjusted significance after 2-way ANOVA with Bonferroni correction for multiple comparisons. 0% power/duty cycle = negative control; 100% power/duty cycle = positive control.

6.5: Discussion

There are substantial challenges to providing denture sterilising in a manner that is easy to use, reliably effective and that does not cause detrimental effects to the denture biomaterial. In the institutionalised care setting, the responsibility of providing denture and oral hygiene may fall upon care staff, adding further barriers of a lack of training, failure to recognise the importance of effective denture care, and potential aversion to providing denture care due to its invasive, time-intensive nature coupled with feelings of disgust towards undertaking denture care (Wårdh et al. 2000; Miegel and Watchel 2009). While efforts to drive behaviour change, improve education and training are one approach to overcoming such barriers (Nicol et al. 2005), an alternative (and potentially complementary) approach is to modify the cleaning process itself.

To this end, the use of a conventional microwave oven to sterilise dentures has been explored by several authors. This option is attractive as domestic microwave ovens are a cheap and familiar appliance, thus offer minimal barrier to use (Augusto Brondani et al. 2012). However, because of the unpredictable distribution of electric field within such devices, high powers and prolonged exposure times are required, which generate considerable heat. This may lead to damage or deformation of denture biomaterials, either acutely if the glass transition temperature of PMMA is exceeded, or over time secondary to thermocycling effects (Sartori et al. 2006).

It was hypothesised that the capability of cavity resonators to generate a uniform, volumetric column of high intensity electric field would allow the use of much lower power, which could translate to reduced heat. Furthermore, it was anticipated that employing pulsed delivery could allow for a high cumulative exposure of microbes to the electric field, while mitigating heat generation. The simulation data presented in Section 6.4.1 predicted a highly efficient transfer of energy to biofilm samples, with concomitant heat generation. The simulation models also suggested that orientation of the sample within the electric field could affect the degree of energy transferred to samples. It was found that the cavity resonator behaved largely as predicted by the simulation models, with a greater biocidal effect and more even temperature distribution in samples oriented parallel to the electric field (Figure 6.4.2A-F). Modulation of power or pulsed delivery of microwave energy did mitigate heating to some extent, although at 10% or greater power levels or duty cycles, there was substantial heat generation. Both pulsed delivery and reductions in power lead to reduced biocidal effect, which suggests that within the limitations of this experiment, the biocidal effect of microwave energy is dependent on heating (Figure 6.4.3A-H).

While simulations of heating in samples oriented perpendicular to the electric field predicted the magnitude of heat generation accurately (Figure 6.4.1A-D, Figure 6.4.2E), there was considerable disparity in the magnitude of heat generation predicted by simulation for samples oriented parallel to the electric field (Figure 6.4.1E-H, Figure 6.4.2F). This is likely due to a combination of factors. Firstly, the COMSOL simulations did not account for conductive or convectional heat loss from the biofilm samples. In reality, heat transfer likely occurred through conduction to the underlying acrylic biomaterial, and evaporation of the sample. Furthermore, changes in sample volume or rapid changes in temperature could lead to loss of critical coupling of the microwave frequency. It was noted that achieving good quality coupling of frequency for samples in the parallel orientation was more challenging, and that this tended to reduce quite dramatically during exposure. In contrast, samples oriented perpendicular to the electric field suffered less reductions in coupling (Data not shown). Another factor which may cofound the apparent results is the accuracy of the thermal imaging camera in measuring each surface orientation. During exposures of samples perpendicular to the electric field, it was possible to orient the camera perpendicularly directly over the sample surface, with a wide aperture afforded by the cavity viewing port. However, during exposures of samples parallel to the field, it was necessary to view the samples at a 45° angle, through a much smaller aperture (approximately 1/3rd the diameter) and at approximately double the distance from the sample. Therefore, measurements recorded may have been less accurate, and more affected by reflection from the aperture periphery. Control of this factor could be achieved through manufacturing a cavity with viewing ports oriented appropriately. Regardless, the predicted distribution of heating was noted across the sample surface, demonstrating the validity of the COMSOL simulations in capturing qualitative, if not quantitative, effects of sample orientation within the field.

Heating and biocidal effects are highly challenging to separate experimentally for a number of reasons (Shazman et al. 2007). There is an interdependence of the two effects: higher power levels result in greater delivery of electric field, which might be expected to result in greater biocidal activity, but also inevitably induces greater heating. Similarly, changing sample orientation provides a greater volumetric exposure of the bulk sample (which can be considered simply as a planar surface) to the electric field, which should result in stronger depolarisation effects, increasing both biocidal activity and heating (Eigen and Schwarz 1957). While this effect was not seen, with the caveats discussed above, there was a more uniform distribution of heating seen across the sample surface in the parallel orientation, which alone may

be sufficient to explain the improve biocidal activity noted. It should also be noted that the thermal imaging camera used offers millimetre resolution and therefore can measure bulk temperature changes but offers no insight regarding heating at a sub-micron scale. The effects seen in differential heating of bulk liquids may also be present within microbial cells themselves, and so even if the relatively low temperatures measured in parallel-oriented samples are accurate, heating at specific points within cells may exceed such temperatures drastically. This possibility has been considered from a conceptual standpoint previously, where it was regarded as unlikely (Sastry and Palaniappan 1991), but at present there is no experimental data to support or refute differential temperature effects within microorganisms.

To ensure total electric field exposure remained constant, while attempting to mitigate heating, both reductions in power and duty cycle were investigated. The total duration of exposure was kept constant, so that experimental run time increased proportionately with decreasing powers or duty cycles. As was hypothesised, there was a reduction in heating noted when power was reduced, or pulsed delivery applied (Figure 6.4.3E - H). However, this did not occur in a linear manner. At either 1% power or a 1% duty cycle, minimal heating occurred. When power or duty cycle were increased to 10%, there was a roughly tenfold increase in heat generation. This is likely due to the capacity of water to retain heat, a function of the increased strength of electrostatic interacting in hydrogen bonds (Silverstein et al. 2000). Consequently, thermal recovery did not occur sufficiently to dissipate heating over time. Beyond 10% power or duty cycle, there was a small, linear increase in heating. There was no biocidal activity seen at 1% power or duty cycle, where heating was not induced (Figure 6.4.3A-D). While there was a reduction in microorganisms recovered by culture at higher powers or duty cycles of approximately 1-log per 10% increase, the clinical relevance of this is questionable.

Limitations of the methods

The work presented in this chapter has several important limitations which would need to be addressed or overcome in future work to enable further development of microwave energy as a means of disinfecting biomedical devices which are temperature sensitive.

The cavity resonator is highly tuneable to ensure maximal distribution of the electric field within a tightly defined volumetric column. However, this coupling is sensitive to changes in temperature. When coupling was remeasured after exposure on a subset of samples, the delivery efficiency was between 10-50% of the starting value. Such a substantial change would likely mean that the electric field was no longer directed

in a well-defined volume, and thus the actual energy delivered to the sample may have been substantially less than this.

Imaging by CLSM following Live/Dead staining of treated microorganisms proved to be infeasible, although the reasons for this remain unknown. It is possible that heat generated caused the acrylic coupons to warp slightly, preventing adequate focus. Alternatively, obliteration of cells by heat may explain why no cellular matter was visible, but this seems unlikely to be the sole reason for the finding, as it was not possible to focus on the acrylic biomaterial surface either. In most experimental runs, the observed temperatures using the thermal imaging camera were well below the glass transition temperature of the acrylic resin used. It could be that actual temperatures were higher, despite calibration of the thermal imaging camera. This may be due to reflection from the metallic surface of the cavity resonator, or higher temperatures generated in deeper regions of the acrylic away from the centre of focus. At present, this finding remains a conundrum, but would be important to solve due to the low precision and possibility of viable but non-culturable microorganisms providing false positive results by culture analysis. Either alternative staining and imaging techniques or an alternative method of reliably assessing biofilm viability may be required, such as a resazurin assay.

The use of swabbing as an alternative to imaging was a crude, imprecise method with risk of contamination, but amenable to high throughput. Because the first experimental series demonstrated improved biocidal activity against biofilm in parallel orientation to the electric field, care was needed to ensure the swab did not contact the sides of the acrylic coupon. Further, while care was taken to ensure a standardised swabbing technique, variability due to operator error will doubtless have induced further variation in the results. The time between swab collection and plating might have affected the results, but swabs were typically processed and plated within 5-10 min of collection, so it is unlikely that this posed a significant problem. However, the process is very time demanding and repetitive, which introduces higher risk of error. For this reason and to improve sensitivity and specificity, a direct assay on the acrylic surface would be preferable.

It can therefore be concluded that while the cavity resonator offers a more efficient and reliable means of using microwave energy for sterilisation of biofilms on denture acrylics, it does not overcome the limitations of a conventional microwave oven, namely generation of excessive heat. There was no clear evidence to support the existence of an athermal biocidal effect of the microwave electric field, although there were a number of unavoidable experimental factors which limited the ability to assess

this to an ideal degree of accuracy and precision. There was a difference in the degree of heating in biofilms depending on the species composition. This likely relates to the specific cell volumes and EPS content and quantity present

Future work could proceed down 3 primary avenues. The application of higher powers of microwave delivery with cavity resonators are possible, which could be pulsed with longer recovery intervals to allow for better heat dissipation. Alternatively, delivery of microwave energy could be provided through a coaxial probe, which removes the requirements for high fidelity frequency coupling, and offers better potential to scale up to the complex 3-D topography of denture surfaces. Finally, exploration of the impact of microwave energy delivery by cavity resonator or coaxial probe on the ease of chemomechanical removal of biofilms from denture acrylic surface could be undertaken.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSIONS

The principle aims of this PhD were twofold:

1. To develop a model of denture-associated biofilms incorporating putative respiratory pathogens and subsequently explore novel antimicrobial and anti-biofilm strategies to eradicate these highly recalcitrant microorganisms.

2. To characterise the oral bacterial communities of denture-wearing individuals in institutional care facilities and with active pneumonia, to evaluate whether changes in the denture-associated oral microbiome reflect pneumonia status.

Initial experiments focussed on the development of a simple biofilm model, using clinically relevant species to represent both normal oral microbes and putative respiratory pathogens (PRPs). Based on previous work from Sands et al. (2016, 2017), *P. aeruginosa* and *S. aureus* were selected as representative respiratory pathogens. Both microorganisms are frequently encountered in the healthcare environment and are responsible for a broad range of medical and device-associated infections including respiratory infection, diabetic foot ulcers, chronic wounds, ventilator-associated pneumonia and central-line associated bloodstream infections (Percival et al. 2015). The two selected PRPs have previously been seen to interact in a complex, dynamic manner in infections, occupying distinct niches within infected tissues (DeLeon et al. 2014). In keeping with this finding, *S. aureus* and *P.* aeruginosa were seen to occupy spatially distinct niches within mixed species biofilms following PNA-FISH labelling (Figure 2.4.3.1). Both microorganisms also have divergent interactions with *C. albicans* (Schlecht et al. 2015; Fourie et al. 2016), which was included in the biofilm model as a representative denture-associated yeast, given the high frequency of isolation of this microorganism in dentures. *Staphylococcus aureus* has been shown to engage in 'microbial hitchiking'; utilising candidal hyphae to gain access to tissues. In contrast, *P. aeruginosa* releases a number of virulence factors and quorum sensing molecules known to inhibit hyphal formation and can even attack and kill Candida in hyphal form.

In the biofilm experiments, recovery of *C. albicans* from mixed species biofilms was rendered impossible as heavy *P. aeruginosa* growth occurred on the SDA plates used to culture the yeast. It is of note that in Live/Dead imaging (Figure 2.4.3.2B&C) of mixed species biofilms, there are few yeast cells evident, and the majority are dead. It may therefore be that the suppression of *C. albicans* is the result of direct killing by *P. aeruginosa* rather than simply a matter of rapid growth of *P. aeruginosa* on SDA. Interestingly, the expression of virulence genes by *P. aeruginosa* in mixed

biofilms was reduced in mixed biofilms, including genes involved in the phenazine biosynthesis pathway thought to be responsible for inhibition of *C. albicans* hyphae. If killing of *C. albicans* by *P. aeruginosa* was a significant factor then it might be expected that the expression level of such genes would be increased. It is difficult to draw meaningful inferences from this data however, as only a small subset of relevant genes was analysed for pragmatic reasons. Furthermore, the translation of proteins does not correlate well with gene expression levels (Liu et al. 2016). This problem can be avoided in future work by addition of a suitable antibiotic to SDA for Candida culture, such as ciprofloxacin.

It was hypothesised that preconditioning of acrylic surfaces by artificial saliva would have divergent effects on the attachment, adherence and subsequent biofilm biomass of the oral commensals compared with respiratory pathogens used in the biofilm model. This was seen most clearly in mixed species inocula during attachment and adhesion experiments, where both *S. sanguinis* and *A. viscosus* were recovered in much greater number from acrylic coupons compared with biomaterials preconditioned with water. However, this trend was not evident when biofilms were examined. As growth curves were not performed, this could potentially be due to faster growth rates of *P. aeruginosa* in particular, which predominated mixed species biofilms, occupying available niches for adhesion. Additionally, with the exception of *A. viscosus*, the strains used were not originally isolated from the oral cavity. Thus this finding may be of little relevance to denture-associated biofilms clinically.

Extraction of microbial RNA from biofilms was highly challenging, as has previously been documented (Cury and Koo 2007; Atshan et al. 2012; Heera et al. 2015). To overcome this, a novel RNA extraction method was employed, utilising a combination of a phenol-chloroform method (FastProBlue, MP Biomedicals, Santa Ana, CA USA) to provide sufficient quantity of RNA and a silica gel membrane spin column (Qiagen RNeasy, Qiagen, Manchester, UK) to ensure high purity and to remove gDNA contamination from RNA. However, there are a huge array of available nucleotide extraction methods which vary by the target species. It is likely that to ensure optimal results from the very different microorganisms *S. aureus* and *P. aeruginosa*, different extraction processes, or at least modifications of a base process would be required. This is especially true in biofilms, where not only the challenge of differing cell wall compositions, but highly dissimilar EPS constituents, many of which can contaminate or inhibit the extraction process, must be overcome. Nonetheless, the method employed provides an excellent starting point for further optimisation of RNA extraction.

While RNA microarrays, or RNA sequencing approaches are increasingly employed in the examination of microbial interactions, this was beyond the remit of the current work. Furthermore, the utilisation of such expensive and intensive processes requires optimisation of RNA extraction and purification to ensure appropriate quality of data is generated. The potential for compound errors to arise from inherent biases in the extraction, reverse transcription and amplification processes is a pitfall for which there presently appears to be no consensus on managing (Gomez-Alvarez et al. 2009).

Following the extensive characterisation of the biofilm model described in Chapter 2, subsequent experiments sought to elucidate the impact of biofilms incorporating PRPs on oral epithelial tissues and explore host-microbial interactions in infection. Initially, a full thickness oral mucosal tissue model (EpiOral FT, MatTek Corporation, Ashland, MA, USA) was employed for tissue infections, as it was expected that the presence of a lamina propria with overlying epithelium would provide a more biologically representative model. However, examination of control tissues demonstrated dysplastic appearance of the tissues, with loss of the normal epithelial stratification, poikilocytosis and acantholysis throughout tissues (data not shown). The RNA extraction protocol used to isolate microbial RNA was the phenol-chloroform-isoamyl alcohol method previously employed with within the group for extraction of *C. albicans* RNA from mixed species biofilms (Cavalcanti et al. 2015). However, this method led to a very low yield of RNA, with nanodrop evaluation suggestive of contamination by guanidine, phenols or carbohydrates (Table 2.4.4.1). These contaminants could arise from reagents used in extraction, or alternative carbohydrates may have been derived from biofilm EPS. Analysis of cytokine profiles in tissues demonstrated elevated levels of pro-inflammatory cytokines in uninfected control tissues which had not been overlaid with PMMA resins, confirming abnormal behaviour of tissues. This artefactual tissue abnormality may have arisen from the 72 h time period of tissue shipment from the USA, followed by further 24 h use for infection experiments, as the accompanying documentation recommends use of the tissues within 72 h of production (including shipping time). Consequently, data from the MatTek tissues was excluded from analysis and an alternative solution was sought.

Although the Human Oral Epithelium (SkinEthic HOE, Episkin, Lyon, France) is a simpler model, consisting simply of keratinocyte layers overlying a polycarbonate insert, this model has been applied extensively to test microbial infections (Lu et al. 2006; Silva et al. 2011; Morse et al. 2018). However, it should be noted that this tissue does not form a keratinised Stratum Corneum, meaning the tissues are far more permeable to microbial insult than typical tissues in denture-bearing regions.

Furthermore, the cells used to seed SkinEthic tissues are from the TR146 cell-line, which is derived from a squamous cell carcinoma. Consequently, cell behaviour may not be representative of normal mucosal epithelium, as metabolic and mitotic activity, signalling behaviour and responses, and tissue integrity may differ in cancer cells (Alberts et al. 2002). Despite these limitations, the SkinEthic HOE modelled offered an extensively validated, commercial tissue model which could be transferred from the manufacturer to the laboratory within 24 h. In addition, histological evaluation of control tissues demonstrated good tissue integrity and no evidence of the dysplastic changes noted in the MatTek tissues (Figure 3.4.1). However, the obliteration of tissues noted in response to biofilms containing *P. aeruginosa* is likely exacerbated by the lack of a resilient, impermeable keratinised layer at the tissue surface. This may in turn have impacted cytokine expression by tissues and explain the absence of the expected rise in IL-6 and IL-8 in response to infection. Alternatively, this change in the expected inflammatory cytokine profiles could be explained by the high constituent expression of such cytokines seen in SCC epithelial cells (Chen et al. 1999), masking the impact of infection.

Despite the above limitations, this study highlighted the key role of *P. aeruginosa* in initiation invasion of epithelial tissues, and potential opportunism was seen by normal oral commensal and *S. aureus* secondary to tissue damage induced by *P. aeruginosa* (Figure 3.4.2). Furthermore, the RNA extraction method developed in experiments described in Chapter 2 was effective in isolating sufficient yield and purity of RNA for subsequent analysis of gene expression. While no clear trend was seen in *S. aureus* gene expression levels, either in biofilms or in response to contact with tissues, only a narrow range of genes was able to be evaluated. It was challenging to identify gene sequences specific to the strain of *S. aureus* employed in these experiments in order to design suitable primers. Consequently, several of the genes initially planned for analysis were not successfully amplified. In contrast, the *P. aeruginosa* genome is extensively mapped for multiple species, making primer design a far simpler task. This work highlighted the potential role of aprA, which encodes alkaline protease, in tissue invasion. Interestingly, expression of this gene was dramatically upregulated within mixed species biofilms only in tissue infections. The implications of this finding are unclear but may reflect competitive behaviour by *P. aeruginosa* to establish a niche deeper within the tissues. Further exploration of this observation is warranted.

The clinical observational study aimed to characterise the oral microbiome of denture wearing individuals in long-term care facilities and in patients who were hospitalised with pneumonia. Previous studies examining the potential for denture-associated biofilms to harbour PRPs have employed both culture-dependent techniques (Russell

et al. 1999; Sumi et al. 2002; Senpuku et al. 2003; El Solh et al. 2004; Daniluk et al. 2006; Sumi et al. 2007) and qPCR identification of species (O'Donnell et al. 2016). However, no studies to date have examined the oral microbiome of denture wearers with pneumonia, and so the composition of denture-associated microbial communities was unknown. Consequently, it was decided to employ a metataxonomic approach in this study, through next generation sequencing of the 16S rRNA gene, to ensure maximal characterisation of the oral microbiome in these previously unexamined populations. Additional culture-dependent isolation of *P. aeruginosa* and *S. aureus* was undertaken as previous work within the group had demonstrated their high prevalence in dental plaque samples of intensive care patients (Sands et al. 2017). This also provided the opportunity to validate the biofilm model employed in Chapters 2 and 3. Finally, *Candida* species were isolated by culture to provide insight into the primary constituent of the mycobiome. An approximately 20-fold increase in PRP bioburden was found to accompany pneumonia status. Importantly this was limited to the denture microbiome, while increases in PRP bioburden in samples from the dorsal tongue and denture-bearing palate were much more modest in magnitude. Salivary pro-inflammatory cytokines were examined and correlated with pneumonia status. However, recovery of sufficient saliva was challenging, the variability between subjects was very high, and there was no difference in expression levels of pro-inflammatory cytokines seen between respiratorily healthy subjects and pneumonia patients.

This study was intended to act as a pilot study, not only to provide proof-of-concept regarding the pathogenic potential of the denture-associated microbiome, but to establish the feasibility of the study protocol in addressing the research questions. There were a number of operational problems encountered during the study. It was challenging to obtain permission from care homes to access residents; only 7 of the 24 approached assented to involvement in the study. This is unsurprising, as care homes in Cardiff are private institutions, and so do not prioritise clinical governance in the same manner as NHS institutions. Additionally, the study necessarily entailed disturbing residents, and placed additional burdens on care home staff, who were required to identify and approach prospective participants. This highlights a major barrier to conducting research in the primary care setting, where there is no clear pathway for remuneration or compensation for care staffs' time. There is also the possibility of selection bias, as only institutions engaged in providing high quality care, which likely includes oral care, might be inclined to grant access to external researchers. Thus, the health status, oral and denture cleanliness, and PRP bioburden may have been underestimated in the control group. Similarly, access to

pneumonia patients was challenging. These individuals were acutely unwell, with competing healthcare needs that superseded the priority of sample collection for this study. Many patients were disinclined to participate simply due to exhaustion, feeling unwell and receiving multiple invasive interventions as part of their care. Both care home residents and pneumonia patients proved challenging to consent for the study. Many care home residents had cognitive impairment and assessing whether this was sufficient to render them unable to provide valid consent was difficult. Similarly, pneumonia patients had both a background of cognitive impairment in some cases, but a number of individuals were also acutely delirious secondary to hospitalisation and infection. The approach taken to consenting was likely over-cautious, and further training in assessing mental capacity and consent would benefit future research endeavours in this population.

This study has a number of strengths which enhance the importance of the findings. To date, this is the first study to evaluate the denture-associated microbiome of patients with active pneumonia. The use of swabs to obtain site-specific samples, rather than an oral rinse, offers insight into the role of the denture biomaterial surface in harbouring PRPs. By employing culture-independent community profiling, compositional shifts across the entire bacteriome could be mapped, and a wide range of PRPs identified semi-quantitatively. This offers an ecological approach to exploring the role of the oral microbiome in seeding respiratory infection, which has not previously been undertaken. Coupling shifts in microbial community composition and pneumonia status with local inflammatory cytokine profiles offered the opportunity to explore the potential of the host response to act as an 'immune fingerprint' to guide diagnosis and therapy in pneumonia.

It was hypothesised that PRP relative abundance would increase and the overall denture-associated microbial community diversity would decrease in patients with pneumonia. The increase in PRP abundance was not immediately apparent when examined at species-level, but became markedly clear when the cumulative relative abundance was examined. Similarly, there was a significant decrease in the Chao and Inverse-Simpson indices in pneumonia patients, indicative of a loss of microbial diversity. However, presence of *S. pneumoniae*, the most frequently isolated bacterial pneumonia pathogen, was detected in 8 care home residents and only 4 pneumonia patients. Similarly, isolation of *S. aureus* and *P. aeruginosa* by culture was approximately equal between cohorts, against expectations. Furthermore, a number of pneumonia patients had low relative abundances of PRPs identified by NGS, while several care home residents had relative abundances of PRPs in excess of 90%. Due to a lack of follow-up data, it is not known if any such individuals subsequently

developed pneumonia. This simply underpins the challenges associated with examining risk factors in multifactorial diseases: the presence of high levels of PRPs in the oral cavity may be a risk factor for pneumonia, but translocation of the bacteria to the respiratory tissues is a necessary event to initiate the disease. Longitudinal follow-up with serial sampling of oral microbes will be important for future work to attempt to evaluate causal relationships.

There were several limitations associated with this study which also deserve examination. The cross-sectional nature of the study limits the observed increase in PRP colonisation of denture biomaterial surfaces to a correlational finding. It could be argued that this change may simply accompany hospitalisation of patients or be driven by selective pressures from antimicrobial therapy. However, as the control cohort were in long-term care facilities, and had a number of comorbid conditions, these individuals also had frequent contact with the hospital environment. Many of the recovered isolates of *S. aureus* were susceptible to beta-lactam antibiotics, despite the empirical treatment of all suspected pneumonia patients with Amoxicillin. In fact, there were higher beta-lactam resistance rates observed in *S. aureus* isolates cultured from the samples of care home residents. To determine a causal relationship between oral colonisation and development of pneumonia, one of two approaches would be necessary. Firstly, consecutive sampling of 'at risk' individuals' oral and denture-associated microbiome, with subsequent follow-up to observe which individuals developed pneumonia. If colonisation of the oral cavity and denture biomaterial surface can be shown to precede development of pneumonia, this would offer strong support in favour of causality. It should be noted that while logistically challenging and expensive to undertake, some cost and resources could be saved by refraining from undertaking DNA extraction from microbial samples and subsequent sequencing until pneumonia cases had been identified. This work could be enhanced by matching control samples by age, gender and a number of key risk and lifestyle factors. Regardless, this would be a substantial undertaking for what remains a purely observational study. An attractive alternative would be to combine the observational methods employed in this study with an interventional trial. An enhanced programme of professionally delivered oral and denture care could be evaluated, with microbiological samples and oral/denture hygiene evaluations undertaken as proof of effectiveness of the intervention and as part of compliance monitoring. The control cohort would receive standard care only but undergo the same sampling methods. This approach could combine evaluation of the effectiveness of enhanced oral/denture care to reduce pneumonia risk with an exploration of the mechanisms of pneumonia aetiopathogenesis.

Salivary cytokines were hypothesised to predict pneumonia status or indicate individuals with a high oral bioburden of PRPs. However, neither pneumonia status nor PRP abundance appeared to correlate with salivary cytokines. It is possible that sampling palatal tissue fluid may give a better estimation of local host-microbiome interactions and thus pro-inflammatory cytokine levels here may more accurately reflect PRP abundance. Such samples have the additional advantage of not being affected by recent meals or drinks, which was difficult to control for in this study. It may still prove challenging to obtain sufficient fluid for analysis however. While the cytokines measured showed limited promise as diagnostic aids, biomarker discovery is an exciting avenue. The ease of access to the oral cavity compared with the lungs, and the acceptability of sampling from this site rather than invasive respiratory or blood samples enhances the appeal of such an approach. Unfortunately, xerostomia associated with age-related changes, comorbid conditions, dehydration and polypharmacy is a major barrier to obtaining suitable samples. It is possible that a brush biopsy, scraping the upper few layers of epithelium with associated tissue fluid, might provide a more reliable means to obtaining immune biomarkers, as these cells are intimately associated with PRPs in the biofilms on denture surfaces. This approach has been explored extensively in relation to oral cancer diagnosis and, while reports are mixed, some modes of brush biopsy have provided promising results (Alsarraf et al. 2018).

The subsequent Chapter aimed to address one approach to reducing colonisation of denture biomaterial surface by PRPs: the production of an antimicrobial or antiadhesive soft denture-liner. As has previously been discussed in Chapter 5, these linings are a useful clinical option, with pertinent applications to residents in long-term care facilities, who often have reduced access to dental care, reduced tolerance to treatment and adaptive capacity, and may receive inadequate oral and denture care. Collaborators from the research group of Professor Ian Fallis produced a library of novel imidazolium derived NHC ligands and corresponding silver complexes which were hypothesised to yield candidate antimicrobials with a broad spectrum of activity against Candida, Gram positive and Gram negative bacteria. In addition, a novel triclosan acetate salt was produced, which was hypothesised to confer physicochemical advantages following incorporation of the antimicrobial into silicone rubbers. These compounds were screened against a range of relevant pathogens frequently isolated form device-associated infections, for inhibitory, biocidal and anti-biofilm activity. The most promising compounds were subsequently loaded into silicone. While triclosan acetate was successfully bulk-loaded into silicone rubbers, the silver-NHC complexes inhibited silicone curing. Consequently, a compatible

material which is used in medical applications; acetoxy resin, was formulated into a fluid dispersion. Loading of the silver-NHC complexes into this material proved successful, and the dispersion was applied to silicone surfaces by dip-coating. The test materials showed no evidence of significant cytotoxicity. However, the antimicrobial activity of silver-NHC complexes was lost following incorporation into acetoxy resins. It is not clear if this was due to lack of release of materials or inactivation of the compounds due to chemical changes. Evaluation by LC would help to determine both the release profile and chemical structure of any released compounds from materials and should be considered in future work. Further work is needed to ensure retention of antimicrobial activity of silver-NHCs in biomaterials. A potential mechanism to achieve this would be immobilisation of the compounds at the surface using conjugation with flexible glycoprotein or peptide ligands.

Finally, an approach to denture sterilisation using microwave delivery was evaluated. The cavity resonator was employed to overcome some of the limitations of domestic (magnetron) microwave ovens, namely imprecise delivery of energy leading to variable biocidal effects, and the requirement for high powers to be employed resulting in excessive heat generation. It was hypothesised that delivery of microwave energy by cavity resonators would kill biofilm microbes on acrylic surfaces, and that by delivering energy in pulsed duty cycles, excessive heat generation could be avoided while retaining anti-biofilm activity. This mode of microwave delivery was found to offer massive increases in efficiency over conventional microwave ovens, with just 43 dBm (20 W) of power required to eradicate viable microorganisms from biofilms on acrylic surfaces in 20 s or less. Furthermore, differential effects dependant on sample orientation within the electric field were noted, which were not entirely explained by changes in temperature distribution alone. While this does not offer conclusive evidence of an athermal effect of microwave energy, this is an avenue that merits further investigation.

The impact of reduced power of microwave energy delivery was explored on both heat generation and biocidal effect. The total electric field exposure was held constant, to attempt to separate thermal effects from biocidal effects. There was a loss of biocidal effect which was roughly proportionate to the degree of heat reduction. Similarly, pulsed delivery was explored, where high power 'spikes' of microwave energy were interspersed with periods of recovery. Again, biocidal effects were not seen in the absence of significant heat generation. The cavity resonator offered an excellent modality to begin exploration of highly controlled delivery of electric field to microbial biofilms on denture acrylic. However, future work should aim to explore the role of higher power microwave energy delivery, delivered by a coaxial

probe to overcome the limitations imposed by the requirement for high-fidelity coupling in the cavity resonator. This work found mixed evidence regarding the existence of an athermal effect of microwave energy, and an integrated approach to experimentation, combining high quality simulations and laboratory studies offers a powerful approach to iteratively explore this possible phenomenon.

Research Conclusions:

A simple model of denture acrylic-associated biofilms was developed incorporating clinically relevant representative oral commensal microbes and putative respiratory pathogens. This model was subsequently validated by a clinical observational study which verified the presence of the selected microbial species on the denture surfaces of long-term care residents and pneumonia patients. The model biofilms were extensively characterised using a range of culture-dependent and molecular techniques. The methods used to generate such biofilms are readily scalable to allow high-throughput experiments such as the screening of antimicrobials or antibiofilm approaches; modelling of biofilm-mediated infections; or assessment of the influence of environmental, nutritive or physical factors such as laminar flow.

The biofilm models developed were used to infect epithelial tissue models and revealed the potential role of *P. aeruginosa* in driving invasion and destruction of tissues, in addition to opportunistic dissemination of commensal oral species and *S. aureus.* The significant upregulation of the aprA gene which encodes for alkaline protease in *P. aeruginosa* was seen in tissue infections with mixed biofilms but was not upregulated in mixed biofilms in the absence of host tissues. This suggests a potential role of this enzyme in securing a competitive advantage in tissue infection, which merits further investigation.

A novel clinical observational study was undertaken to characterise the oral microbiome of denture wearing individuals in long-term care facilities compared with patients hospitalised with pneumonia. This study employed next generation sequencing of the bacterial 16S rRNA gene to provide an unrestricted evaluation of oral bacterial community profiles associated with respiratory health in a population at-risk of pneumonia, and in patients with active pneumonia. Denture-associated microbial communities showed a significant increase in the relative abundance of putative respiratory pathogens, as well as loss of species richness and diversity, which are the hallmarks of dysbiosis. The magnitude of this difference was substantially higher in the denture-associated microbiota compared with either the denture-bearing palatal mucosa or dorsal tongue, underpinning the importance of the denture biomaterial surface as a potential nidus for respiratory pathogens.

Two novel approaches to controlling colonisation of denture biomaterials were investigated. Triclosan and triclosan acetate were successfully incorporated into silicone rubbers and demonstrated strong activity against *Staphylococci*, with only slight detrimental effects to the biomaterials physicomechanical properties.. While novel silver-imidazole NHC complexes failed to retain antimicrobial activity once incorporated into biomaterials, these compounds offer promise, as broad-spectrum biocidal activity was seen against Gram-positive and Gram-negative bacteria and *C. albicans.* Exploration of the antibiofilm effects of microwave energy delivered in a highly controlled manner by cavity resonator demonstrated the biocidal effectiveness of this sterilisation modality. However, further work is required to optimise delivery to mitigate heat generation. A possible observation of an athermal effect of the electric field was seen, which offers an exciting avenue for further research.

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Appendix I

Supporting documents for clinical study

- University sponsorship letter
- Ethical approval
- Substantive amendment to ethical approval
- NHS Research and Development permission letter
- Research participant information sheet
- Study protocol
- Participant consent form
- Care home letter of access

University sponsorship letter

Research and Innovation Services
Director Geraint W Jones
Gwasanaethau Ymchwil ac Arloesi
Cyfarwyddwr Geraint W Jones

CARDIFF
UNIVERSITY

PRIFYSGOL
CAERDYⅮⅮ

Cardiff University
7th Floor
30 - 36 Newport Road
Cardiff CF24 0DE
Wales UK

Tel *Ffôn* +44(0)29 2087 5834
Fax *Ffacs* +44(0)29 2087 4189

Prifysgol Caerdydd
Llawr 7
30 - 36 Heol Casnewydd
Caerdydd CF24 0DE
Cymru Y Deyrnas Unedig

08 July, 2016

Professor David Williams
School of Dentistry
Cardiff University
Dental Hospital
4th Floor,
Heath Park
Cardiff , CF14 4XY

Dear Professor Williams,

Title: Analysis of denture biofilms and associated salivary parameters in individuals with or at risk of respiratory infection

Short title: Saliva, denture plaque and pneumonia

I understand that you are acting as Chief Investigator for PhD project to be conducted by Dr Joshua Twigg.

I confirm that Cardiff University agrees in principle to act as Sponsor for the above project, as required by the Research Governance Framework for Health and Social Care.

<u>Scientific Review</u>
I can also confirm that Scientific Review has been obtained from Professor David Williams and Dr Melanie Wilson – School of Dentistry, Cardiff University.

<u>Insurance</u>
The necessary insurance provisions will be in place prior to the project commencement. Cardiff University is insured with UMAL. Copies of the insurance certificate are attached to this letter.

<u>Approvals</u>

On completion of your IRAS form (for NHS REC and NHS R&D approvals), you will be required to obtain signature from the Sponsor ('Declaration by the Sponsor Representative').

Please then submit the project to the following organisations for approvals:

- NHS Research Ethics Committee(s);
- Health & Care Research Wales Permissions Coordinating Unit (formerly known as NISCHR PCU) - to arrange host organisation R&D approval for Welsh NHS sites.
- Care Homes involved in study.

Once Research & Innovation Services has received evidence of the above approvals, the University is considered to have accepted Sponsorship and your project may commence.

THE QUEEN'S
ANNIVERSARY PRIZES
2013

INVESTORS
IN PEOPLE

Registered Charity, 1136855 *Elusen Gofrestredig*

Roles and Responsibilities

As Chief Investigator you have signed a Declaration with the Sponsor to confirm that you will adhere to the standard responsibilities as set out by the Research Governance Framework for Health and Social Care. In accordance with the University's Research Governance Framework, the Chief Investigator is also responsible for ensuring that each research team member is qualified and experienced to fulfill his delegated roles including ensuring adequate supervision, support and training.

If your study is adopted onto Health & Care Research Wales Clinical Research Portfolio you are required to upload recruitment data onto the portfolio database.

<u>Contracts</u>

The following contracts will be in place prior to research commencing:

- Sire agreement with Cardiff and Vale UHB (to include MTA provisions);
- MRA with Research and Testing Lab (Texas, U.S.A.) – for DNA testing.

May I take this opportunity to remind you that, as Chief Investigator, you are required to:

- ensure you are familiar with your responsibilities under the Research Governance Framework for Health and Social Care;
- undertake the study in accordance with Cardiff University's Research Governance Framework and the principles of Good Clinical Practice;
- ensure the Research complies with the Data Protection Act 1998;
- inform Research and Innovation Services of any amendments to the protocol or study design, including changes to start /end dates;
- co-operate with any audit inspection of the project files or any requests from Research and Innovation Services for further information.

You should quote the following unique reference number in any correspondence relating to Sponsorship for the above project:

SPON 1509-16

This reference number should be quoted on all documentation associated with this project.

Yours sincerely

Dr K J Pittard Davies
Head of Research Governance and Contracts
Direct line: +44 (0) 29208 79274
Email: resgov@cardiff.ac.uk

Cc   Dr Joshuan Twigg

Ethical approval

Gwasanaeth Moeseg Ymchwil
Research Ethics Service

Wales REC 6
First Floor
Institute of Life Science 2
Swansea University
Singleton Park
Swansea
SA2 8PP

Telephone : 01792 606334
E-mail : penny.beresford@wales.nhs.uk
Website : www.hra.nhs.uk

12 October 2016

Dr David Williams
Reader in Microbiology
Oral Surgery Medicine and Pathology
Cardiff University School of Dentistry
Heath Park, Cardiff
CF14 4XY

Dear Dr Williams

Study title:	**Analysis of denture biofilms and associated salivary parameters in individuals with or at risk of respiratory infection**
REC reference:	16/WA/0317
Protocol number:	NA
IRAS project ID:	200133

Thank you for your email of 11/10/2016, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Ms Penny Beresford, penny.beresford@wales.nhs.uk. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

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

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).
Guidance on applying for HRA Approval (England)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.
There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

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

Approved documents

The documents reviewed and approved by the Committee are:

Document	Version	Date
Covering letter on headed paper [REC cover letter]	NA	26 September 2016
Covering letter on headed paper [REC cover letter]		05 October 2016
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Sponsor insurance letter]		26 July 2016
IRAS Checklist XML [Checklist_11102016]		11 October 2016
Letter from sponsor [Letter from sponsor]		08 July 2016
Other [consent form for respiratory ward patients]	1.3	05 October 2016
Other [Information sheet for respiratory ward patients]	1.3	05 October 2016
Participant consent form [consent form for care home residents]	1.3	05 October 2016
Participant information sheet (PIS) [Information sheet for care home residents]	1.3	05 October 2016
REC Application Form [REC_Form_26092016]		26 September 2016
Research protocol or project proposal [Study Protocol]	1.2	27 June 2016

Summary CV for Chief Investigator (CI) [Prof David Williams outline CV]	1.0	07 September 2016
Summary CV for student [Joshua Twigg outline CV]		
Summary CV for supervisor (student research) [Dr Melanie Wilson outline CV]		13 September 2016

Statement of compliance

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

After ethical review

<u>Reporting requirements</u>

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

<u>Feedback</u>

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

16/WA/0317	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Penny Beresford

pp
Prof Roy Evans
Chairman

Email: penny.beresford@wales.nhs.uk

Enclosures: *"After ethical review – guidance for researchers" [SL-AR2]*

Copy to: *Mrs Helen Falconer, Cardiff University*
Ms Mital Patel, Cardiff And Vale University Health Board
Dr Joshua Twigg, Cardiff University

Substantive amendment to ethical approval

Ymchwil Iechyd a Gofal Cymru / Health and Care Research Wales

**Gwasanaeth Moeseg Ymchwil
Research Ethics Service**

*Ariennir gan Lywodraeth Cymru
Funded by Welsh Government*

Wales REC 6
First Floor
Institute of Life Science 2
Swansea University
Singleton Park
Swansea
SA2 8PP

Telephone : 01792 606334
E-mail : penny.beresford@wales.nhs.uk
Website : www.hra.nhs.uk

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

22 May 2017

Dr David Williams
Reader in Microbiology
Oral Surgery Medicine and Pathology
Cardiff University School of Dentistry
Heath Park, Cardiff
CF14 4XY

Dear Dr Williams

Study title:	Analysis of denture biofilms and associated salivary parameters in individuals with or at risk of respiratory infection
REC reference:	16/WA/0317
Protocol number:	NA
Amendment number:	1
Amendment date:	24 April 2017
IRAS project ID:	200133

The above amendment was reviewed at the meeting of the Committee held on 17 May 2017.

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

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Notice of Substantial Amendment (non-CTIMP) [Summary Participant Information sheet]	1	24 April 2017
Participant information sheet (PIS) [Summary participant information sheet]	1	24 April 2017
Research protocol or project proposal	1.3	24 April 2017

Membership of the Committee

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

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

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

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

16/WA/0317:	**Please quote this number on all correspondence**

Yours sincerely

pp
Dr M J Lawrence
Vice Chair

E-mail: penny.beresford@wales.nhs.uk

Enclosures:	*List of names and professions of members who took part in the review*
Copy to:	*Ms Mital Patel, Cardiff And Vale University Health Board*
	Mrs Helen Falconer, Cardiff University

NHS Research and Development permission letter

GIG | Bwrdd Iechyd Prifysgol
NHS | Caerdydd a'r Fro
CYMRU | Cardiff and Vale
WALES | University Health Board

**Ysbyty Athrofaol Cymru
University Hospital of Wales**

Heath Park,   Parc Y Mynydd Bychan,
Cardiff, CF14 4XW   Caerdydd, CF14 4XW
Phone 029 2074 7747   Ffôn 029 2074 7747
Fax 029 2074 3838   Ffacs 029 20743838
Minicom 029 2074 3632 Minicom 029 2074 3632

Tel: 029 20746986

CAV_Research.Development@wales.nhs.uk

From: Professor C Fegan
R&D Director
R&D Office, 2nd Floor TB2
University Hospital of Wales
Cardiff
CF14 4XW

09 January 2017

Dr Joshua Twigg
Tissue Engineering and Regenerative Dentistry
University Dental Hospital
Heath Park
Cardiff
CF14 4XY

Dear Dr Twigg

Cardiff and Vale UHB Ref: 16/SEP/6649
IRAS Project ID: 200133
Title: Analysis of denture bio films and associated salivary parameters in individuals with or at risk of respiratory infection

The above project was forwarded to Cardiff and Vale University Health Board R&D Office by the Health and Care Research Wales Permissions Service. A Governance Review has now been completed on the project.

Documents approved for use in this study are:

Document	Version	Date
R & D Form	5.3.2	26/09/2016
SSI Form	5.3.2	23/09/2016
Protocol	1.2	27/06/2016
Participant Information Sheet (Care Home Residents)	1.3	05/10/2016
Participant Consent Form (Care Home Residents)	1.3	05/10/2016
Participant Consent Form (Respiratory Ward Patient)	1.3	05/10/2016
Participant Information Sheet (Respiratory Ward Patient)	1.4	05/11/2016

Bwrdd Iechyd Prifysgol Caerdydd a'r Fro yw enw gweithredol Bwrdd Iechyd Lleol Prifysgol Caerdydd a'r Fro
Cardiff and Vale University Health Board is the operational name of Cardiff and Vale University Local Health Board

Ymchwil Iechyd
a Gofal Cymru
Health and Care
Research Wales

I am pleased to inform you that the UHB has no objection to your proposal subject to the following:

- The consent form should be amended to refer to NHS Health Board rather than NHS Trust.
- You should obtain agreement from applicable wards before involving patients from those wards.
- This R&D approval letter gives permission for UHB secondary care sites only. This permission does not cover care homes, for which you will need to seek approval from those homes.
- Appropriate agreements should be implemented between the study sponsor and the Research and Testing Laboratory (Texas, USA).

You have informed us that Cardiff University is willing to act as Sponsor under the Research Governance Framework for Health and Social Care. Please accept this letter as confirmation of permission for the project to begin within this UHB.

I note that this study is being considered for adoption onto the Central Portfolio Management System (CPMS) and/or the Health and Care Research Wales Clinical Research Portfolio. Adoption is important so that the UHB can receive funding to support this study. If you wish to begin the study before it has been adopted, you must first obtain permission to do so from the Dental Directorate R&D Lead.

If your study is adopted onto the portfolio, it will be a condition of this NHS research permission that you will be required to upload recruitment data onto the portfolio database, or forward recruitment data to the Chief Investigator to be uploaded.

During recruitment to portfolio adopted studies accrual data will need to be submitted on a monthly basis to the CPMS database. Failure to do so may result in the withdrawal of R&D approval. Systems have been set up to streamline and make this process as automated as possible. Details on how to upload accrual data are available at http://www.crn.nihr.ac.uk/can-help/funders-academics/nihrcrn-portfolio/portfolio-user-guides/. Please contact portfolio@Wales.nhs.uk if help is required.

Your Directorate R&D Lead has raised no objection to providing Directorate support for this study. If your study is not eligible for adoption onto the Health and Care Research Wales portfolio, you should liaise with him to ensure that he is satisfied that arrangements are in place for meeting any costs from outside of the R&D Activity Based Funding allocation.

May I take this opportunity to wish you success with the project and remind you that as Chief / Principal Investigator you are required to:

- Inform the Health and Care Research Wales Permissions Service and the UHB R&D Office if any external or additional funding is awarded for this project in the future
- Ensure that all study amendments are submitted to the Health and Care Research Wales Permissions Service

- Ensure the Health and Care Research Wales Permissions Service is notified of the study's closure
- Ensure that the study is conducted in accordance with all relevant policies, procedures and legislation
- Provide information on the project to the UHB R&D Office as requested from time to time.

Yours sincerely,

Professor Christopher Fegan
R&D Director / Chair of the Cardiff and Vale Research Review Service (CaRRS)

CC   R&D Lead Dr Vaseekaran Sivarajasingam
CC   Anthony Williams, Finance
CC   Clinical Board, Assistant Head of Finance, Philip Rowles
CC   Chief Investigator, Professor David Williams
CC   Sponsor contact, Helen Falconer, Cardiff University
CC   Academic supervisor, Dr Melanie Wilson

Research participant information sheet

SCHOOL OF DENTISTRY
Dean: Professor Michael Lewis
YSGOL AM DEINTYDDIAETH
Deon: Yr Athro Michael Lewis

CARDIFF
UNIVERSITY

PRIFYSGOL
CAERDYD

Cardiff University
School of Dentistry
Heath Park, Cardiff CF14 4XY, UK
Tel Ffon: +44(0)29 2074 2470
Fax Ffacs: +44(0)29 2074 8274
E-mail E-bost
Lewismao@cardiff.ac.uk
Prifysgol Caerdydd
Coleg Meddygaeth Cymru
Ysgol am Deintyddiaeth
Mynydd Bychan
Caerdydd CF14 4XY

RESEARCH PARTICIPANT INFORMATION SHEET

The role of denture bacteria and saliva in chest infection.
Site: Care Home

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.

- Part 1 tells you the purpose of the study and what will happen to you if you take part.
- Part 2 gives you more detailed information about the conduct of the study.

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 your interest in this research study.

PART 1

What is the purpose of the study?

The aim of this study is to collect samples of saliva; and bacteria and other microorganisms from peoples mouths and dentures, to see if they might change between people with and without pneumonia.

Why have I been chosen?

You have been chosen because you meet the study entry criteria of having a denture and are at least 18 years of age.

Altogether, a maximum of 100 people will take part in this study. If you agree to be involved we will take a sample of the bacteria present on your mouth and denture, and a sample of your saliva (spit). .

Do I have to take part in this study?

No. You are entirely free to choose whether or not to take part. If you do decide to take part, you will be asked to sign a consent form, of which you will be given a copy along with this information sheet to keep. You are still free to withdraw at any time without giving a reason.

What will happen to me if I take part in this study?

If you agree to participate in this study, you must read this Research Participant Information Sheet and then sign and date the Informed Consent Form before any study procedures begin.

The activities to be performed if you agree to participate will be as detailed below:

After reading through this information sheet you will be given plenty of time to ask the clinical staff in private about any questions you may have regarding the study, before deciding whether or not to participate. After you have provided your written informed consent to participate in this study your date of birth, gender, race and smoking status will be recorded in your study booklet. The study dentist will also ask you about your medical and dental history, (including the age of your denture) and whether or not you use denture adhesive and cleansers and record this in your study booklet, along with any medications you may be taking. The dentist may also ask to look at your medical notes. Assuming you meet the study eligibility criteria, the study dentist will then take a look inside and carry out an examination of your mouth.

The study dentist will then take samples from your mouth and will then remove your denture to sample it for the bacteria present.

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You can choose whether to give consent and have the sample taken on one visit, or if you would prefer to have time to reflect and discuss with others, the consent process and sampling may be undertaken at a second visit by the dentist.

Procedure No.	Procedure/assessment name	Why is the procedure / assessment being performed?	What will procedure/ assessment involve
1	Imprint culture	To collect a sample of the different bacteria and other microorganisms which live in your mouth and on your denture.	The study dentist will place a foam square (moistened in salt water) on the surface of your denture, palate and tongue for 30 seconds.
2	Swab	These samples will be processed to allow us to know exactly which species of microorganism is in your mouth.	The study dentist will gently swab the surface of your denture, palate and tongue with cotton buds.
3.	Saliva sample	This will be used to see if your saliva changes in response to the bacteria in your mouth.	A cotton roll will be placed in your mouth for a minute by the study dentist.

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PRIFYSGOL CAERDYDD

Cardiff University
School of Dentistry
Heath Park, Cardiff CF14 4XY, UK
Tel Ffon +44(0)29 2074 2470
Fax Ffacs +44(0)29 2074 8274
E-mail E-bost
Lewismao@cardiff.ac.uk
Prifysgol Caerdydd
Coleg Meddygaeth Cymru
Ysgol am Deintyddiaeth
Mynydd Bychan
Caerdydd CF14 4XY

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What do I have to do?

- Attend Visit 2 (if separate from visit 1) at the allocated time.
- Complete all study assessments as directed.
- You must **not** use denture adhesive on the day you are providing the samples.
- It is important that you report any concerns to the study staff both during the study and for 5 days after your visit. The contact numbers are at the end of this information sheet.
- Inform one of the study staff if you are taking part in another clinical study at present.

What are the possible disadvantages and risks of taking part?
The procedures and assessments that you will undergo are similar to those you would experience during a routine dental check-up. Completion of these procedures should require around 10 minutes of your time.

If you choose to take part in the study, you will be required to swill salty mouthwash and chew on a cotton wool roll, which some people find unpleasant. If you experience any discomfort while taking part in the study, you can speak to the Study Dentist.

Are there any side effects?
There are no known side effects of providing samples of your saliva, or mouth and denture swabs. However, if you experience any discomfort or have any other concerns as a result of taking part in the study, you should speak to the Study Dentist (details listed on this Participant Information Sheet) or your own dentist.

Are there any benefits in taking part?
If you choose to take part in this study, there will be no direct benefit to you. The results of your swabs and samples will contribute to our understanding of the possible links between the health of your mouth and chest infections such as pneumonia, which may benefit others in the future.

What will happen to any samples I give?
The samples taken will be transferred to a laboratory where they will be used to grow the bacteria for identification and further study. Once grown the bacteria and other microbes will be identified and used to try and replicate infection in laboratory models where they will be grown on plastic surfaces. Some of the samples will be sent to a laboratory in the USA to allow identification of microorganisms present within them. Your samples may be retained at the end of this study for use in future research within the UK and abroad. At this stage we do not know what the research will involve but some of it could include DNA analysis. On the consent form you will be given the option to exclude your samples from future research. Your samples will not be sold for profit or be used in genetic research, but could be used in animal research or the commercial sector in future research. Once collected, all samples will be fully anonymised; meaning no one including the researchers involved in the main study will be able to identify which samples you donated. Once we have hidden your identity for the samples you will not be able to withdraw your consent for their future use.

What happens when the research study stops?
The study dentist will not be taking over the care of your mouth and throughout the proejct and afterwards you can receive treatment as normal from your dental care team.

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What if there is a problem?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in the study be kept confidential?
Yes. All the information about your participation in this study will be kept confidential. These details are included in Part 2.

Contact details
If you have any further questions concerning the study, or in case of any difficulty during the study, please contact:

Dr Joshua Twigg   Tel: 02920742539
Prof David Williams   Tel: 02920 742448
Dr Melanie Wilson   Tel: 02920742442

School of Dentistry, Cardiff University, Heath Park, Cardiff, CF14 4XY, UK.

If you want to contact someone other than the research team regarding any concerns with the study, please contact:

Prof Michael Lewis   Tel: 02920742541

School of Dentistry, Cardiff University, Heath Park, Cardiff, CF14 4XY, UK.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making a decision.

PART 2

What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available about the treatment that is being studied. If this happens, your study dentist will tell you about it and discuss whether you want to or should continue in the study. If you decide to continue in the study you will be asked to sign an updated consent form. Also, on receiving new information your study dentist might consider it to be in your best interests to withdraw you from the study. He will explain the reasons why. If the study is stopped for any other reason, you will be told why.

What will happen if I don't want to carry on with the study?
You can withdraw from the study at any time without your medical/dental care or legal rights being affected. If you withdraw from the study we will not use the data collected.

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SCHOOL OF DENTISTRY
Dean: Professor Michael Lewis
YSGOL AM DEINTYDDIAETH
Deon: Yr Athro Michael Lewis

Cardiff University
School of Dentistry
Heath Park, Cardiff CF14 4XY, UK
Tel Ffon: +44(0)29 2074 2470
Fax Ffacs: +44(0)29 2074 8274
E-bost E-mail:
Lewismao@cardiff.ac.uk
Ysgol y Deintyddiaeth
Coleg Meddygaeth Cymru
Ysgol am Deintyddiaeth
Mynedfa Parc Y
Caerdydd CF14 4XY

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What happens if something goes wrong?

If you have concerns related to any aspect of the study and your involvement, you may contact the Study Dentist or the Senior Investigator to discuss this. If you wish to contact someone who is not part of the research team, then their details are also provided to allow this above. If you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the Cardiff University complaints mechanisms will also be available to you. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it.

Will my taking part in this study be kept confidential?

The results will be analysed by Cardiff University researchers. and in due course may be reviewed by the regulatory and claims approval authorities. This may involve inspection of all study-related information but your identity will always remain strictly confidential. Your name or initials will not appear on any information collected.

Your data will be made available (within and outside the European Union) to the sponsor's employees, investigators, members of the ethics committee and/or employees of the competent supervisory authorities exclusively in anonymised form for the purposes of examining data. Your participation in the study will be treated as confidential, that is, any personally identifiable information will be held and processed under secure conditions Data will be kept on file for 15 years. You will not be referred to by name in any report or publication (in a scientific journal) of the study. Your identity will not be disclosed to any person, except in the event of a medical emergency or if required by law. You are entitled under law to access your personal data and to have any justifiable corrections made. If you wish to do so, you should request this from the investigator conducting the study.

Your anonymised data will be processed electronically to determine the outcome of this study, and to provide it to health authorities/drug regulatory agencies. Your data, which is anonymised, will not be used for any other research.

Who is organising and funding the research?

The sponsor organising and funding this study is Cardiff University.

What will happen to the results of the research study?

It is possible that the results of the study will be made available in the public domain through scientific publications and/or presentations as appropriate. The main use of the results of this study will be to form part of a thesis for a PhD. In both cases any information about you will be anonymised as detailed in 'Confidentiality' above. Your name or details will not be associated with the research.

Who has reviewed the study?

This study has been reviewed and given ethical approval by a Research Ethics Committee (Wales Research Ethics Committee 6 Proportionate Review Sub Committee).

Thank you for your help.

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Study protocol

PhD Studentship Proposal: Supervisors Dr David W Williams, Dr Melanie Wilson; School of Dentistry, College of Biomedical and Life Sciences, Cardiff University, Heath Park, Cardiff, CF14 4XY

Background:

Biofilms are diverse communities of microorganisms which adhere to both biotic and abiotic surfaces and produce an extra-cellular polysaccharide matrix, which can provide structure and allow regulation of the environment. Complex interactions between microbes within biofilms enable synergistic nutrient metabolism, detoxification of agents including antimicrobials, and coordination of disparate species to promote overall survival of the community. Consequently, are often highly resistant to eradication. This is particularly problematic in healthcare settings, where a large proportion (up to 65 %) of infections are related to microbial biofilms.

The oral microbiome is one of the most diverse examples of a biofilm, with estimates of at least 700 different species present. Up to 70 % of the species present have not been amenable to culture in the laboratory. While most notably found as dental plaque, oral biofilms can form on any surface, including the soft tissues and any prostheses such as dentures or endotracheal tubes that may be present or contiguous to the oral cavity. Importantly, patients with poor oral health have been demonstrated to be at risk of aspiration pneumonia, in part due to changes in the oral microflora. It has thus been suggested that oral biofilms may act as a reservoir for potential respiratory pathogens in at risk patients.

The aim of this project is to compare the composition of the denture associated oral microflora of patients with respiratory infection to dentures wearers without respiratory infection. We hypothesise that the presence of an artificial surface in the oral cavity increases the likelihood of colonisation by atypical microbes, and may promote the presence of respiratory pathogens in at-risk individuals. By profiling the microbial communities of denture patients with and without respiratory infection (WP1), and comparing this to microbial communities of mechanically ventilated patients (previous research; WP3), using next generation sequencing; we hope to identify possible patterns in ecological shifts towards respiratory pathogens. Collection of saliva samples will allow analysis of salivary cytokine profiles (WP2), with the aim of providing potential diagnostic information and further understanding of the immune response in different patient cohorts. Taken together, this information will be used to inform an *in vitro* biofilm model to allow testing of a novel microwave technology to eradicate biofilms (WP4) and develop antimicrobial silicone surfaces to combat endotracheal tube biofilms (WP5).

Samples will be collected from two distinct groups of participants – residents in both private and NHS carehomes within the Cardiff and Vale area (at-risk, non-pneumonia group), and patients admitted to the University Hospital Wales (UHW) with a current diagnosis of pneumonia. Patients of at least 18 years of age; are able to provide valid informed consent; and who wear either a full or partial denture will be included. In carehome residents, a

diagnosis of respiratory infection within the past 30 days will result in exclusion from the study. For both participant groups, severely immunocompromised patients, such as those with HIV, medically immunosuppressed eg. after solid organ transplant, or undergoing chemotherapy, will be excluded.

Work Packages and Protocols

Recruitment and Patient Consent

Carehomes will be initially approached via telephone, drawing from a list of carehomes which have previously aided another research group within the University Dental Hospital. Further carehomes will be approached if an inadequate number of residents is recruited from this initial list. Following approval by the manager, the study dentist shall attend each carehome. A member of the residents normal care team will be asked to approach potential participants to ensure they are happy to discuss the study further with the study dentist. Assenting residents will then be provided a written patient information sheet and given ample time to discuss any concerns or further questions with the study dentist. If necessary, a return visit can be arranged at another date to allow participants further time to consider their involvement in the study.

Recruitment of patients from the University Hospital Wales will first be approved by the consultants responsible for these patients. Following approval, ward nurses responsible for the patients will be asked to approach patients to confirm they are willing to discuss involvement in the study with the study dentist. If agreeable, the same consent procedure outlined above will be used.

In all cases, it will initially be confirmed that potential participants meet the study inclusion criteria. Patient information leaflets can be translated into Welsh upon request, and large print versions will also be available.

WP1 Community profiling of denture biofilms from individuals with/without respiratory infection

While there have been several studies demonstrating that the presence of dentures, as well as poor oral hygiene appear to be correlated with risk of respiratory infection; little has been done to examine the microbial communities in at risk individuals compared to those with respiratory infection. To date, one study has suggested that the denture surface may provide a reservoir for known respiratory pathogens. However, this was limited by the use of species-specific probes for only a small number of microbes.

To complement and enhance these findings, next generation sequencing (NGS) will be used, allowing identification of a large proportion of the microorganisms present within oral biofilms of denture-wearing individuals. Samples for NGS will be collected by swabs and imprint cultures from denture fitting surfaces, the palate and tongue. DNA exraction will be performed in the University Dental Hospital, Cardiff. The extracted DNA will the be sent to a specialist laboratory: Research and Testing Laboratory (4321 Marsha Sharp FWY, Door #2 Lubbock, Texas 79407, USA) for sequencing. This is particularly important given the complex interactions between different microbial species within the biofilm community, and may

identify previously unconsidered factors which predispose to pathogenic colonisation, in addition to a diverse array of putative pathogens.

To conduct NGS, bacterial DNA will be extracted from a foam imprint culture of dentures surface and mucosa using Qiagen DNA extraction kit. PCR amplicon libraries will be generated using a variety primers including covering the V1-V5 hypervariable regions of the bacterial 16S rRNA gene. Identification of the microorganisms will be conducted using the MOTHUR suite of programmes (Schloss et al., 2009). Taxonomic composition and relative abundance of phyla, genera and species within the microbial communities will be analysed using appropriate software.

Molecular analysis will be performed alongside cultural analysis for comparison; to determine antimicrobial susceptibility of isolates and to obtain microbial isolates to be used in subsequent in vitro biofilm modelling.

WP2 Analysis of salivary cytokine profiles from individuals (MV patients and denture wearers)

Saliva has an important role in mediating the oral microbiome, and alterations in saliva composition, pH and rate of flow have been implicated in permitting an ecological shift towards more virulent species in the mouths of mechanically ventilated patients. Saliva also is rich in cytokines derived from local epithelial cells in response to virulent strains of microbes. Sampling of saliva is a convenient and non-invasive means of gleaning potential diagnostic markers for respiratory pathogen colonisation, and further analysis facilitate early identification of at-risk patients. Both the overall level of expression and patterns of cytokine expression will be examined using a cytokine array including the inflammatory set IL-1β, IL-8, IL-17, IFN-γ, TNFα, TGFβ and IL-10. The presence of a range antimicrobial factors such as antimicrobial peptides will also be determined. In addition, microbiological analysis of saliva will be undertaken. The samples will be destroyed at or prior to completion of the PhD (August 2018) and will not be used in any other research.

WP3 Comparison of results with those from previous studies of plaque and saliva from mechanically ventilated patients

Previous work within our research group involved a community profiling of endotracheal tube biofilms and cytokine analysis of saliva from these individuals. The aims within this work package are to compare the community profiling of the samples from patients with and without respiratory infection to the findingsexpand on this to identify pathogenic changes in the context of the overall microbial ecology. In addition, this information with complement that of the previous work package; allowing comparisons between the two patient cohorts that will elucidate commonalities and contrasting factors between these populations. Using the same NGS methods as in WP1, plaque samples, and samples from ETT biofilms will be collected after extubation for DNA extraction as described above.

WP4 Development of microwave technology to combat denture biofilms

Community profile of denture biofilms will inform an *in vitro* model cultured on denture acrylics, to enable the development of a novel microwave technology to eradicate denture

biofilms, sparing the need for chemotherapeutic measures. This cavity resonator allows precise control of electromagnetic radiation delivery, and will be aimed at overcoming the shortfalls of microwave ovens commonly used in denture sterilisation – namely the deforming effects of heat generated.

Once a kill effect without excessive heat delivery is demonstrated, the microwave technology will be tested on relevant materials such as the silicones used in endotracheal tubes and catheters, as a potential means of sterilising these devices in situ.

WP5 Development of antimicrobial silicone surfaces to combat ETT biofilms

Endotracheal tube silicone materials will be modified using silver nanoparticles and other antimicrobial agents to prevent the growth of microbes. Using information obtained through community profiling in WP2, these can be tested against relevant organisms both in isolation and within a complex biofilm where resistance to antimicrobials is significantly reduced and difficult to predict.

Participant consent form

Screening Number: | S | | | | |

INFORMED CONSENT FORM (Site: Care Home)

The role of denture bacteria and saliva in chest infection

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Please initial box

I confirm that I have read and understood the information sheet version 1.2 dated 27/6/16 for the above study. I have had the opportunity to consider the information, ask questions and have had these questions answered satisfactorily.

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical/ dental care or legal rights being affected.

I understand that relevant sections of any of my medical/dental notes and data collected during the study may be looked at by responsible individuals from regulatory authorities, Cardiff University or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

I give permission for my sample(s) to be used for DNA analysis, I understand my samples will be anonymised and I will not receive any feedback regarding the results of the analysis.

I agree for my sample(s) to be used for future use by researchers in the UK and abroad, I understand the research may involve DNA analysis, animal research or use by the commercial sector and that researchers will not be able to identify me from my sample.

I agree for my anonymous data to be stored in an internet repository for future sharing with researchers as part of an open science initiative.

I agree to take part in the above study.

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Screening Number:	S				

INFORMED CONSENT FORM (Site: Care Home)

The role of denture bacteria and saliva in chest infection

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Cardiff University
School of Dentistry
Heath Park, Cardiff CF14 4XY, UK
Tel Ffôn +44(0)29 2074 2470
Fax Ffacs +44(0)29 2074 8274
E-bost
Lewismao@cardiff.ac.uk

Prifysgol Caerdydd
Ysgol Deintyddiaeth
Ysbyty am Deintyddiaeth
Heath Park
Caerdydd CF14 4XY

_____   _____

Name of Subject (print)   Signature

Date (dd/mmm/yyyy): _____

_____   _____

Name of Person taking consent (print)   Signature

Date (dd/mmm/yyyy): _____
For office use only (tick appropriate box):
Subject Copy ☐   *Study Site File Copy* ☐

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Care home letter of access

Dear Sirs

Letter of access for Cardiff University research study

You have notified us that Joshua Twigg, a PhD Student at Cardiff University(**"Student"**) is conducting a research study entitled, ***"Analysis of denture bio films and associated salivary parameters in individuals with or at risk of respiratory infection"*** (**"Study"**). We understand that the Student would like to access to our premises in order to recruit participants to the Study to collect saliva samples. We acknowledge that the Study is being supervised by Professor David Williams at Cardiff University's School of Dentistry (**"Supervisor"**).

You have supplied us with the Study details, including the Research Participant Information Sheet, and we have a clear understanding of the activities the Student wishes to undertake.

We are satisfied that we have the necessary rights and authority to permit the Student to access our premises and residents. We confirm that the Student has a right of access to undertake the activities as set out in the Research Participant Information Sheet (**"Activities"**) for the purposes of the Study on the terms and conditions set out below.

The right of access is to our premises at [insert address/es] (**"Premises"**) and commences on [insert date] until [insert date] unless terminated earlier in accordance with the provisions below.

You shall ensure that the Student has sufficient training and instruction in order to carry out the Activities and that the Activities are performed with reasonable skill and care in accordance with all applicable legislation, regulations, approvals, codes of conduct, policies and procedures.

The Student is required to co-operate with us in discharging our duties under the Health and Safety at Work etc Act 1974 and other health and safety legislation and to take reasonable care for the health and safety of himself and others while on the Premises. You must ensure that the Student observes the same standards of care and propriety in dealing with our residents, staff, visitors, equipment and premises as is expected of any other contract holder and you must ensure that the Student acts appropriately, responsibly and professionally at all times.

While undertaking Activities, the Student will be considered a legal visitor to the Premises and you are responsible for his conduct during this Study. The Student will remain accountable to you at all times, other than when required to follow our reasonable instructions in relation to the terms of this right of access.

Where any third party claim is made, whether or not legal proceedings are issued, arising out of or | in connection with the right of access granted in this letter, you are required to co-operate fully with any investigation we may conduct in connection with any such claim and to give all such assistance as may reasonably be required regarding the conduct of any investigations and/or legal proceedings.

You are required to ensure that all information regarding residents or staff remains secure and *strictly confidential* at all times and to comply fully with the Data Protection Act 1998 and any other legislation regarding the security, management and handling of personal data.

You should ensure that, where the Student is issued with an identity or security card, these are returned upon termination of this arrangement. Please also ensure that while on the Premises the Student wears his University ID badge at all times and is able to prove his identity if challenged. Please note that we do not accept responsibility for damage to or loss of personal property.

We may revoke this letter and may terminate the right to attend our Premises at any time either by giving the Supervisor e-mail notice at (WilliamsDD@cardiff.ac.uk) or immediately without any notice if you or the Student are in breach of any of the terms or conditions described in this letter or if you or the Student commit any act that we reasonably consider to amount to serious misconduct, or to be disruptive and/or prejudicial to our interests and/or business or if the Student is convicted of any criminal offence.

If there are changes to the Student or the Activites, you shall notify us immediately and shall not assume any continued right of access pending our further written confirmation. We acknowledge that if we have any further questions concerning the Study, or in case of any difficulty relating to the Study we can contact: (i) Dr Joshua Twigg Tel: 02920742539; (ii) Prof David Williams Tel: 02920 742448 or (iii) Prof Michael Lewis Tel: 02920742541 who is outside of the research team. All personnel are located at School of Dentistry, Cardiff University, Heath Park, Cardiff, CF14 4XY, UK.

Yours faithfully

Read, acknowledged and agreed:

For and on behalf of Cardiff University: Date:

By the Student: Date: