Proinflammatory cytokines as mediators of phenotypic change in bacteria implicated in ventilator-associated pneumonia

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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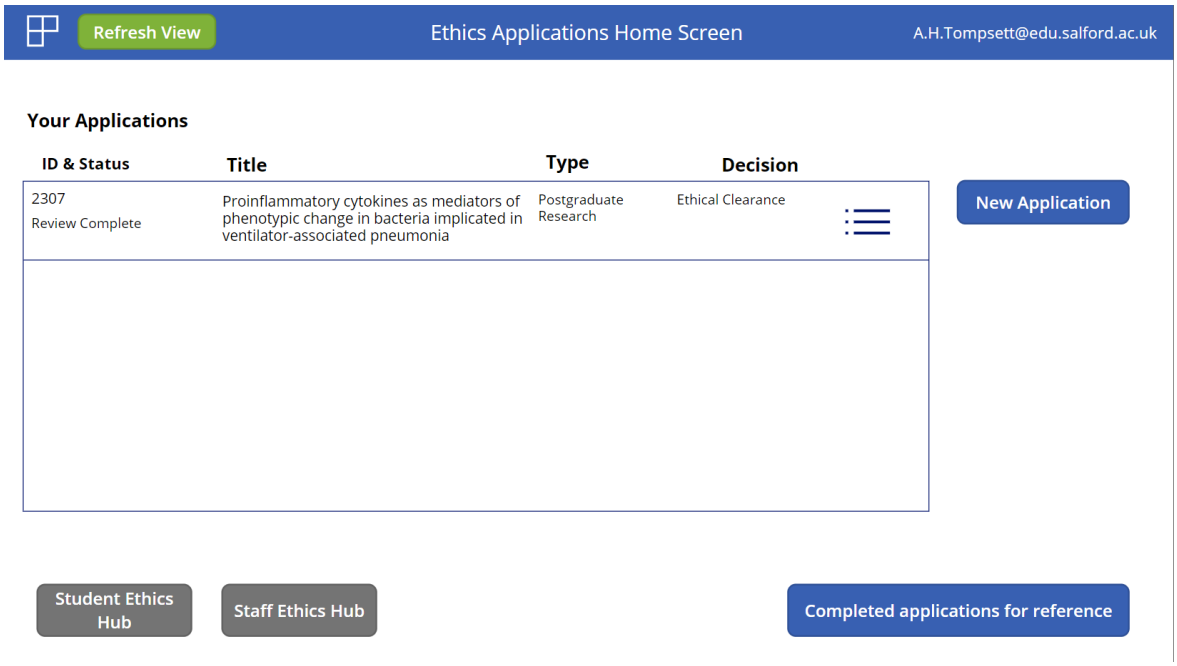
I greatly appreciate the consistent support and love from my family over the past four years. My mother, father, and two brothers have provided continuous encouragement and understanding over the past four years.

v | Declaration

This thesis is a product of the candidate's own study and research. It contains work previously presented as a poster at the Microbiology Society Annual Conference 2019. The abstract submitted to this conference is publicly available online (Tompsett *et al*., 2019).

vi | Ethical approval

Below is a screen capture of the University of Salford's online ethics application system, with confirmed ethical clearance for this project.



vii | List of acronyms and abbreviations

AHL	Acyl homoserine lactones
AMC	Amoxicillin-clavulanic acid
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
APACHE II	Acute physiology and chronic health evaluation II
ARDS	Acute respiratory distress syndrome
ASC	Apoptosis-associated speck-like protein containing a CARD
BAL	Bronchiolar lavage
BFU	Biofilm forming units
BPC	Penicillin G
cAMP	Cyclic adenosine monophosphate
CAMP	Cationic antimicrobial peptide
CAP	Community-acquired pneumonia
CCL	CC chemokine ligand
CDC	Centers for disease control
CFU	Colony forming units
CIP	Ciprofloxacin
CLR	Clarithromycin
CMV	Cytomegalovirus

COPD	Chronic obstructive pulmonary disease
CPD	Cefpodoxime
CPIS	Clinical pulmonary infection score
CPS	Capsular polysaccharide
CTX	Cefotaxime
CXM	Cefuroxime
CYLD	CYLD lysine 63 deubiquitinase
DOX	Doxycycline
DSI	Daily sedation interruption
EGFR	Epidermal growth factor receptor
EPS	Extracellular polymeric substances
ESBL	Extended spectrum β-lactamase
ESKAPE	*Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species
ETA	Endotracheal aspirate
ETT	Endotracheal tube
EUCAST	European committee on antimicrobial susceptibility testing
E-VAP	Early-onset VAP
EVPL	*Ex vivo* pig lung
ExoS/T/U/Y	Exoenzyme S/T/U/Y

FOX	Cefoxitin
HAP	Hospital-acquired pneumonia
HELICS	Hospitals in Europe link for infection control through surveillance
Hla	Alpha-hemolysin
HMI	Host-microbe interactions
HSV	Herpes simplex virus
HV	Hypermucoviscosity
ICE	Interleukin-1 converting enzyme
ICL	International clonal lineage
ICU	Intensive care unit
IL	Interleukin
IL-1R	Interleukin-1 receptor
IPM	Imipenem
LB	Lysogeny broth
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
LukAB	Leukocidin A/B
L-VAP	Late-onset VAP
LVX	Levofloxacin

LZD	Linezolid
MDR	Multi-drug resistance
MEM	Meropenem
MHA	Mueller-Hinton agar
MIC	Minimum inhibitory concentration
MMI	Microbe-microbe interactions
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMM	Microbial surface components recognising adhesive surface matrix molecules
MSSA	Methicillin-susceptible *Staphylococcus aureus*
MV	Mechanical ventilation
MyD88	Myeloid differentiation factor 88
NHSC	National healthcare safety network
NICE	National Institute for Health and Care Excellence
NLR	Nod-like receptor
OD_{600}	Optical density at 600nm
OXA	Oxacillin
PAI	*Pseudomonas* autoinducer
PAMP	Pathogen-associated molecular pattern
PBP	Penicillin-binding protein

PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-OH kinase
PRR	Pattern recognition receptors
PSM	Phenol-soluble modulin
PVL	Panton-Valentine leucocidin
RIP	Receptor-interacting protein kinase
RSV	Respiratory syncytial virus
SAM	Ampicillin-sulbactam
SOFA	Sequential organ failure assessment
SP	Surfactant protein
STAT	Signal transducer and activator of transcription
SXT	Trimethoprim-sulfamethoxazole
T3SS	Type 3 secretion system
TET	Tetracycline
TLR	Toll-like receptor
TNFR1	Tumour necrosis factor receptor 1
TNFα	Tumour necrosis factor alpha
TZP	Piperacillin-tazobactam
VAC	Ventilator-associated complication
VAN	Vancomycin

VAP	Ventilator-associated pneumonia

viii | Abstract

Ventilator-associated pneumonia (VAP) is a common hospital-acquired infection in intensive care. VAP is associated with increased hospital stay, healthcare costs, and high mortality. VAP is difficult to diagnose and manage appropriately, leading to widespread empirical broad-spectrum antibiotic use, which contributes to development of antimicrobial resistance in hospitals. We hypothesise that greater understanding of host-microbe interactions (HMI) in the pathogenesis of VAP should dictate appropriate treatment. This thesis begins to address this by investigating the role of host immunity on bacterial physiology.

We measured the effects of proinflammatory cytokines (IL-1β, IL-6, TNFα) on three species common in VAP: *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa*. The ability of these cytokines to influence growth, biofilm formation, antimicrobial sensitivity, and rate of antibiotic adaptation was investigated *in vitro*. The insect infection model *Galleria mellonella* was modified to investigate antibiotic adaptation *in vivo*.

TNFα significantly increased planktonic growth of all three bacteria, and increased biofilm formation by *K. pneumoniae*. TNFα was also found to significantly increase minimum inhibitory concentration of meropenem for *P. aeruginosa*. There was no effect of TNFα on the overall rate of bacterial adaption to meropenem in any species when measured *in vitro*. However, in *G. mellonella*, which represents a complex host environment, elevated concentrations of TNFα significantly reduced the rate of *S. aureus* adaptation to meropenem.

These results demonstrate that proinflammatory cytokines may modify clinically important phenotypic characteristics of bacteria implicated in VAP. This work

provides a foundation for further development and application of models of increasing complexity to better understand HMI in these infections.

ix | COVID-19 affected thesis statement

ix.i | COVID-19 impact on research

The COVID-19 pandemic disrupted access to the laboratory to perform research in support of the thesis. Between the dates of 18/03/2020 and 07/09/2020 the candidate was in residence in Rugby, Warwickshire, and not in proximity to the University of Salford. Due to the closure of the university at this time, it was not possible to collect data for the thesis, at a time where data acquisition was the main priority.

During university closure, the candidate was able to work on outstanding thesis writing. However, at this time the thesis was short on data, and most writing that took place were amendments to literature review or rewriting of earlier results.

Upon return to the university, there was no extension of funding to cover the candidate's living costs. This represented a significant loss of time in the laboratory, as whilst deadline extensions were available, taking that extra time was not financially realistic. Despite residing away from Salford during the initial national lockdown and university closure, the candidate was still required to meet maintenance costs on their rented property in Salford.

As a consequence, the thesis may be somewhat data-lite, as not all planned experiments were possible.

ix.ii | How planned work would fit trajectory of thesis

Two major series of experiments were not completed due to COVID-19 and are thus omitted from the thesis. Firstly, investigation of proinflammatory-cytokine

induced changes in biofilm growth in bacteria implicated in VAP, on *ex vivo* lung tissue. These experiments represented a logical progression of *in vitro* investigations. TNFα was demonstrated to significantly enhance biofilm productivity of *Klebsiella pneumonia*. We had hypothesised that these interactions would translate into an *ex vivo* model for biofilm growth, and the results collected in this more complex mammalian model would increase the validity of these findings.

Secondly, transcriptomic analysis of bacteria implicated in VAP after culture with TNFα. There were significant TNFα-mediated enhancements of planktonic growth in all of *Staphylococcus aureus*, *K. pneumoniae*, and *Pseudomonas aeruginosa;* biofilm formation in *K. pneumoniae*; and minimum inhibitory concentration of meropenem for *P. aeruginosa*. Despite these observations, the mechanisms underlying these changes are unknown. The intention was to use RNA-Seq to look at changes in expression levels of potential gene targets for TNFα-binding to bacterial DNA which could explain the observed effects.

ix.iii | Decisions and actions taken to mitigate for prevented work

Experiment plans were changed to focus on expanding upon existing *in vitro* work. This was done out of a desire to prioritise methods which could generate useful data quickly and inexpensively. Prior to the pandemic, the effects of proinflammatory cytokines on bacterial growth, biofilms, and antimicrobial sensitivity had already been investigated. These methods were revisited to complete outstanding replicates and expanded to investigate changes in rate of adaptation to antibiotics.

The *Galleria mellonella* infection model was adapted to develop a model for investigating *in vivo* antibiotic adaptation, and how this may be influenced by proinflammatory cytokines. Development of this model provided the basis for Chapter 5. The other thesis chapters required restructuring to reflect the work performed. Originally, a chapter covering cytokine-mediated changes in biofilm formation *ex vivo* had been planned. The results chapters in this thesis now report cytokine-mediated changes in growth and biofilm production in VAP isolates; cytokine-mediated changes in AMR in VAP; and *in vivo* modelling of antibiotic adaptation.

Chapter 1 | Introduction

1.1 | VAP

Ventilator-associated pneumonia (VAP) is an infectious complication of mechanical ventilation (MV) in hospitals. VAP is amongst the most common hospital-acquired infections occurring in adult intensive care units (Vincent *et al.*, 2006). The prevalence of VAP is due to the frequent necessitation of MV to support breathing and gas exchange in critically ill patients (Charles *et al.*, 2014). VAP occurs following ≥48 hours of MV, and is associated with increased length of hospital stay, increased duration of ventilation, and increased risk of mortality (Vincent *et al.*, 2009). The mean duration of VAP is 5-7 days, resulting in significantly increased time under MV, time in intensive care, and time admitted to hospital (Rello *et al.*, 2002). Estimations of mortality attributable to VAP are variable. High mortality rates have been reported, commonly around 30% (Kollef *et al.*, 2005). However attributable mortality, those deaths that would not have occurred in absence of the infection are debated, with a lower estimated rate ranging from 9-13% (Melsen *et al.*, 2011, 2013). This variability in mortality is due to difficulty in determining cause of mortality in those with multiple risk factors for death. There appears to be variation in considerations of attributable mortality on a geographical level. In the United States, VAP mortality is reported at 13% (Kalil *et al.*, 2016). In Europe, mortality is closer to the higher reported figures, at 29.9% (Martin-Loeches, Rodriguez and Torres, 2018).

VAP is a major contributor to the ongoing antibiotic crisis; approximately 50% of antibiotics given across all of intensive care are for treatment of VAP (Vincent *et al.*, 1995; Fihman *et al.*, 2015). These high antibiotic usage figures represent a risk

to other patients in intensive care, increasing the rates of multi-drug resistance (MDR) in these settings. Other patients, potentially with nosocomial infections of their own, admitted to the intensive care unit (ICU) are at greater risk of infection with MDR bacteria if VAP rates are high. This is due to the significant selective survival pressure on bacteria as a consequence of large prescription of antimicrobials (Vincent *et al.*, 1995).

VAP represents a significant economic burden on healthcare institutions. Longer durations of patient stay increases cost of care and increases risk of acquiring further infections (Warren *et al.*, 2003). Estimates of economic cost per incidence of VAP range from £368.72 to £31823 (figures converted to GBP) (Safdar *et al*., 2005; Restrepo *et al*., 2010; Kollef *et al*., 2012; Dogru *et al*., 2015; Luckraz *et al*., 2018). The true cost is difficult to estimate and depends on which outgoings are considered as VAP expenses. In the UK, variations in cost are suggested to be due to the scrutiny of tracking costs, which is observed more closely in private healthcare institutions for reimbursement purposes (Luckraz *et al*., 2018).

1.1.1 | Incidence and epidemiology

To date there remains a lack of a “gold standard” definition for VAP, which complicates diagnosis and incidence reporting (Hunter, 2012). As such, both under-diagnosis and over-diagnosis are common. Furthermore, a lack of standardised definitions confounds the accuracy and reliability of diagnostic tests for VAP (Rea-Neto *et al*., 2008).

Lack of consensus on definition results in wide variation in incidence rates between studies. An estimated 9-27% of mechanically ventilated patients develop

VAP, with increased risk during the days immediately following admission (American Thoracic Society and Infectious Diseases Society of America, 2005). Estimated rates in the US are reported as between 1.2 and 8.5 cases per 1000 ventilator days (Skrupky *et al.*, 2012). The International Nosocomial Infection Control Consortium, encompassing over 50 countries worldwide, report rates of 13.6 cases per 1000 ventilator days (Kherallah *et al.*, 2012). There are geographical trends in incidence too. Incidence in developing healthcare institutions tends to be much higher, as many as 50 cases per 1000 ventilator days (Joseph *et al.*, 2009). VAP in tertiary care hospitals in India reaches reported incidences of 22.94 per 1000 ventilator days (Joseph *et al.*, 2009), whilst in China a recent meta-analysis estimated incidence of VAP at 23.8% of MV patients (Ding *et al.*, 2017).

The use of VAP as a means to track quality of ventilated patient care in ICU has been debated. However, variability in definitions and between observers may lead under or over-estimations of the quality of patient care (Schurink *et al*., 2004; Klompas, 2010; Ego *et al*., 2015). This metric may fall under further scrutiny when also accounting for the frequent under or overdiagnosis of VAP by techniques such as endotracheal aspirate (ETA) versus bronchoalveolar lavage (BAL) (Nora and Póvoa, 2017).

VAP has traditionally been divided into early-onset VAP (E-VAP) and late-onset VAP (L-VAP). E-VAP encompasses pneumonias occurring within the first four days of MV, and L-VAP encompasses those occurring five days or later (Nair and Niederman, 2015). E-VAP is traditionally caused by organisms sensitive to common antibiotics, with a bias towards Gram-positive cocci, such as methicillin-susceptible and resistant *Staphylococcus aureus* (MSSA/MRSA) (Golia et al.,

2013). Organisms implicated in L-VAP were more frequently MDR, with a bias towards Gram-negative bacilli, such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Acinetobacter baumannii* (Giard et al., 2008; Golia et al., 2013; Nair and Niederman, 2015). This classification is controversial. VAP patients admitted to ICU for ≥2 days prior to onset of MV were found more likely to become infected with bacteria associated greatly with L-VAP, irrespective of intubation time (Hunter, 2012). This suggests that extended ICU admission is the major determinant of infection with MDR pathogens, rather than duration of MV. Studies have reported that contribution of MDR pathogens to E-VAP and L-VAP are very similar (Giantsou *et al*., 2005; Gastmeier *et al*., 2009). Gram-negative bacilli cause a majority of respiratory infection in ICU, with minimal differences between hospital-acquired and ventilator associated pneumonias (Esperatti *et al*., 2010; Koulenti *et al*., 2017). This implies that MDR is widely prevalent in all cases of VAP, and the frequency of each species' implication is uniform despite time of onset. Together, these findings beg the question as to the usefulness of early or late classification in current practice.

This trend away from the classical concept of: E-VAP = sensitive, L-VAP = resistant, is concerning for the future of VAP aetiology, with a move towards MDR pathogens as the norm in all cases of infection. The detrimental impact this could have on empirical therapy and patient outcomes is profound. Greater prevalence of MDR bacteria in VAP would lead to greater incidence of treatment failure, and increased patient morbidity and mortality.

1.2 | Aetiology of VAP

The composition of the human microbiome changes during admission to intensive care. This is a direct consequence of large scale antibiotic administration in ICU, and the prevalence of MDR bacteria that reside there as a consequence of this antibiotic use (Zaborin *et al.*, 2014; McDonald *et al.*, 2016; Akrami and Sweeney, 2017). Nosocomial pathogens and the microflora of healthcare workers and other patients can readily be transmitted to a patient undergoing MV, with an associated risk of VAP.

The most frequent causative agents in VAP are bacteria common to the hospital setting. These include *S. aureus; P. aeruginosa*; Enterobacteriaceae (particularly *K. pneumoniae* and *E. coli*); *Enterobacter* spp.; *A. baumannii*; *Stenotrophomonas maltophilia*; *Haemophilus influenzae*; and *Serratia marcescens* (Gastmeier *et al.*, 2009; Chi *et al.*, 2012). This list is not exhaustive but accounts for the most common and clinically relevant causative bacteria. The most frequently implicated bacteria in VAP in Europe are *S. aureus* (24.9% of cases), *P. aeruginosa* (17.4% of cases), and *Enterobacteriaceae* (32.9% of cases) (Koulenti *et al*., 2017). However, the prevalence of different organisms can vary between and within institutions, which emphasises the importance of local epidemiological knowledge. This is particularly important, as a large proportion of studies are performed in Western or developed healthcare institutions (Cook *et al*., 1998; American Thoracic Society and Infectious Diseases Society of America, 2005; Chi *et al*., 2012).

VAP is commonly polymicrobial, with infections caused by as many as four pathogens concurrently frequently reported (Gastmeier *et al.*, 2009). The impairment and breakdown of usual host defences during MV and critical illness

provides a permissive and accessible environment within which multiple bacterial species can thrive (Gunasekera and Gratrix, 2016). However, the polymicrobial nature of VAP samples may disguise the true causative agents, as it becomes exceedingly difficult to distinguish causative organisms from simple opportunistic colonisers (Gastmeier *et al.*, 2009). The conundrum being that whilst a particular species may be capable of causing VAP, it may not be actively contributing to an ongoing infection in every instance.

ICUs are increasingly associated with antimicrobial resistance (AMR) and MDR, associated with frequent use of broad spectrum antimicrobials and their associated selective pressure (Moolchandani et al., 2017). Thus, the usual suspect bacteria causing VAP in intensive care have almost universally developed resistance to commonly-used broad-spectrum antimicrobials (Park, 2005a). The following subsections pertain to species commonly implicated in VAP, and which have been used as part of this study.

1.2.1 | *Staphylococcus aureus*

S. aureus is a Gram-positive, round-shaped (coccus), facultatively anaerobic bacterium. A common coloniser of the skin and upper respiratory tract, it is an opportunistic pathogen capable of causing a range of infections (Horn *et al.*, 2018). This includes but is not limited to: abscesses, wound infections, endocarditis, toxic shock syndrome, pneumonia, bacteraemia and sepsis (Lowy, 1998). Treatment of *S. aureus* can provide a complex challenge to clinicians. In VAP, the rate of methicillin resistance, mediated by the *mecA* gene, ranges from 50-100% (Vincent *et al*., 1995; Chi *et al*., 2012). In their study, Chi *et al.* (2012)

found all *S. aureus* isolated from VAP patients in a South Korean hospital were methicillin resistant. This has obvious consequences for treatment, with increased risk of treatment failures and patient mortality.

S. aureus secretes a range of pore-forming toxins in pneumonia. These toxins, as the name implies, form pores in the membrane of host cells leading to their rupture by cytolysis (Grousd, Rich and Alcorn, 2019). The importance of the pore-forming toxin alpha-haemolysin (Hla) in the pathogenesis of pneumonia has been well characterised in murine models (Kebaier *et al.*, 2012). Hla is capable of activating the NLRP3 inflammasome, leading to production of interleukin-1β (IL-1β) and induction of programmed necrotic death in lung tissues (Kebaier *et al.*, 2012). To challenge eukaryotic cells, Hla assembles into heptamers on the cell surface by binding to ADAM10. Hla then inserts into the cell membrane, forming a small pore which allows for movement of ions across the membrane (Wilke and Bubeck Wardenburg, 2010). Dysregulation of ion transport abolishes normal internal cell signalling, leading to cytokine release and necrotic cell death, and impairment of mucociliary clearing in the respiratory tract (Becker *et al.*, 2014). Airway colonising strains of *S. aureus* with high Hla activity have been demonstrated to predict progression to VAP (Stulik *et al*., 2014). As a result, Hla has been identified as a potential therapeutic target in the treatment of VAP (Tabor *et al*., 2016).

Panton-Valentine leukocidin (PVL) is another pore-forming toxin expressed by *S. aureus*. It targets the human complement 5a receptors, and thus its specific target is phagocytic cells (Spaan *et al.*, 2013). PVL is not produced by all strains of *S. aureus*, and rates vary worldwide. In 2012, Brown *et al.* reported rates of PVL expression in *S. aureus* in the US as 36% to 98% (Brown *et al.*, 2012). PVL positive strains are more associated with extremely severe disease, such as

necrotising pneumonias, and accelerated disease progression (Gillet *et al*., 2002; Zhang *et al*., 2016a). However the presence of the gene encoding PVL does not correlate directly with severity or patient outcome (Peyrani *et al*., 2011). Insertion of PVL into phagocytic membranes leads to disruption of K^+ efflux, leading to widespread IL-1β release and activation of the NLRP3 inflammasome, in a manner similar to Hla (Grousd, Rich and Alcorn, 2019).

Leukocidin A/B (LukAB) is a more recently discovered toxin of *S. aureus*, which kills macrophages, monocytes, neutrophils and dendritic cells, all professional immune cells (DuMont *et al.*, 2013). It binds to CD11b on the cell surface, and as a pore-forming toxin, it induces cell death through activation of the NLRP3 inflammasome (DuMont *et al.*, 2013). Induction of IL-1β and IL-18 release and necrotic cell death by *S. aureus* is thus dependent on interleukin-1 converting enzyme (ICE) and apoptosis-associated speck-like protein containing a CARD (ASC) (Melehani *et al.*, 2015). The exception is when *S. aureus* is localised intracellularly, here LukAB can bind to CD11b independently of NLRP3 and ASC (Melehani *et al.*, 2015).

S. aureus pore-forming toxins are also capable of inducing necroptosis in lung epithelial and immune cells. Necroptosis is a programmed cell death pathway, but unlike apoptosis it does require the activation of caspases (Sridharan and Upton, 2014). The necrosome forms in response to death receptor signalling activated by pore-forming toxins, which consists of receptor-interacting protein kinase 1 (RIP1) and RIP3 (Sridharan and Upton, 2014). The necrosome phosphorylates downstream targets, chiefly mixed lineage kinase domain like pseudokinase, ultimately leading to cell lysis via ion flux (Sridharan and Upton, 2014). Necroptosis is potentiated by tumour necrosis factor alpha (TNFα), which is

elevated during *S. aureus* pneumonia by the inflammatory response. Signalling via TNF receptor 1 (TNFR1) recruits RIP1, which in turn recruits RIP3, leading to rapid formation of the necrosome and increasing the numbers of necroptotic cells (Linkermann and Green, 2014). Necroptosis of alveolar macrophages appears to be reliant on Hla, as murine models deficient in ADAM10 exhibit no cellular death by necroptosis (Becker *et al.*, 2014).

Numerous cell surface components have been implicated in *S. aureus* adhesion and invasion of respiratory epithelium, and stimulation of the innate immune system (Ferry *et al*., 2005). This includes lipoteichoic acid, peptidoglycan, and microbial surface components recognising adhesive surface matrix molecules (MSCRAMM). *S. aureus* protein A and β-toxin both mediate cell damage in necrotising pneumonia (Gómez *et al*., 2004; Hayashida *et al*., 2009). Protein A binds to TNFα receptor 1, mediating the inflammatory response and impairing phagocytic clearance by binding to antibodies (Gómez *et al*., 2004).

S. aureus can cause cell death in neutrophils through the secretion of phenol-soluble modulins (PSMs). PSMs are also pore-forming toxins, and induce cytolysis through nonspecific receptor-independent mechanisms (Otto, 2014). The receptor for PSM, formyl peptide receptor 2, is not necessary for cytolysis. PSMs are also involved in other processes of benefit to *S. aureus*, including biofilm formation and protection from antimicrobials (Figure 1.1).

S. aureus deploys numerous tools to circumvent host defences, in addition to toxin-mediated cytolytic killing of immune cells. Staphylokinase is secreted to degrade complement protein C3 and antimicrobial peptides by complexing with plasminogen (Mölkänen *et al.*, 2002; Jin *et al.*, 2004). CHIPS protein inhibits

neutrophil chemotaxis by blocking the C5a receptor and formyl peptide receptor on the neutrophil surface (Tawk *et al.*, 2015). Furthermore, *S. aureus* biofilms are able to subvert reactive oxygen species killing of bacteria by limiting neutrophil access to oxygen (Lodge *et al.*, 2017). Secretion of Nuc1, a nuclease enzyme, allows *S. aureus* to degrade neutrophil extracellular traps (Schilcher *et al.*, 2014). These are formed from neutrophil DNA mixed with proteases and antimicrobial peptides from neutrophil granules, and are released upon cell lysis to trap and degrade bacteria (Pleskova, Gorshkova and Kriukov, 2018). Biofilm formation by *S. aureus* has also been shown to effectively reduce the efficiency of neutrophil extracellular traps (Bhattacharya *et al.*, 2018).

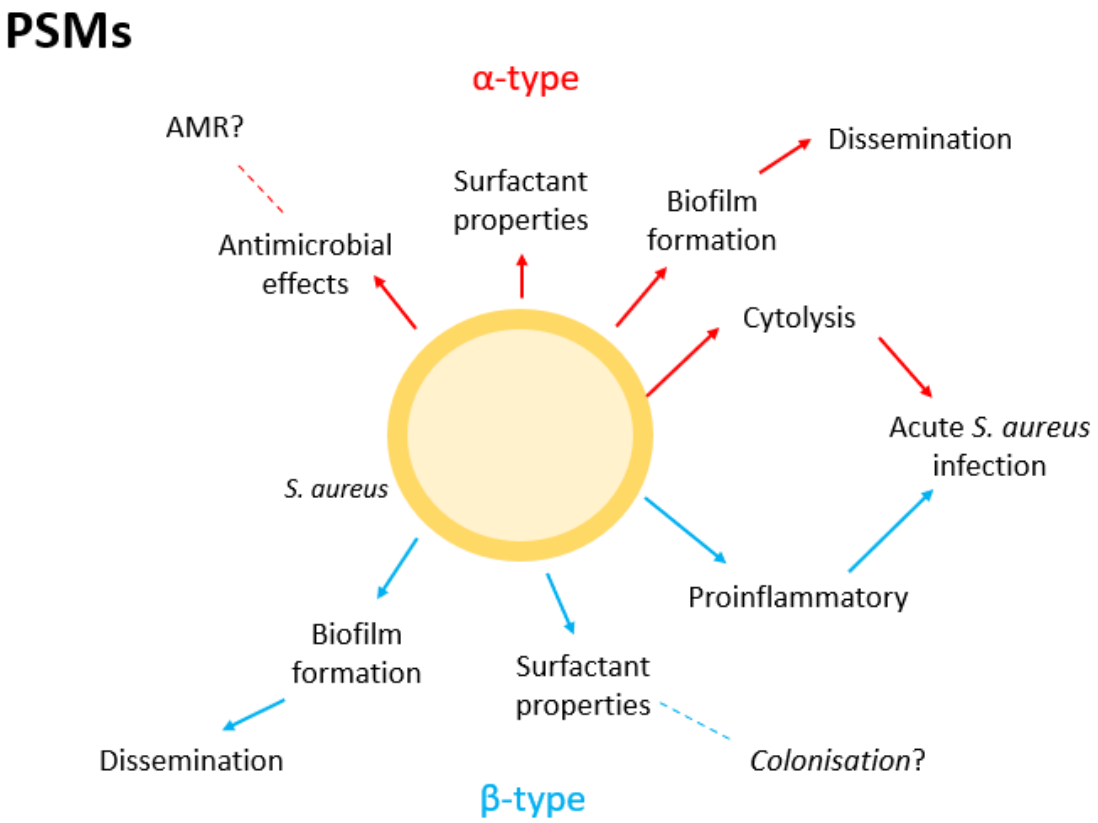


Figure 1.1: *In vitro* effects of phenol-soluble modulins (PSMs) of *S. aureus*. Arrow coloration corresponds to activity by α or β PSMs. Many functions of PSMs are shared between α and β type. Speculated and confirmed *in vivo* effects of PSMs are also listed.

1.2.2 | *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative, rod-shaped, encapsulated, aerobic, and non-fermenting bacterium. Amongst all bacterial causes of VAP, it has historically been the most consistently associated with mortality (Brewer *et al.*, 1996; Rello *et al.*, 1996). It is highly ubiquitous within the environment, able to colonise humans but also occurring in soil and water. It also has few nutrient requirements, and can survive long-term in moist conditions, where it can act as a potential reservoir for infection (Lewenza *et al*., 2018). In addition to VAP, it is a major pathogen in other respiratory diseases, such as cystic fibrosis and chronic obstructive pulmonary disease (COPD) (Nixon *et al*., 2001; Rodrigo-Troyano *et al*., 2018). *P. aeruginosa* intrinsically resists many antibiotics, for example aminoglycosides and quinolones. Strains with acquired resistance have been reported to all common antibiotics used in intensive care, with the exception of polymyxin B (Murray *et al.*, 2015). The resistance profile of *P. aeruginosa* does not appear to impact treatment failure in VAP, but greater resistance rates do result in increased length of hospital admission (Planquette *et al*., 2013).

P. aeruginosa can attribute its ability to thrive under hostile conditions to its large genome. The respiratory tract expresses numerous antimicrobial factors to combat colonisation of the mucosal surfaces, including antimicrobial peptides, neutrophil elastase, reactive oxygen species, and lactoferrin (Sadikot *et al.*, 2005). Due to its afforded genetic diversity, *P. aeruginosa* mutants capable of overcoming these challenges are selected more readily than other bacteria.

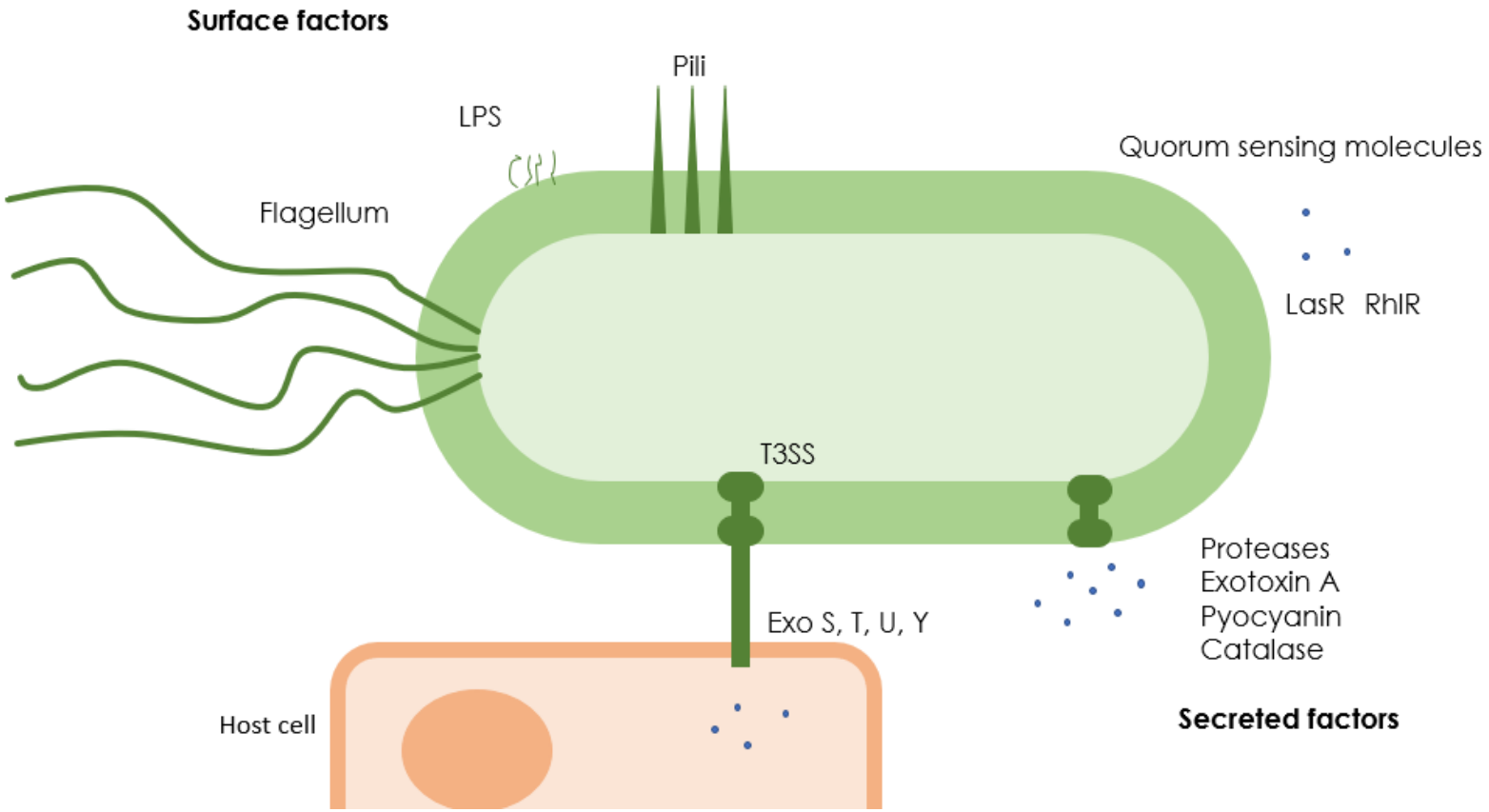


Figure 1.2: Virulence factors of *P. aeruginosa*. Includes surface factors (flagellum, pili, lipopolysaccharide (LPS)) and secreted factors (extracellular products and toxins, type 3 secretion system (T3SS) proteins, quorum-sensing molecules).

P. aeruginosa expresses a diverse range of virulence factors (Figure 1.2) to support its pathogenesis. On the bacterium's surface, polar pili mediate attachment to eukaryotic cells through recognition of GalNacβ1-4 receptor in asialylated glycolipids (Krivan *et al*., 1988). Type IV pilus also enable twitching motility vital to biofilm formation, are involved in attachment and movement across host surfaces, and activate the inflammasome in a manner similar to flagellin (Merz et al., 2000; Maier et al., 2004; Lindestam Arlehamn and Evans, 2011). Also expressed on the bacterial surface are polar flagella. These long protrusions enable *P. aeruginosa* motility, facilitating tissue invasion, but are also highly immunogenic and readily recognised by TLR5 (Adamo *et al.*, 2004).

The type 3 secretion system (T3SS) is a major virulence determinant of *P. aeruginosa*, allowing for direct injection of toxins into host cells. The T3SS is associated with poorer outcomes in VAP patients (Hauser *et al*., 2002). T3SS is activated upon pilin-mediated contact with eukaryotic cells and uses secretion and translocation machinery to inject effector proteins directly into host cytoplasm. Four anti-host exotoxins secreted by T3SS are known to be encoded by *P. aeruginosa* , the exoenzymes S, T, U, and Y (Yahr *et al*., 1998; Stover *et al*., 2000). ExoS and ExoT possess GTPase activating protein activity, and function as ADP-ribosyltransferases. Their actions abolish host cell signalling, leading to disruption of the actin cytoskeleton and apoptotic cell death (Pederson *et al*., 1999; Krall *et al*., 2000). ExoU is a phospholipase A2, which promotes inflammation by catalysing the arachidonic acid pathway, leading to acute rapid cytotoxicity and necrotic cell death (Sato *et al*., 2006). ExoY is an adenylate cyclase but is poorly understood. Its eukaryotic cofactor was recently identified as

filamentous actin, thus it is speculated to contribute to pathogenesis similarly to ExoS and ExoT (Belyy *et al*., 2016).

P. aeruginosa readily adapts to its environment through coordinated cell-to-cell signalling, controlled by quorum-sensing. Quorum-sensing allows *P. aeruginosa* to regulate expression of its virulence genes via synthesis of a diffusible molecule called *Pseudomonas* autoinducer (PAI) (Passador *et al*., 1993, 1996; Li *et al*., 2017). The secretion of PAI is required for the expression of protease, elastase, neuraminidase, alkaline protease, and for coordinating biofilm formation (Pearson *et al*., 1994; Ochsner and Reiser, 1995; Singh *et al*., 2000). Quorum-sensing signalling molecules are acyl homoserine lactones (AHL), freely diffusible molecules. AHL binds to LasR/RhlR transcriptional activators at high concentrations, which leads to the production of two PAIs: 3-O-C12-HSL and C4-HSL (Gambello and Iglewski, 1991; Pearson *et al*., 1995). 3-O-C12-HSL works in concert with LasR to activate transcription of LasA protease, LasB elastase and exotoxin A, which directly contribute to host tissue damage and necrosis (Casilag *et al.*, 2016). Exotoxin A is an ADP-ribosylating enzyme that inhibits protein synthesis in eukaryotic cells, through ADP-ribosylation of eukaryotic elongation factor-2 (Yates and Merrill, 2001). This results in cellular death, and facilitation of *P. aeruginosa* dissemination. C4-HSL and RhlR cooperate to activate transcription of LasA protease, LasB protease elastase, and pyocyanin.

Rhamnolipids are glycolipids which aid infiltration of primary human airway epithelia by *P. aeruginosa*, and contribute to necrosis of polymorphonuclear leukocytes (Zulianello *et al*., 2006). Production of rhamnolipids is controlled by Rhl quorum-sensing and is positively correlated with VAP (Köhler *et al*., 2010).

P. aeruginosa is capable of iron scavenging. Iron is sequestered tightly by host tissues during infection, bound to transferrin in the blood or lactoferrin in the airways (Xiao and Kisaalita, 1997). *P. aeruginosa* produces two siderophores, pyochelin and pyoverdine, which allow it to compete with the host for iron acquisition (Cox, 1980; Cox *et al*., 1981; Cox and Adams, 1985). The *Pseudomonas* siderophores are also involved in controlling expression of other virulence factors, including exotoxin A and endoprotease, and thus indirectly contribute to necrotic cell death in pneumonia (Lamont *et al.*, 2002). Pyoverdine does not appear to be essential in VAP. Pyoverdine-deficient *P. aeruginosa* mutants have been isolated from late-stage VAP patients (Wang *et al*., 2017b).

Pyocyanin is a pigment secreted by the majority of *P. aeruginosa*, and is the source of its characteristic blue-green colouration (Denning *et al.*, 1998). Pyocyanin exerts a proinflammatory effect on bronchial epithelium and exerts oxidative stress on the host. This is mediated through inhibition of catalase activity in host lung epithelial cells, diminishing their ability to decompose reactive oxygen species (O'Malley *et al.*, 2003).

1.2.3 | *Klebsiella pneumonia*

K. pneumoniae is a Gram-negative, rod-shaped, encapsulated, facultatively anaerobic, and non-motile bacterium (Ranjbar *et al*., 2019). Considered an opportunistic pathogen, it is a frequent coloniser of mucosal surfaces, from which it can disseminate and cause infection (Dao *et al*., 2014). It is implicated not only in VAP, but in a range of nosocomial infections, including bacteraemia, urinary tract infections, liver abscesses, and wound infections (Lin *et al.*, 2010). *K.*

pneumoniae causing nosocomial infections are considered hypervirulent, and have drawn increasing attention as a consequence of rising infection rates and antibiotic resistance (Holt *et al*., 2015). This species is a reservoir for antibiotic resistance, and can spread resistance genes to other Gram-negative bacteria, with high rates of resistance to fluoroquinolones, cephalosporins, and aminoglycosides (EARS-net, 2020). The majority of EU/EEA population exhibited greater incidence of MDR (28.6%) than single resistance (8%) in the 2019 EARS-Net annual epidemiological report. Furthermore, *Klebsiella* species may exhibit resistance to carbapenems, making these bacteria incredibly challenging to treat, requiring combination therapy or the much maligned colistin (Kumarasamy *et al*., 2010; Capone *et al*., 2013). Hypervirulent carbapenemase-producing *K. pneumoniae* have been described and isolated from clinical infections in China, capable of causing near-untreatable disease in the immunocompetent (Zhang *et al*., 2015, 2016b; Gu *et al*., 2018).

A major reservoir of *K. pneumoniae* within the healthcare setting is carriage within the gastrointestinal tract (Gorrie *et al.*, 2017). The gut microbiota is demonstrably protective against *K. pneumoniae* pneumonias, as shown in germfree mice (Fagundes *et al.*, 2012). In these models, IL-10 in the lungs attenuates the inflammatory response, allowing *K. pneumoniae* to establish infection (Fagundes *et al.*, 2012). Common gastrointestinal colonisers *Lactobacillus reuteri*, *Enterococcus fecalis*, *Lactobacillus crispatus* and *Clostridium orbiscindens* can trigger IL-17-GM-CSF within the lung microenvironment. This stimulates the alveolar macrophages to resolve the infection in a MAPK-dependent manner (Clarke, 2014; Brown *et al*., 2017). The mechanism by which gut microflora can

govern IL-17 production in the lung is unknown, and the role that upper respiratory microflora play in protection against infection remains unclear.

K. pneumoniae has classically been considered a stealth pathogen, capable of masking its immunogenic features to avoid stimulating an immune response. *K. pneumoniae* outer membrane proteins and lipopolysaccharide (LPS) are immunogenic and activate the classical complement pathway. OmpF and OmpC bind to Cq1 and activate complement (Albertí *et al.*, 1993). Activation and progression of the complement pathway leads to opsonisation of *K. pneumoniae* by C3b and clearance by lung epithelial cells, neutrophils, and macrophages (Domenico *et al*., 1994; de Astorza *et al*., 2004). *K. pneumoniae* protects itself from C3b deposition with its capsular polysaccharide (CPS). Strains deficient in CPS are more susceptible to the actions of complement, and protection afforded by CPS appears to be thickness-dependent (Alvarez *et al.*, 2000; de Astorza *et al.*, 2004). CPS also confers protection from the surfactant proteins, SP-A and SP-D, by physically blocking their binding to LPS (Kostina *et al.*, 2005). By preventing surfactant protein binding, CPS shields *K. pneumoniae* from bacterial agglutination and phagocytosis by macrophages (Tino and Wright, 1996). CPS is also induced by surfactant challenge, which increases virulence and biofilm formation in pneumonia (Willsey *et al.*, 2018).

K. pneumoniae is capable of secreting free CPS, which is released from the bacterial surface. Free CPS is able to sequester and neutralise cationic antimicrobial peptides (CAMPs) such as defensins, as well as certain antibiotics such as polymyxins (Llobet *et al*., 2008). The ability to secrete free CPS is shared by *P. aeruginosa* (Llobet *et al*., 2008). The action of CAMPs and polymyxins is also subverted through the remodelling of LPS. *K. pneumoniae* rapidly adjusts to

the presence of CAMPs through modification of lipid A, regulated by the actions of the PhoPQ, PmrAB and Rcs systems (Llobet *et al.*, 2011). OmpA is also involved in regulation of resistance to antimicrobial peptides (Llobet *et al.*, 2009).

K. pneumoniae is capable of exerting cytotoxic effects when engulfed by airway epithelial cells, which damages the lung environment during infection (Cano *et al*., 2009; Leone *et al*., 2016). This engulfment of bacteria is a constituent of the host defence, and upregulates the inflammatory response through NF-κB signalling (Regueiro *et al*., 2006). Cytotoxicity is dependent on live bacteria and CPS, as non-capsulated bacteria how been demonstrated to have no cytotoxic effect (Cano *et al.*, 2009).

Hypermucoviscosity (HV) is another important virulence factor of *K. pneumoniae* (Okada *et al*., 1994; Wiskur *et al*., 2008). HV is associated with unusually aggressive, virulent, and invasive *K. pneumoniae* infections (Lee *et al*., 2006a). HV is associated with higher incidence of VAP, development of bacteraemia, and mortality (Russo *et al*., 2014; Guo *et al*., 2016).

Control of *K. pneumoniae* by the host is largely mediated through downstream effects of NF-κB (Wieland *et al.*, 2011; Tomás *et al.*, 2015). *K. pneumoniae* avoids activation of NF-κB by blocking its own uptake by epithelial cells, through the expression of CPS. This results in reduced expression of defensins, the chemokine IL-8, and intercellular adhesion molecules involved in the recruitment of immune effectors (Regueiro *et al*., 2006; Moranta *et al*., 2010). *K. pneumoniae* is also capable of active suppression of NF-κB signalling by manipulating CYLD lysine 63 deubiquitinase (CYLD) (Frank *et al.*, 2013). CYLD is a deubiquitinating enzyme and dispels activation of NF-κB signalling. Manipulation of CYLD is

through the activation of a NOD1 signalling pathway and an epidermal growth factor receptor (EGFR)-phosphatidylinositol 3-OH kinase (PI3K)-AKT-PAK4-ERK-GSK3β signalling pathway (Regueiro *et al*., 2011; Frank *et al*., 2013). Activation of the EGFR pathway by *K. pneumoniae* is reliant on the CPS, and *cps* mutants are incapable of abrogating NF-κB signalling (Frank *et al.*, 2013).

K. pneumoniae is capable of disrupting resolution of infection. It is able to block efferocytosis, clearance of apoptotic neutrophils, by macrophages (Angsana *et al.*, 2016; Jondle *et al.*, 2018). *K. pneumoniae*, through deployment of presently unknown factors, decreases exposure of phosphatidylserine, which stimulates efferocytosis by phagocytic cells (Jondle *et al.*, 2018). Neutrophil death by necroptosis is upregulated instead, which results in increased inflammation at a time when the immune response is trying to resolve the infection (Pasparakis and Vandenabeele, 2015).

Through exploitation of the anti-inflammatory cytokine IL-10, *K. pneumoniae* is able to attenuate the immune response (Dolgachev *et al*., 2014). Overexpression of IL-10 within the lungs interferes with normal stimulation of macrophages, and results in decreased intracellular killing of *K. pneumoniae* (Dolgachev *et al*., 2014). IL-10 stimulates the anti-inflammatory response through the actions of the IL-10 receptor and activation of signal transducer and activator of transcription (STAT) 3. In mouse models, IL-10 is highly elevated in individuals infected with wild-type *K. pneumoniae*, though not in *cps* mutants (Lawlor *et al*., 2006). The implication is that the CPS is important for manipulation of IL-10 expression, although the signalling pathways and factors involved remain open questions.

1.2.4 | Other bacterial causes of VAP

The range of pathogens capable of causing VAP in ICU is broad. One of the most significant pathogens not previously discussed is *Acinetobacter baumannii*. This is a Gram-negative, coccobacillus, motile, opportunistic pathogen. *A. baumannii* is responsible for up to 10% of Gram-negative nosocomial infections (Sieniawski *et al*., 2013). Typically less virulent than other pathogens commonly implicated in VAP, it does not contribute as significantly to mortality in intensive care (Garnacho *et al*., 2003). Longstanding *A. baumannii* infections are difficult to treat, and can progress to bacteraemia and septic shock, an extremely serious condition frequently resulting in multiple organ failure and death (Cisneros *et al.*, 1996). This species is also frequently resistant to imipenem, with rates estimated at 11%, making these infections a therapeutic challenge (Shete *et al.*, 2010).

A. baumannii is capable of surviving on non-organic surfaces, which allows it to persist in the hospital environment, and conveys excellent capability to spread within hospitals (Shete *et al.*, 2010). Some strains of this bacterium are capable of surviving on dry surfaces in hospitals for periods of months if not disinfected properly, as they are able to survive desiccation (Wendt *et al.*, 1997; Jawad *et al.*, 1998). The increasing clinical relevance of *A. baumannii* does not seem to be due to the acquisition of new virulence traits. Within the core genomes of the predominant international clonal lineages (ICL) 1 and ICL2, no toxin, hydrolytic enzymes or adhesins were detected that would account for the bacteria's clinical significance (Imperi *et al.*, 2011). It is perhaps its ability to adapt to the hospital environment that has allowed for its success as a pathogen in VAP.

Very little is known about the pathogenesis of *E. coli* respiratory infections (Messika *et al*., 2012). Most *E. coli* VAP are caused by highly virulent

extraintestinal strains (Messika *et al*., 2012). The K54 capsule and O4-antigen are important virulence factors in pneumonia, and directly mediate lung injury in a bacterial load-dependent manner (Russo *et al*., 2003)

Enterococcus faecium is a significant nosocomial pathogen, and frequently causes bloodstream infections. This species is rarely isolated from VAP patients, but has been documented in case studies (Kimura and Kobayashi, 2011). This species is frequently vancomycin resistant, which may be problematic for cases of VAP in which is it implicated (Diaz Granados *et al*., 2005; Kampmeier *et al*., 2018).

S. maltophilia VAP is a low prevalence infection which develops in high-risk patient groups, i.e. those with long durations of hospital stay and mechanical ventilation (Guerci et al., 2019). It is most frequent in those exposed to prior broad-spectrum antimicrobials, such as carbapenems (Ibn Saied et al., 2020). Interestingly, treatment delays for *S. maltophilia* are not associated with mortality despite high death rates in these infections, suggesting low virulence and a greater role for the underlying condition (Guerci et al., 2019).

H. influenzae is a species more commonly associated with community-acquired pneumonias, the majority of these infections caused by non-typeable strains due to widespread vaccination against type b strains (Slack, 2015). In intensive care, *H. influenzae* has been associated with development of acute lung injury in trauma patients with VAP (Stéphan et al., 2006).

1.2.5 | Viral and fungal causes of VAP

Although this thesis is focused on bacterial causes and cases of VAP, not all cases are bacterial in origin. Numerous viruses have been reported causing VAP.

The Herpesviridae herpes simplex virus 1 and 2 (HSV), and cytomegalovirus (CMV) are those most frequently detected in the lungs of ventilated patients, in 64% and 55% respectively (Luyt *et al*., 2007; Osman *et al*., 2014). Other viruses causing reactivation pneumonia in ventilated patients include adenovirus, respiratory syncytial virus (RSV), and metapneumovirus (Vaideeswar *et al*., 2011). RSV has been associated with increased respiratory colonisation with *Streptococcus pneumoniae* in mouse models (Yan *et al*., 2020). Due to rarity in cases and uncertainty around clinical relevance even when present, understanding of viruses in VAP is poor, and literature is limited (Daubin *et al*., 2005). Fungal cases of VAP are similarly uncommon; *Candida* species, *Aspergillus* species, and *Pneumocystis carinii* have all been implicated in individuals undergoing immunosuppressive therapy or with autoimmune disease (Delisle *et al*., 2011; Tejerina *et al*., 2019).

1.3 | Pathogenesis of VAP

In the healthy lung, the normal physiological clearance of the upper respiratory tract is mediated by the cough reflex and mucociliary action (Girod *et al*., 1991). Particulates are trapped in mucus secretions and forced out of the respiratory system by the beating motion of cilia. In the MV patient this clearance does not occur. The cough reflex is suppressed by unconsciousness, and immune mediated defences are downregulated with the reduced immune response common amongst anaesthetised individuals (O'Donnell et al., 1992; Jensen et al., 1993; Heine et al., 1996). The MV patient is thus predisposed to microbial infection of the lower respiratory tract. The presence of the endotracheal tube (ETT) also allows bacteria to directly bypass host defences by travelling down the

lumen of the tube. The insertion of the ETT also suppresses the normal defensive reflexes of the upper respiratory tract. The term VAP is a misnomer, the presence of the ETT is more significant to the development of pneumonia than the action of the ventilator, and the longer the patient remains intubated the greater the risk of VAP (Awasthi *et al*., 2013).

Bacterial infection in VAP is driven by very general pathogenic mechanisms in bacteria. Bacteria adhere to host cells through a mixture of simple biophysical means and specific adaptations, such as the actions of type IV pili. Bacterial adhesins recognise a large variety of host cell structures and components. For example, the RrgA protein of type I pili in *Streptococcus pneumoniae*, which binds to toll-like receptor 2, located on the surface of epithelial respiratory cells, pulmonary alveoli, and immune effectors (Basset *et al*., 2013).

Following adhesion, pathogens may physically enter and colonise the host. Some will colonise intercellularly through active processes, for example injection of effector proteins by T3SS to permit uptake, or by rearrangement of the cellular actin cytoskeleton (Rosqvist *et al*., 1991; Adam *et al*., 1995). Other bacteria will colonise the extracellular space, including *S. aureus* and *P. aeruginosa*, which are strongly associated with VAP (Van Baarlen *et al*., 2007). Once established within the host, pathogenic bacteria are then able to damage host cells and tissues, mediated through a combination of pathogenic and host factors.

Bacterial multiplication in the lower airways may be expedited by underlying lung pathology, for example pulmonary oedema or trauma from previous lung infections. The insertion of the ETT also results in pooling of respiratory secretions around the cuff, which is inflated to hold the airways open during MV. ETT cuffs

frequently provide an imperfect seal between the ETT and tracheal wall. This allows for gradual microaspiration of colonised secretions into the lower respiratory tract (Figure 1.3) (Hamilton and Grap, 2012).

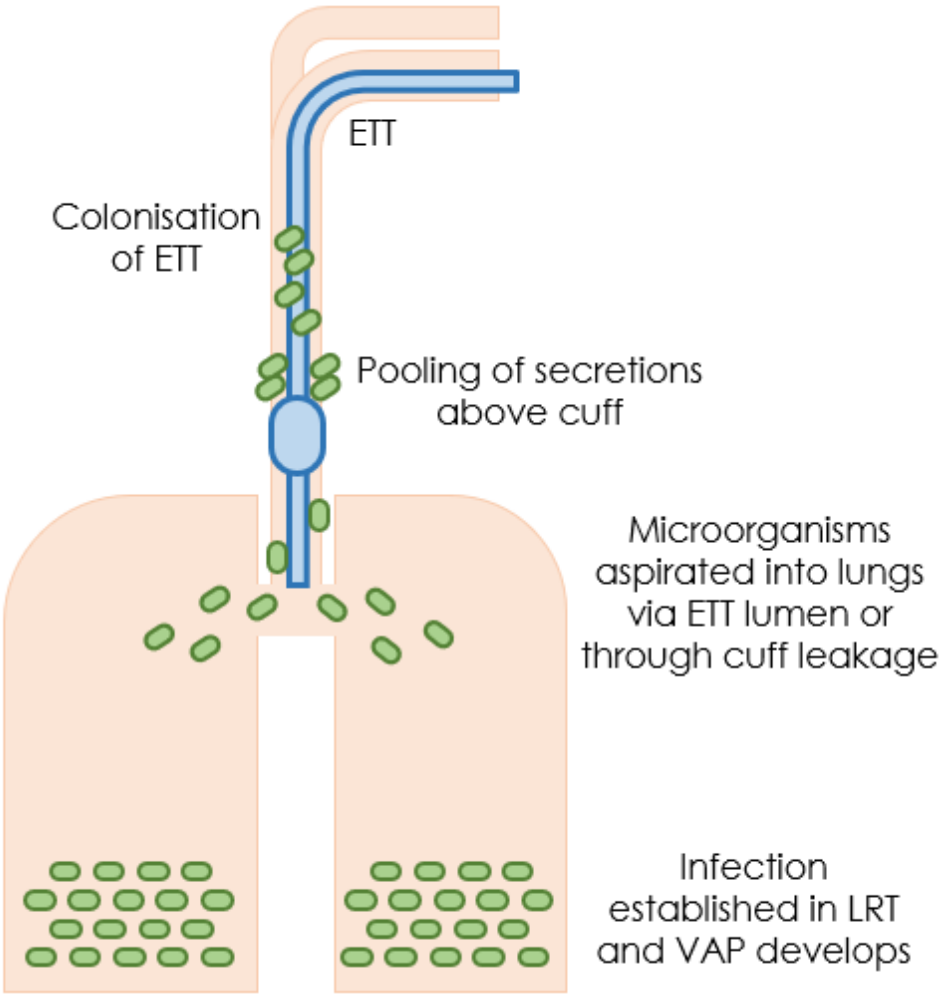


Figure 1.3: Major pathogenic events during the development of VAP. Mechanical ventilation with insertion of an endotracheal tube (ETT) leads to impairment of physiological defences. Consequently, respiratory secretions begin to pool above the ETT cuff, due to ineffective clearance. Additionally, the lumen of the ETT is colonised by bacterial biofilms. Pooled secretions and colonising bacteria are then aspirated into the lungs through leakage and cycling of the ventilator circuit, leading to development of infection.

Most understanding of the pathogenesis of VAP is of the healthcare interventions that provide bacteria with an entryway into the lower respiratory tract. The mechanisms by which bacteria cause VAP once established, particularly the important virulence determinants, are less clear. The important host factors and their role in mediating development of VAP are even more poorly understood. For example, while the mechanisms by which *A. baumannii* invade human cells is reasonably well characterised (Lee *et al*., 2006b; Choi *et al*., 2008). Those mediating cell death are not established beyond a few factors, including the involvement of proinflammatory cytokines and oxidative stress (Smani *et al*., 2011). There is value to be found in the increased usage of pneumonia and sepsis models, as well as models of virulence, e.g. the larvae of the greater wax moth *Galleria mellonella* (Mortensen and Skaar, 2012). These insects can be used to model the innate host immune response to infection; however, it is of limited use in the study of host signalling networks.

1.3.1 | Bacterial biofilms

The key phenomenon in the pathogenesis of bacterial VAP is the formation of biofilms within the ETT (Figure 1.4) (Adair *et al*., 2002). These are complex microbiological consortiums, frequently polymicrobial, which form readily on many medical devices. This includes ETTs, venous catheters, heart valves, pacemakers, contact lenses, and prosthetic joints, amongst others (Raad *et al*., 1993; McLaughlin-Borlace *et al*., 1998; Tunney *et al*., 1999). Biofilms form on the interior surfaces of ETTs within hours of mechanical ventilation (Adair *et al*., 2002).

After coming into contact with a suitable site, bacterial cells attach reversibly at their poles via the actions of flagella, pili, and fimbriae (O'Toole and Kolter, 1998b, 1998a). This attachment becomes irreversible following the action of surface proteins such as LapA and SadB (Hinsa *et al*., 2003; Caiazza and O'Toole, 2004). Transporters also play an important role, for example the *lapBCE*-encoded ATP-binding cassette transporter (Hinsa *et al.*, 2003). Once irreversibly attached, bacteria within a biofilm secrete an encapsulating polysaccharide matrix of extracellular polymeric substances (EPS). Biofilm intercellular communication networks form within a self-produced matrix of EPS, for example the *P. aeruginosa* exopolysaccharide PsI (Zhao *et al.*, 2013). This allows for the aggregation of irreversibly attached cells into microcolonies. Once established, these microcolonies can undergo exponential division and begin the formation of a mature biofilm (Zhao *et al.*, 2013).

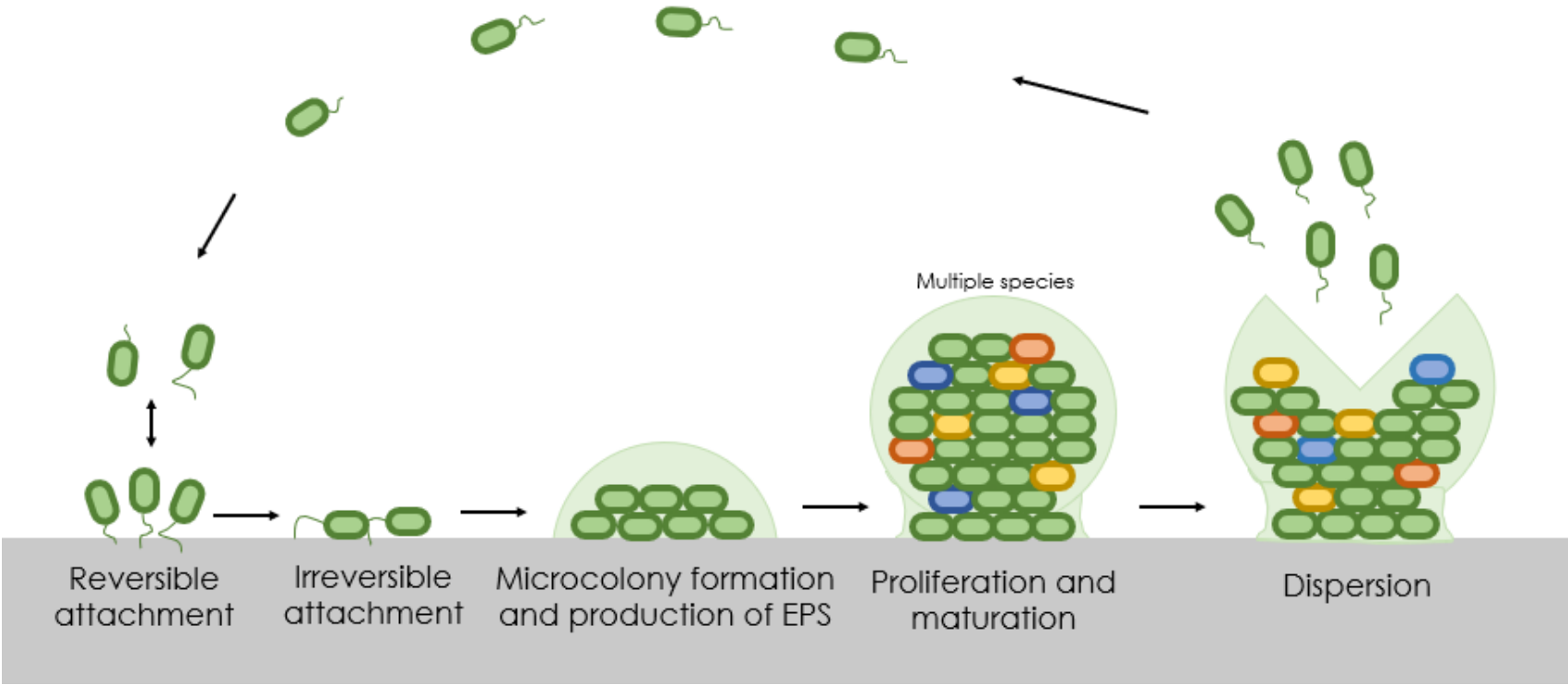


Figure 1.4: Major stages of biofilm formation. Planktonic bacteria adhere to a surface and become irreversibly attached in response to environmental stimuli. Action of secondary messengers (e.g. cyclic AMP) drive biofilm formation factors and lead to aggregation of bacteria into microcolonies. The biofilm becomes encased in a polysaccharide matrix as it matures, which offers protection from antimicrobials and provides a medium for extensive cell-cell communication. As the biofilm matures, other species are acquired as the community becomes polymicrobial. Some cells return to planktonic phase during dispersion events to seed further surfaces.

After reaching maturity, dispersal events occur, transferring some bacteria to the planktonic phase. These bacteria are then free to travel and seed further surfaces for the formation of new biofilms. Dispersal events can be active or passive. In active dispersal, environmental triggers upregulate genes linked to motility, chemotaxis and EPS degradation (Webb *et al*., 2003; Purevdorj-Gage *et al*., 2005). Genes involved in EPS production and cellular attachment are downregulated. Active dispersal can be triggered by changes in temperature, nutrients, oxygen levels, and toxic metabolites. These changes in environment control the levels of intracellular cyclic dimeric-GMP in bacteria, a second messenger which serves as a control switch between the planktonic and biofilm phenotypes (Fang and Gomelsky, 2010). Passive dispersal is governed by physical factors, such as physical abrasion of the biofilm or shear forces (Wimpenny and Colasanti, 2006).

The cradle of the biofilm provides an ideal niche for intercellular communication between bacteria. Within the biofilm, cellular processes such as toxin formation, regulation of enzymes, and horizontal gene transfer can synchronise through the action of quorum signalling (Rutherford and Bassler, 2012). These processes are more efficient within highly regulated biofilm communities than in planktonic phase. Extracellular DNA is an established component of the extracellular matrix, and is important in biofilm formation (Whitchurch *et al*., 2002). *P. aeruginosa* extracellular DNA contributes to decreased susceptibility to aminoglycosides in biofilms through sequestration of the antibiotic (Chiang *et al*., 2013). This protective effect is conferred to other species present within polymicrobial biofilms.

ETT biofilms are polymicrobial, and their composition is difficult to report as the constituent bacteria cannot necessarily be cultured (Li *et al*., 2015; Bardes *et al*.,

2016). The ESKAPE pathogens (*Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species) are frequently identified among biofilm communities in VAP, playing the greatest role in disease. Oral microflora are often present, but are thought to contribute more to initial biofilm formation than the development of VAP (Vandecandelaere and Coenye, 2015). Some studies have indicated no association between duration of intubation and the number of bacterial species identified in ETT biofilms, suggesting that diversity in ETT biofilm communities do not change over the course of MV (Cairns *et al*., 2011; Bardes *et al*., 2016).

Biofilms offer significant protection to the resident microorganisms from host defences and antimicrobials. The encapsulating matrix physically blocks these factors, and bacteria within biofilms are more metabolically quiescent than those in planktonic phase, which reduces the rate of antimicrobial uptake (Donlan, 2001). Anchored to the inner lumen of the ETT, the biofilm becomes inaccessible to systemic antibiotics and host immune and mucosal clearance (Adair *et al*., 1999). The cycling of the ventilator circuit repeatedly forces bacteria dispersed from biofilms into the lower respiratory tract, where they can establish pneumonia (Sottile *et al*., 1986; Park, 2005a; Gunasekera and Gratrix, 2016).

1.3.2 | Risk factors

Risk factors influencing the development or mortality of VAP can broadly be divided into those associated with the host and with interventions. Host risk factors will differ on a case-by-case basis, whilst intervention risk factors are largely consistent based on the clinical actions being taken.

1.3.2.1 | Host risk factors

The role of advanced age in the development of VAP is controversial (Ding *et al*., 2017; Zubair *et al*., 2018). Mortality from VAP is greater in elderly patients, believed to be due to decline in immune function, decreased elasticity of lung tissue, and weakened cough reflex (Blot *et al*., 2014; Liu *et al*., 2017). Elderly patients are more susceptible to infections with Enterobacteriaceae, which cause 43% of VAP in the elderly compared to 24% in those middle-aged (Blot *et al*., 2014).

Another risk factor for VAP is male sex. This is believed to be due to differences in sex hormones, differences in pathogen profiles between sex, and differences in complications (Forel *et al*., 2012). 81% of acute respiratory distress syndrome (ARDS) patients who develop VAP are male, compared to 63% of ARDS patients with no VAP, illustrating the disparity in this patient group (Forel *et al*., 2012). There are higher incidences of VAP in male trauma patients than female, 3.8% to 2.6% respectively (Sharpe *et al*., 2014).

Trauma, including burns, is a significant risk factor. 21.6% of trauma patients and 10-65% of burns patients develop VAP (Jaimes *et al*., 2007; Latenser *et al*., 2007). Burns patients become susceptible through inhalation injury resulting in immune dysfunction, acute respiratory failure, and impaired bacterial clearance from the lungs (Sen *et al*., 2016). Chronic trauma patients are at greater risk of VAP caused by MDR pathogens, due to long term antibiotic use in this group (Singh *et al*., 1998). Another significant risk factor in trauma patients is the development of secondary bacteraemia, wherein bacteria access the bloodstream after infection at another site. This occurs in 13%-40% of trauma patients and increases their risk of mortality (Kunac *et al.*, 2014; Karakuzu *et al.*, 2018).

Patients with COPD are at increased risk of VAP, and are twice as likely to develop this infection than those without COPD (Rouzé *et al*., 2014; Liu *et al*., 2017). These individuals commonly require MV and are often of advanced age due to usual late onset of COPD in life. Rates of lower respiratory tract colonisation are high in these individuals, and they frequently suffer inhibited mucociliary action as a consequence of smoking (Rouzé, Cottereau and Nseir, 2014). Consequently, clearance of bacteria from the respiratory tract and lungs is reduced. COPD patients are also at particular risk of infections caused by *P. aeruginosa*, MRSA, and *H. influenzae*, all of which are frequently implicated in VAP (Rouzé, Cottereau and Nseir, 2014). VAP-related mortality in COPD patients is significantly higher than in those without COPD, 60% to 43% (Makris *et al*., 2011).

ARDS patients are at increased risk of VAP due to increased ventilation rates, with 55% developing VAP (Chastre *et al*., 1998; American Thoracic Society and Infectious Diseases Society of America, 2005). The rate of recurring VAP is also high in these patients, at 61% compared to 33% those with VAP alone (Stéphan et al., 2006). The impact of VAP on ARDS mortality rates is contentious. Differences in ARDS mortality with and without VAP frequently appear insignificant. Ayzac *et al*. (2016) contest this, and demonstrated that when VAP is considered as a time-dependent variable, it becomes a significant predictor of mortality in ARDS patients (Ayzac *et al.*, 2016).

Underlying cardiac disease is another risk factor in the development of VAP (Perbet *et al*., 2011). Approximately one third of patients requiring MV following cardiac arrest develop VAP (François *et al*., 2019). This may be linked to targeted

temperature management in out-of-hospital cardiac arrest, which as been linked to increased risk of secondary infections (Polderman, 2009; Perbet *et al*., 2011).

Table 1.1: Criteria measured by Acute Physiology and Chronic Health Evaluation II for classification of disease severity. Measurements are taken upon admission into intensive care, using the worst value for each variable within the prior 24 hours.	
General	Age (years) Glasgow coma scale score (3-15) Severe organ failure or immunodeficiency
Vital signs	Body temperature (°C) Mean arterial pressure (mmHg) Heart rate (bpm) Respiratory rate (bpm)
Blood oxygenation	Fraction of inspired oxygen (FiO_2) (%) Partial pressure of oxygen (PaO_2) (mmHg) Arterial pH
Blood chemistry	Sodium (mmol/L) Potassium (mmol/L) Creatinine (µmol/L) Presence of acute renal failure
Haematology	Haematocrit (%) White blood count ($x10^9$/L)

Those with high Acute Physiology and Chronic Health Evaluation II (APACHE II), and Sequential Organ Failure Assessment (SOFA) scores are also at increased risk of mortality in VAP. This is a consequence of the severe disease in those who score highly by these criteria. APACHE II is a score of disease severity taken upon admission into ICU, using the worst value for each variable measured within the prior 24 hours (Table 1.1) (Knaus *et al*., 1985). Newer versions of this scoring system have been developed, APACHE III and APACHE IV, but are not as

commonly used due to copyright issues with the statistical methods employed. SOFA measures the risk of organ failure as a consequence of sepsis (Table 1.2) (Jones *et al*., 2009). SOFA scores are taken upon admission into intensive care but are recalculated throughout the duration of stay. As for APACHE II, the worst value for each variable within the prior 24 hours is used.

Table 1.2: Criteria measured by Sequential Organ Failure Assessment for prediction of intensive care mortality. Measurements can be taken on any patient upon admission to intensive care and can be repeated throughout the duration of stay, using the worst value for each variable within the prior 24 hours.

General	Glasgow coma scale score (3-15) Requirement of mechanical ventilation
Vital signs	Mean arterial pressure (mmHg) Required vasoactive agents (mg/kg/min)
Blood oxygenation	FiO_2 (%) PaO_2 (mmHg)
Blood chemistry	Platelets ($x10^3$/µL) Bilirubin (µmol/L) Creatinine (µmol/L)

1.3.2.2 | Role of the microbiome

Long standing colonisation of the upper respiratory tract and oral cavity is a risk factor for the development of VAP. Oral colonisation with *Enterococcus fecalis, Fusobacterium periodonticum*, *Gemella morbillorum*, *Neisseria mucosa*, *Propionibacterium acnes*, *Prevotella melaninogenica*, *Streptococcus oralis*, *Streptococcus sanguinis*, *Treponema denticola*, or *Veillonella parvula* are all statistically significantly related with development of VAP (de Carvalho Baptista *et al.*, 2018). These colonisers of the mouth are frequently benign, but can establish

themselves within the oropharynx and subsequently an inserted ETT, allowing aspiration into the lungs (Hamilton and Grap, 2012). The initial seeding of biofilm in the ETT by oral microbiota provides a suitable environment for the adherence and propagation of potential pathogens such as *S. aureus* and *P. aeruginosa* (Vandecandelaere *et al*., 2012).

There may be evidence that the oral microbiome acts as a reservoir for recolonisation in VAP. In one study, a “microbial shift” in dental plaque occurred during MV, notably in favour of common VAP pathogens, including *S. aureus* and *P. aeruginosa* (Sands *et al*., 2017).

The lungs are themselves home to a complex and diverse microbiome, despite previous belief of sterility (Dickson *et al*., 2016). In health, the lungs are commonly colonised by *Bacteroides*, *Firmicutes,* and *Proteobacteria*, including common genera *Prevotella*, *Veillonella*, *Streptococcus* and *Pseudomonas* (Erb-Downward *et al*., 2011). There is natural variability in the lung microbiome between individuals, and this can vary between different states of disease and health. The microbiome of the lower respiratory tract bears more similarity to the upper respiratory tract of the same individual, than it does to the lung microbiome of a different individual (Charlson *et al*., 2012).

Variations in lung microbiota are a useful predictor of ICU outcomes in MV patients. Poor outcomes are associated with higher bacterial burdens, as well as the presence of gastrointestinal taxa, such as Lachnospiraceae and Enterobacteriaceae (Dickson *et al*., 2020). The understanding of the role the lung microbiota actively plays in VAP remains underdeveloped (Alagna *et al*., 2019). Throughout MV, its composition changes, regardless of whether VAP develops,

with increases in Gram-negative bacteria, such as *P. aeruginosa* (Zakharkina *et al*., 2017). There is a greater degree of dysbiosis in VAP patients than those without (Zakharkina *et al*., 2017). Lower alpha diversity is linked to the development and duration of VAP, independent of antibiotic use (Kelly *et al*., 2016). The presence of certain species allows for cooperative action, for example *S. aureus* colonisation and established biofilm formation allowing for *P. aeruginosa* to attach and establish itself (Alves *et al*., 2018).

1.3.2.3 | Intervention risk factors

Intervention risk factors are associated with the clinical actions taken upon patients in intensive care. Enteral feeding by nasogastric tube may increase risk of VAP by increasing gastric pH. This compromises the natural hostility of the stomach afforded by its acidic environment, enabling colonisation by Gram-negative pathogens. The aspiration of colonised stomach contents is a significant cause of pneumonia, particularly in patients rested in the supine position (Keyt, Faverio and Restrepo, 2014). Reintubation increases the risk of developing VAP, as the nasogastric tube is in place during this procedure. Thus there is an elevated risk of aspirating gastric contents during the reintubation process (Chastre and Fagon, 2002).

Frequent changing of the ventilator circuit has been linked with increased rates of VAP. Fink *et al.* (1998) reported that longer use of a single ventilator circuit is associated with fewer incidents of VAP. They found that a 2-day change interval resulted in 11.88 cases of VAP per 1000 ventilator days. This is compared to 3.34 cases with a 7 day change interval (Fink *et al*., 1998). Hess *et al.* (2003)

determined that closed suction catheters could be used to assist in preventing the development of VAP, as they do not necessitate daily changeover. They did not however conclude the maximal safe duration of closed suction catheters (Hess *et al*., 2003). A later study suggested that given the risks associated with changing ventilator circuits, they should be left in place indefinitely unless soiled or damaged to reduce risk of VAP in the intubated patient (Han and Liu, 2010).

Extended duration of VAP has previously associated with increased risk of VAP caused by *P. aeruginosa* and MRSA (Ibrahim *et al*., 2000). More recent findings suggest that increased duration of MV before onset of VAP does not significantly affect the causative pathogens encountered, or their resistance profiles (Giantsou *et al*., 2005; Gastmeier *et al*., 2009).

Local microbiological and susceptibility data must be considered when assessing the potential risk of VAP and during selection of empirical therapies (Park, 2005b). Inappropriate selection of antibiotics can increase VAP mortality by as much as 90% (Karakuzu *et al*., 2018). This is a consequence of high MDR rates in common causative agents of VAP, leading to increased risk of treatment failure. By considering local data, clinicians are more informed when prescribing empirical therapy, thus reducing the risk of treatment failure.

1.3.3 | Host response

The signalling cascades which initiate the host response to infection are activated upon contact between bacteria and the airway epithelium. The insertion of the ETT or the inflation of the cuff often results in the impairment of physical barriers and the denudation of ciliated epithelial cells (Ballinger and Standiford, 2013).

Microorganisms display conserved structures, known as pathogen-associated molecular patterns (PAMPs) (Lavoie, Wangdi and Kazmierczak, 2011). PAMPs are detected by the host through binding to pattern recognition receptors (PRRs). This includes Toll-like receptors (TLRs), found on the cell surface and within the endosome, Nod-like receptors (NLRs), and the TNF receptor 1 (Lavoie, Wangdi and Kazmierczak, 2011). TLR2 can detect PAMPs such as cell wall components, toxins, and genetic material, and subsequently initiate immune responses to a broad range of bacterial species, including *P. aeruginosa* and *S. aureus* (Soong et al., 2004; Oliveira-Nascimento et al., 2012). TLR2 interacts with the receptor asialoGM1, a glycolipid which is known to act as a receptor for *P. aeruginosa* (de Bentzmann *et al.*, 1996). When ligated, these receptors activate transcription factors that trigger events such as the NF-κB signalling cascade and cyclic adenosine monophosphate (cAMP) response element binding protein (Prince *et al.*, 2006). This results in the release of cytokine mediators of the innate immune response, and MUC2, which upregulates mucus secretion (Prince *et al.*, 2006). The mucus response covers the airway epithelium in a viscous colloid, which traps invasive infectious particles (Rogers, 2007). For the control of *P. aeruginosa* in the lung, almost all TLRs require an adaptor molecule, myeloid differentiation factor 88 (MyD88) (Skerrett *et al.*, 2004). MyD88 knockout mice are susceptible to lung infections with *P. aeruginosa*, but not to *S. aureus* (Skerrett *et al*., 2004).

TLR4 recognises bacterial LPS, large lipid molecules embedded within the outer membrane of Gram-negative bacteria. TLR4 signalling of LPS-induced inflammatory responses is essential to defence against Gram-negative infection (Chow *et al.*, 1999). This has been demonstrated in murine models, where TLR4 deficiency is associated with reduced *K. pneumoniae* pneumonia survival rates

(Branger *et al.*, 2004). Gram-positive bacterial components, such as soluble peptidoglycan and lipoteichoic acid (LTA), are recognised predominantly via TLR2 (Schwandner *et al.*, 1999; Yoshimura *et al.*, 1999). TLR5 recognises flagellin, the protein which comprises bacterial flagella. These organelles are important in biofilms, bacterial motility and chemotaxis, and are thus very well conserved (Feldman *et al.*, 1998). They are essential to pathogenesis in several organisms, for example *P. aeruginosa*, where they allow the bacteria to spread through the respiratory tract to colonise and acquire nutrients (Feldman *et al.*, 1998). Flagella are also an important ligand for the activation of the innate immune response in the respiratory tract (Balloy *et al.*, 2007). Its importance as a ligand is demonstrated in TLR2,4$^{-/-}$ mice. *P. aeruginosa* LPS is recognised by these receptors, but knockout mice are still able to clear infection via TLR5 recognition of flagellin (Skerrett *et al.*, 2007). When a mutant strain of *P. aeruginosa* without flagella is introduced, these knockout mice become hypersusceptible (Ramphal *et al.*, 2008). This redundancy between TLRs is important for adequately defending from acute respiratory pathogens, as not all invasive bacterial species may be flagellated, display LPS, or display LTA.

When immune stimulators such as LPS, LTA or flagellin are detected, the formation of diverse cytokine networks is elicited, frequently with proinflammatory effects (Liu *et al*., 2018). The host cytokine response to VAP-associated pathogens comprises both proinflammatory and anti-inflammatory components. Perhaps the most prominent early response is the activation and secretion of the proinflammatory cytokine, IL-1β (Wangdi *et al*., 2010). IL-1β is activated by the action of caspase-1, otherwise known as ICE (Li *et al.*, 1995). ICE is formed within the inflammasome, large multiprotein complexes of NLRs which recruit and

activate procaspase-1, the ICE progenitor (Yang *et al*., 1998). Activation of ICE will also trigger cell death via pyroptosis, a poorly understood process that is believed to trigger pore-induced intracellular traps to clear bacteria by efferocytosis (Jorgensen *et al.*, 2016).

The production of IL-1β immediately results in an increase in IL-1 receptor (IL-1R) signalling. The actions of IL-1R mediate the localisation of neutrophils to the infection site (Mijares *et al.*, 2011). The importance of IL-1R signalling in the clearance of infectious agents is demonstrated again in knockout mice. Those deficient of IL-1R signalling are much less capable of adequate bacterial clearance and are more susceptible to infection resulting from small inoculum dosages (Schultz *et al.*, 2002). Not all proinflammatory responses are beneficial though, an extended inappropriate or greatly increased proinflammatory response has been associated with poor treatment responses and thus increased mortality in VAP (Ioanas *et al.*, 2004). It is suggested that a regulated inflammatory response clears bacterial infection, whilst unregulated inflammation may actually enhance growth of bacteria (Meduri, 2002).

The IL-1 and TNF families of cytokines are largely similar in effector function. They are unique amongst the cytokines for their exceptionally broad effects and involvement in initiating all key host responses to infection (March *et al*., 1985; Schoenfeld *et al*., 1991). The significance of each cytokine differs depending on the causative agent of VAP. Murine models deficient in receptors for TNFα, IL-1α, and IL-1β have shown to still be protected from *E. coli* infection, despite a diminished inflammatory response (Mizgerd *et al*., 2004). Conversely, murine models of *Streptococcus pneumoniae* deficient for the same receptors showed largely inadequate activation of NF-κB. This is a highly conserved protein complex

with many functions, including regulation of immunity, and these mice were unable to clear *S. pneumoniae* infection (Jones *et al.*, 2005). These contrasting findings illustrate the differing importance of TNFα and IL-1 in the mouse immune response to pneumonias of differing aetiology. In the context of community-acquired pneumonias, pneumococcal pneumonias are most associated with elevations in IL-6, IL-8, IL-10 and TNFα (Karhu *et al.*, 2018). Gram-negative community pneumonias are most associated with elevations in IL-8, and *K. pneumoniae* also elevates IL-10 (Karhu *et al.*, 2018).

The proinflammatory cytokines selected for use in this thesis, TNFα, IL-1β, and IL-6, and the reasoning behind their selection, are individually covered in more detail in Chapter 3.

Alveolar macrophages are fixed resident immune cells present in the lung microenvironment (Guilliams *et al*., 2013). Macrophages are a key component of the innate immune response. They directly kill pathogens through phagocytic internalisation and subsequent breakdown but are also sentinel cells which sense their environment and can activate the immune response. Alveolar macrophages can recruit neutrophils to the infection site through the production of chemokines, induced by TLR activation by bacterial PAMPS (Raoust *et al.*, 2009). Alveolar macrophages also produce IL-1β, which will stimulate airway epithelium to produce further chemokines, recruiting further neutrophils (Mijares *et al.*, 2011). Accessory to their antibacterial responsibilities, alveolar macrophages are responsible for resolution and repair of the infection site. They phagocytose dead neutrophils and clear the infection site of cellular debris (Amano *et al*., 2004).

Very early following inflammasome induction, neutrophils are rapidly recruited to the infected airways. These granulocytes directly kill invasive pathogens through multiple mechanisms whilst also producing further cytokines to stimulate further immune effectors (Ear and McDonald, 2008). Neutrophils progress the immune response to microbes through the production of CC chemokine ligands (CCL), including CCL3, CCL4, CCL5 and CCL20. These chemokines attract immature dendritic cells to the infection site, antigen-presenting cells that bridge the gap to adaptive immunity through processing and presentation of antigenic material to T cells (Bennouna *et al.*, 2003). Production of the cytokine TNFα by neutrophils also stimulates the activation of immature dendritic cells (Bennouna *et al.*, 2003). Other proinflammatory cytokines released by neutrophils include IL-6 and IL-1β, which further mediate leukocyte localisation to the infection site (Xing *et al.*, 1994). This positive feedback and further neutrophil recruitment is important for adequate control of VAP, due the saturable nature of granulocyte killing in pneumonia (Drusano *et al.*, 2011). Small infectious inocula are more easily cleared, large infectious dosages of bacteria lead to increasing burden of illness if neutrophil recruitment is inadequate.

In addition to previously outlined effects, neutrophils also produce a range of serine proteases. The importance of these in clearing infection has been demonstrated in murine studies. Neutrophil elastase knockout mice have impaired ability to clear *P. aeruginosa* from their lungs and are at increased risk of acute infection (Hirche *et al.*, 2008). Neutrophil elastase targets the outer membrane porin OprF, increasing bacterial membrane permeability (Hirche *et al.*, 2008). Neutrophil elastase and other serine proteases, such as proteinase 3, are nonspecific, and can target host cellular processes as well as bacteria (Dahlen *et*

al., 1999). This can result in potentiation of the inflammatory response and damage the cells and tissues of the lung. This targeting of the host is not always detrimental however, as protease-activated receptors on lung epithelial cells and phagocytic cells are targeted by the action of neutrophil serine proteases (Moraes *et al.*, 2008).

Neutrophils are potent producers of nitric oxide, a free radical and powerful reactive species (Wink *et al.*, 2011). The production of nitric oxide within the lungs generates an environment rapidly hostile to invasive bacteria not expressing nitric oxide reductase (Kakishima *et al.*, 2007). The importance of nitric oxide induction has been demonstrated in murine models, where neutrophils of knockout mice were able to phagocytose *P. aeruginosa*, but failed to clear the internalised bacteria (Zhang *et al.*, 2011).

Airway epithelial cells respond to invasive bacteria through the release of chemokines and cytokines. They are, for example, capable of recognising LPS and flagellin of *P. aeruginosa* via TLRs, and release immunomodulatory proteins in a MyD88-dependent manner (Raoust *et al.*, 2009). They may also take more direct action through the secretion of anti-microbial peptides. Airway epithelium can secrete surfactant proteins (SP), of which SP-A and SP-D act as opsonins through binding to surface carbohydrate in pathogens (Giannoni *et al.*, 2006). SP-C deficiency in mouse models has been associated with mildly defective bacterial clearance, but is not an opsonin or direct killer of bacteria (Glasser *et al.*, 2008). SP-B is not currently understood as having any immune function (Weaver and Conkright, 2001). Lung surfactant proteins are also immunomodulatory, with some control over the inflammatory response and phagocytosis (SANO and KUROKI, 2005; Kuroki, Takahashi and Nishitani, 2007). Epithelial cells also produce

defensins and the human cathelicidin LL-37/hCAP-18, which are strongly antimicrobial. Further to this, defensins and cathelicidins are involved in processes related to infection resolution, including cell proliferation, immune modulation and angiogenesis (Ganz, 2003; Bowdish, Davidson and Hancock, 2006). It is fair to conclude that despite not being professional immune cells, lung epithelial cells have a decisive role to play in the host immune response.

1.4 | Diagnosis of VAP

Community-acquired pneumonia (CAP) is a well-defined illness and is easily diagnosed (Pugin, 2002). The identification of VAP is much more problematic. Current methods for diagnosis of VAP lack sensitivity and specificity, and there is no gold standard consensus definition of what constitutes a case of VAP (Klompas, 2007). Due to the low sensitivity and specificity of individual criteria, VAP is diagnosed using a combination of clinical findings, chest radiology, and microbiological testing of samples (Klompas, 2007). The issue arises when considering the time-consuming nature of performing so many tests, especially considering the severity of these infections.

The use of clinical criteria is complicated by a range of conditions which present similarly to VAP, including ARDS, pulmonary oedema, pulmonary contusion, tracheobronchitis, and thromboembolism (Stewart and Cuthbertson, 2009). Nonspecific signs of lung pathology can result in overtreatment of those who do not have VAP, or undertreatment of those who do (Fagon *et al*., 1993). Overtreatment of patients without the condition is concerning, especially

considering the large contribution of antibiotics prescribed for VAP treatment to the development of AMR in intensive care.

Using different diagnostic criteria in combination has been shown to have more merit. Radiological microbiological lung infiltrates concurrent with two of fever (>38°C), purulent respiratory secretions, and leucocytosis (>12 x 10^9 leukocytes/mL), is suggestive of VAP (69% sensitivity, 75% specificity) (Torres *et al*., 1994). Many clinical criteria are subjective and susceptible to intra- and inter-observer variation, for example changes in appearance of respiratory secretions (Stewart and Cuthbertson, 2009).

Radiological findings are difficult for clinicians to interpret. The presence of new infiltrates on x-ray is a sensitive criterion but is lacking in specificity (Stewart and Cuthbertson, 2009). Those requiring MV often have abnormalities on chest x-ray secondary to another condition, and the presence of the ETT causes non-infectious opacity (Pugin, 2002).

There are limitations to both invasive and non-invasive sampling methods when diagnosing VAP. Invasive sampling methods are limited by poor access to the areas of the lung affected in VAP, hampering sensitivity. Non-invasive methods are at increased likelihood of contamination with normal oropharyngeal colonisers. Invasive sampling methods are associated with increases in modified antibiotic therapy (Sanchez-Nieto *et al*., 1998; Fagon *et al*., 2000). Fagon *et al*. (2000) state that mortality at 14 days is reduced with invasive sampling. This is contested by a large trial of 739 patients by the Canadian Critical Care Trials Group, who found that invasive BAL and non-invasive culture ETA have similar clinical outcomes and overall antibiotic usage. This Canadian study emphasised to clinicians to modify

antibiotic therapy in response to culture findings (Canadian Critical Care Trials Group, 2006). Given the strength of the Canadian study and a more recent meta-analysis (Berton et al., 2014) it appears a majority of more recent literature supports the notion that invasive sampling confers no tangible benefit to the VAP patient.

1.4.1 | VAP definitions

There is a lack of consensus definition of VAP. Numerous definitions are used in healthcare worldwide. This complicates reporting of VAP incidence, as well as diagnosis, as what one institution considers VAP may be ignored by another.

The clinical pulmonary infection score (CPIS) was devised in 1991, and uses clinical variables to diagnose VAP (Pugin *et al.*, 1991). CPIS has been found to have low sensitivity and specificity for diagnosis of VAP, far lower than originally reported (Schurink *et al*., 2004). CPIS criteria are also subject to inter-observer variability, especially when considering tracheal secretions and chest x-ray. Use of CPIS criteria has also been associated with over-prescription of antibiotics to patients with suspected VAP (Luyt *et al*., 2004). In a meta-analysis by Shan *et al.* (2011), CPIS was estimated to have sensitivity and specificity of 65% and 64% respectively. The authors concluded that whilst the diagnostic viability is only moderate, CPID remains viable due to its ease of use, with the recommendation that it is combined with other more rigorous diagnostic methods (Shan *et al*., 2011)

The US Centers for Disease Control (CDC) National Healthcare Safety Network (NHSN) algorithm is used for the clinical definition of healthcare-associated pneumonias. It is not specific for VAP and is not designed for diagnosing

pneumonia, however this has not stopped it being used for this purpose (Horan, Andrus and Dudeck, 2008). Assessment of the CDC definition of VAP has reported an acceptable sensitivity of 84% but a poor specificity of 69% (Miller *et al.*, 2006).

The Johanson criteria were devised in 1972, working off the presence of or absence of new or progressive infiltrates on chest x-ray (Johanson *et al.*, 1972). Radiography results are considered in conjunction with common clinical signs: fever, leucocytosis, and changes in respiratory secretions. However this definition suffers from very poor sensitivity and specificity, as chest x-ray is difficult to interpret, leading to great observer variability (Shaw *et al*., 1990). As such, they have fallen largely out of favour as a diagnostic tool.

The Hospitals in Europe Link for Infection Control through Surveillance (HELICS) criteria are used for surveillance of VAP in Europe (Wilson, 2004). HELICS classifies categories of VAP based on the microbiological method used for identification (Stewart and Cuthbertson, 2009). Criticism of these criteria are levied at surveillance definitions failing to identify many cases where antibiotics were prescribed for a suspected case of VAP (Pugh *et al*., 2016). This is due to clinicians not performing chest x-rays in close to half of treated cases, therefore precluding them from meeting the HELICS definition of VAP (Pugh *et al*., 2016). With application of HELICS alone, many patients with a legitimate case of VAP would not be prescribed antibiotic treatment.

Recently, there has been revision of ICU surveillance methods in the US, moving away from methods with high subjectivity in interpretation, such as chest radiography (Magill *et al.*, 2013). This has led to the introduction of the Ventilator-

associated event, with a deterioration in oxygenation comprising a Ventilator-associated complication (VAC). A VAC is associated with poor clinical outcome, however a large number of different conditions would satisfy the criteria for VAC, aside from VAP (Klompas *et al.*, 2011; Pugh *et al.*, 2016). Furthermore, only a minority of VAP patients meet the diagnostic criteria for VAC, due to stringent time criteria and frequently, insufficient deterioration of oxygen (Muscedere *et al*., 2013). Consequently, many episodes of VAP are missed when applying this definition, which is again of detriment to those with legitimate VAP.

The lack of a gold-standard and universal definition for the diagnosis of VAP makes it difficult to accurately measure true VAP incidence, leads to frequent over- and under-diagnosis of VAP, increases inappropriate antibiotic use, and makes it incredibly challenging to quantify the success of new intervention strategies. Current research directions into improved diagnosis are predicated on understanding the unique events constituting a case of VAP.

1.5 | Management of VAP

1.5.1 | Antimicrobials for treatment of VAP

Antibiotics previously considered as last resort, particularly carbapenems, are common choices for empirical therapy in VAP. This is due to the risks associated with inappropriate or ineffective antibiotherapy. The selection of an appropriate empirical antibiotic can decrease mortality by as much as 38%, whilst use of an inappropriate antibiotic can increase mortality by up to 90% (Kollef *et al*., 1999; American Thoracic Society and Infectious Diseases Society of America, 2005).

Broad spectrum antibiotics are currently necessitated by severity of infection in VAP. De-escalation is possible but only following microbiological results. As no rapid or accurate diagnostic test exists, clinicians must consider other data, for example local epidemiology, prior airway cultures, and results of Gram-staining, when selecting an empirical antibiotic. Unfortunately, empirical therapy is not always of definite benefit, and no regimen is proven to be optimal for every healthcare institution (Swanson and Wells, 2013).

The recommended duration of antibiotics for hospital-acquired pneumonia (HAP) is 14-21 days. VAP is currently excluded from National Institute for Health and Care Excellence (NICE) prescribing criteria for hospital-acquired pneumonia (NICE, 2019). The optimal duration of treatment for VAP is unclear, though there is mounting support for shorter antibiotic regimens (Yu and Singh, 2004). Antibiotic de-escalation is not always feasible, as in many cases it would be of unacceptable detriment to the patient. One study found that in cases caused by a potentially MDR pathogen, only 2.7% could feasibly be de-escalated (Rello *et al*., 2004). De-escalation could ultimately result in delayed resolution of infection and worsen patient outcome, even if it would be beneficial for preventing the development of AMR.

The subject of combination therapy for treatment of VAP is controversial. It is widely recommended for empirical therapy, but due to risks of interference between antibiotics its use after acquisition of microbiology data is less popular (Bernabeu-Wittel *et al*., 2005). Further data concerning the use of combination therapy specifically for VAP is scarce.

1.5.2 | AMR

Antibiotic resistance rates are rising in all VAP pathogens. A good example of this is MRSA, its resistance to penicillins is clearly implied in the name, but MRSA is also commonly resistant to aminoglycosides, macrolides, tetracycline, chloramphenicol, and lincosamides (Nikaido, 2009). More recently, Gram-negative bacteria resistant to virtually all available antibiotics have emerged, including strains of *P. aeruginosa* and *A. baumannii* (Nikaido, 2009). Resistance can arise in numerous ways, for example alteration of drug targets by random mutation. Fluoroquinolones, which target topoisomerase IV and DNA gyrase, can become ineffectual following the mutation of these enzymes (Hooper, 2000). Mutational change of drug targets is the most frequent resistance mechanism encountered for synthetic drugs.

Some antibiotics are inactivated through more direct enzymatic means, most commonly those of natural origin. Aminoglycosides, such as streptomycin and gentamicin, are inactivated through removal of their net positive charge (Wright, 1999). β-lactam antibiotics are cleaved by β-lactamase enzymes, which become prevalent shortly following administration of penicillins. There are numerous classes of β-lactamase enzymes. In Gram-negative bacteria, the TEM variant is commonly expressed (Datta and Kontomichalou, 1965). Development of later generations of β-lactam antibiotics led to rise of mutant β-lactamases with activity against third and fourth generation cephalosporins, the extended spectrum β-lactamases (ESBL) (Jacoby and Medeiros, 1991).Carbapenems, β-lactams with a new nucleus retaining much of the clinical effectiveness, are increasingly challenged by carbapenemase enzymes (Queenan and Bush, 2007).

Resistance may also arise through direct blockage of antibiotics from their targets. Drug-specific efflux pumps expel antibiotic molecules from the bacterial cell in a proton force motive dependent manner (Tamura *et al*., 2003). The efflux systems of *P. aeruginosa* extrude antibiotics of many classes, including quinolones, macrolides, tetracycline, penicillins, and meropenem (Masuda *et al*., 2000). Some bacteria may resist the killing effect of β-lactams through development of porin-deficient phenotypes, for example some Enterobacteriaceae (Harder *et al*., 1981). Whilst porin deficiency comes at cost of nutrient influx which would compromise fitness, under great selective pressure these mutations can give pathogenic bacteria and competitive edge.

The significance of AMR in the treatment of VAP is covered in more detail in Chapter 4. This includes the effects on prescription of empirical antimicrobials, treatment failure, as well as the impact that prescriptions for VAP impact AMR in intensive care.

1.5.3 | Prevention of VAP

Numerous strategies have been implemented to try and prevent VAP in intensive care. Foremost amongst these is the “care bundle”, a package of interventions applied together to prevent disease. In 2007, the department of health launched an initiative entitled “Saving lives: reducing infection, delivering clean and safe care”, which outlined high impact interventions for VAP (Dept of Health, 2007). This included: bed head elevation; daily sedation interruption (DSI); antiseptic oral hygiene; drainage of subglottic secretions; ETT cuff pressure control; and prophylaxis for gastric ulcers (Dept of Health, 2007). These measures resulted in a

marked reduction of VAP from 31 to 12 cases per 1000 ventilator days, even without total compliance (Morris *et al*., 2011).

Elevation of the bed head to 30-45° has been recommended (Hellyer *et al*., 2016). This intervention is criticised due to low quality of evidence, and due to risks of increased aspiration of subglottic secretions due to gravity (Bassi *et al*., 2008; Póvoa *et al*., 2017). Elevation of bed head is complicated by poor compliance and lack of feasibility in some patient groups (Van Nieuwenhoven *et al*., 2006).

The evidence linking DSI alone to reductions in MV, mortality, and length of stay is poor (Burry *et al*., 2014). When combined with spontaneous breathing trials, DSI reduces days spent under MV over 28 days of ICU admission, from 14.7 to 11.6 (Girard *et al*., 2008).

Subglottic secretion drainage removes pathogens that may bypass the cuff if improperly inflated or deflated (Nseir *et al*., 2011). A number of meta-analyses on subglottic secretion drainage have confirmed its effectiveness for reducing VAP incidence (Dezfulian *et al*., 2005; Muscedere *et al*., 2011; Wang *et al*., 2012; Pozuelo-Carrascosa *et al*., 2020). Modern ETT cuffs incorporate a suction catheter to allow for drainage of secretions, and some tubes integrate automatic measurement of cuff pressure to adjust and prevent aspiration (Nseir *et al*., 2011; NICE, 2020).

Chlorhexidine has been used in the past for decontamination of the oropharynx, but this is no longer recommended by NICE or The Intensive Care Society as a preventative strategy for VAP (NICE, 2008; Hellyer *et al*., 2016). In a recent review, oral chlorhexidine was found to be potentially harmful to the patient, further supporting the decision against its use in VAP (Bouadma and Klompas, 2018).

Current prevention strategies for VAP reduce incidence, particularly when used in tandem. However, these do not translate well to direct benefit of the patient e.g., reduced duration of stay, reduced MV, or reduced mortality. Despite simplicity in their implementation, the benefit of VAP prevention strategies is difficult to determine, due to a mix of poor clinical compliance, patient heterogeneity, and poor quality of evidence for their use.

1.6 | Host-microbe interactions

Host-microbe interactions dictate how microorganisms survive and sustain themselves within their host organisms. They are critical to the persistence of these microorganisms, and underpin the pathogenesis and development of infectious disease (Proal *et al*., 2017). Despite the clear importance of HMI in the development and pathogenesis of VAP, they are very poorly understood in this context. HMI comprise a range of complex interactions between pathogenic microorganisms, the host, the extant microbiome, the immune system, and antimicrobial interventions (Figure 1.5).

Loss of diversity in the extant microbiome has been linked to the development of VAP (Zakharkina et al., 2017). The healthy lung microbiome predominantly comprises species from the Firmicutes and Bacteroidetes phyla, with the gut-lung axis believed to be important in this colonisation (Takahashi *et al*., 2018; Chellappan *et al*., 2019). Variations in the specific composition of the lung microbiome has been linked to differences in severity of lung infections such as asthma and cystic fibrosis (Hilty *et al*., 2010; Hector *et al*., 2016).

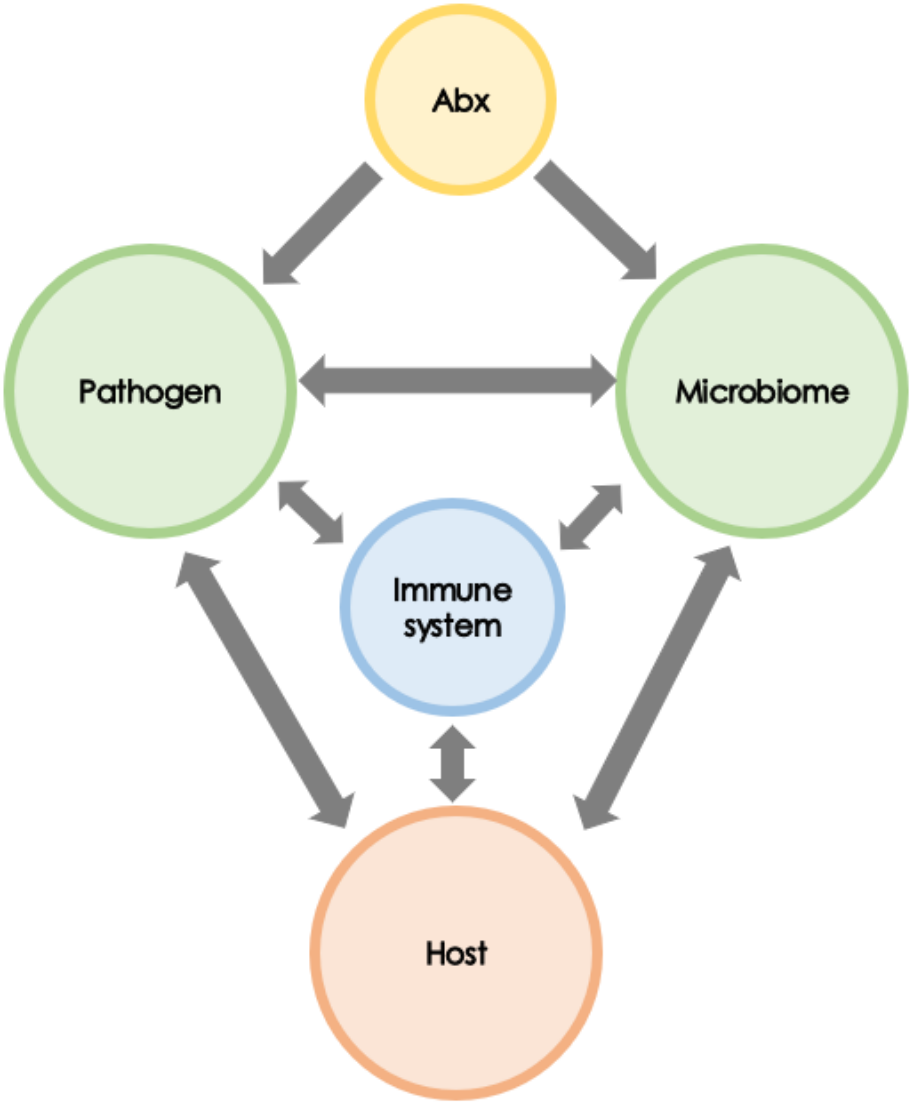


Figure 1.5: Host-microbe interactions underlying development of disease. VAP arises from interactions between numerous elements, including those of the host, pathogen, and the effects of clinical interventions. There is a lack of understanding of the mechanisms by which VAP develops, and how these interactions are involved.

Colonisation with certain species profiles can result in competition and subsequent induction of inflammatory events. The role of diversity of the microbiome is yet to be explicitly defined, but recently reduced alpha-diversity was found to be associated with improved recovery from VAP (Woo *et al*., 2020). Lower or loss of diversity has traditionally been associated with poorer health and outcome of

disease (Flight *et al*., 2015; Takahashi *et al*., 2018). The findings of Woo *et al.* (2020) demonstrate this may not be broadly applicable across different diseases.

Long durations of MV have been linked previously with gradual development of dysbiosis, and increased colonisation with pathobionts as diversity is lost (Zakharkina *et al*., 2017; Woo *et al*., 2020). The source of the infectious is not always clear however, in many cases the causative organism originates in the oropharynx, and the pathogen and microbiome should not always be considered discrete entities when considering HMI (Zakharkina *et al*., 2017; Bos and Kalil, 2019).

Constituents of the microbiome can be suppressed by numerous factors. These may be environmental, such as pollution or diet, or clinical, such as administration of antibiotics (Rylance *et al*., 2016; Hosgood *et al*., 2019; Gesell Salazar *et al*., 2020). Antibiotic use can play a role in VAP even before admission to ICU. Prior antibiotic use is a risk factor for VAP, as its use can select for pathobionts in the nasopharyngeal microbiome (Prina *et al*., 2015; Teo *et al*., 2015; Lepainteur *et al*., 2019). These can then lead to serious lower-respiratory tract infections at a later point. More research into the contribution of prior antibiotic therapy to dysbiosis, and how this can influence VAP, is required.

In gut microbiota and urinary tract infections, certain antibiotic classes have been associated with the promotion of certain bacterial species. Aminoglycosides can increase the risk of colonisation with vancomycin resistant *Enterococcus*, *A. baumannii*, *Proteus mirabilis*, and *P. aeruginosa* (Wang *et al*., 2017a). Additionally, carbapenems have been associated with promoting *P. aeruginosa* colonisation (Wang *et al*., 2017a). Whilst these findings are not in the context of lung infections,

the species and antibiotics discussed are clinically relevant in VAP. Similar if not identical phenomena may be involved in driving the development and pathogenesis of VAP and warrant further exploration.

A large proportion of VAP cases involve co-infection with multiple pathogens (Ramírez-Estrada *et al*., 2016). Microbe-microbe interactions (MMI) are therefore of clear importance in the pathogenesis of VAP. Bacteria in VAP commonly exist in biofilms, as previously discussed, and provide a unique niche in which MMI are abundant. Bacteria such as *P. aeruginosa* persist within biofilms over many generations. During this time, divergence can result in formation of cooperative metabolic pathways with other species, for example *S. aureus*. This can occur despite antagonism in prior generations of these bacteria (Michelsen *et al*., 2016). Coinfection of airway epithelium has shown reduction of IL-8 levels through the actions of *S. aureus*, which reduces burden on *P. aeruginosa* colonisation (Chekabab *et al*., 2015). Changes in the levels of markers such as IL-8 may be indicative of co-infection with these species, but this requires further study.

The composition of immune responses to VAP may be influenced by interventions. High volume ventilation settings have been demonstrated to increase levels of proinflammatory cytokines in BAL from mice models (Nickles *et al*., 2014). IL-6 is increased in patients colonised with *S. aureus* and *E. coli* following 6 hours of MV (Dhanireddy *et al*., 2006). MV has also been demonstrated to induce inflammation and lung injury in pneumonia (Dhanireddy *et al*., 2006).

1.7 | Case for the study

VAP is not a simple case of infection with a single pathogen causing pneumonia. It is a consequence of disruptions to many or all of the bacteriome, virome, mycobiome, host, and immune response. The myriad interactions underpinning VAP development and progression remain unclear and render it difficult to investigate and understand. Regardless, extensive further study is necessary to better improve understanding, improve diagnostics, treatment, and outcomes of these infections.

VAP remains highly problematic within ICU, occurring in in 10-20% of MV patients in the NHS, and costing £10000-20000 per episode (NHS England, 2016). The infected patient is at substantial risk of death, with the mortality rate in the NHS estimated at 30%. The prompt diagnosis and treatment of VAP is complicated by inconsistencies in definitions, which vary between different institutions. Without a gold-standard definition, the selection and timely administration of an appropriate antibiotherapy is a frequent challenge. Management of VAP is further limited by lack of knowledge surrounding the HMI and MMI involved. Much existing work is focused on the microbiological or physiological elements of the illness in isolation.

There is little existing research on the role of HMI in VAP. Much existing work is modelling in rodents, selected for the small size, high reproductive rate, and ease of use (Pulido *et al*., 2017). These models are disadvantageous for the study of VAP. They differ significantly anatomically and physiologically from humans, and have markedly different immune compositions, such as PAMPS, and lack a direct IL-8 homolog (Baron *et al*., 2012). The use of live pig models for study of spontaneous and induced VAP has increased in popularity. This includes the use

of anaesthetised piglets, and carries significant ethical concerns (Luna *et al*., 2009).

Due to this lack of meaningful data in literature, this project begun by utilising high-throughput *in vitro* study to identify potentially relevant HMI in VAP. Broth microdilution assay is commonly and routinely performed for the identification of minimum concentrations of antibiotic required to inhibit bacterial growth. This study modifies broth microdilution to incorporate recombinant human cytokines of interest and investigate changes in bacterial sensitivity. Previous literature has demonstrated that the presence of cytokine can influence bacterial growth rates, and this is explored in more detail in Chapter 3 (Meduri *et al*., 1999). This study aims to identify if other phenotypic characteristics, namely antimicrobial sensitivity, rate of antibiotic adaptation, and biofilm formation, can be modified in a similar manner. Results observed *in vitro* will assist in the selection of interactions to model *ex vivo*. This method is limited by its heterogeneity to the human lung, with the effect of single cytokines in isolation being tested against a single bacterial species in the presence of a single antibiotic. In reality, a myriad of host factors would be present during infection, infection may be polymicrobial, and the treatment used may be in combination. The advantage of testing *in vitro* is its high-throughput nature and ease of use, allowing the identification of HMI of interest from a wider panel of tested interactions.

This project used the *Galleria mellonella* insect model for investigation of bacterial virulence, and as a novel model for studying *in vivo* adaptation to antibiotics (Glavis-Bloom *et al*., 2012; Tsai *et al*., 2016). This model is advantaged over other models such as *Caenorhabditis elegans* and *Drosophila melanogaster* as *G. mellonella* will survive at 37°C and allows for great control over timing and density

of inocula. *G. mellonella* have a rudimentary yet robust innate immune system which produces responses similar to mouse pneumonia models, whilst being much easier to handle. Unlike rodent models, this model does not require ethical approval, and the larvae are sold commercially as bait food for fishing or for feeding pets (Insua *et al*., 2013; Tsai *et al*., 2016). The use of *G. mellonella* in this project is based upon rapid acquisition of data and cost-effectiveness.

1.7.1 | Aims and objectives

The overall aims of this thesis were to begin to explore and define the host-microbe interactions in VAP. In this thesis this will take the form of investigating proinflammatory cytokines IL-1β, IL-6, and TNFα, and how they may influence phenotypic change in common VAP bacteria: *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*. This will be investigated through a mixture of high-throughput *in vitro* models, before applying findings to an *in vivo* insect model of infection.

In vitro modified broth microdilution assay will be used for high-throughput investigation of cytokine effects on phenotypic traits of VAP bacteria. The aim of these experiments is to identify which HMI elicit positive changes in these characteristics, so that they can be adapted to more appropriate models. Changes in growth, antimicrobial sensitivity, and biofilm productivity will be tested when exposed to TNFα, IL-6 and IL-1β. The hypothesis is that exposure to proinflammatory cytokines will increase rates of growth, decrease susceptibility to antimicrobials, and modify biofilm formation.

Larvae of *G. mellonella* will be used to investigate the virulence of VAP bacteria, and to model cytokine effects on bacterial adaptation to antibiotics *in vivo*. The aim

of these experiments is to determine the virulence of VAP isolates, and to determine if cytokines can modify rate of bacterial adaptation.

The work undertaken for this project will serve as initial preliminary work for a larger multidisciplinary project investigating HMI in the context of VAP. This larger project will use the work in this thesis as a foundation as it explores more complex mammalian *in vivo* models to study HMI in the context of VAP. Before looking to recruit VAP patients and investigate their infections using methods and models developed throughout. Ethical approval has been obtained for this larger study. The section of work detailed in this thesis has not involved the recruitment of any patients for the direct acquisition of samples.

Chapter 2 | Methods

2.1 | Acquisition, culture, and identification of bacterial isolates

Bacterial samples were isolated from VAP patients at The Royal Liverpool University Hospital. These samples were sub-cultured and grown, and colonies of *Staphylococcus aureus*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* were picked from these and grown into pure culture. Pure cultures were then kindly donated to us for use in this study. These species are all commonly implicated in VAP and are easy to culture and use for experimentation.

For virulence testing of bacterial isolates, PA01 a common reference strain of *P. aeruginosa* was also used. A frozen stock of this reference strain was donated from another research group within the University of Salford.

The bacterial isolates used in this project are pure bacterial cultures. They have no patient information or patient identification. They contain no patient sample of any kind. They contain no patient cells and contain no patient DNA. Results from the experimental use of these isolates are not being used to inform the direct clinical intervention or treatment of the patients from which they originated. The use of these isolates is for the preliminary establishment of models and experiments for use as part of a larger project. As such, their usage as part of this project is not ethically contentious. Full ethical clearance was obtained from the University of Salford to reflect this, under application ID:2307. Evidence of ethical clearance is included in the front matter.

Throughout the study, bacterial stocks were stored at -80°C in Luria-Bertani (LB) broth (Oxoid, UK) with 50% glycerol (Sigma, UK). Growth of organisms from stock cultures, viable counts and antimicrobial susceptibility tests were all performed on

Mueller-Hinton agar (Oxoid, UK) as growth of all samples was permissible using this media. Liquid cultures were prepared in LB broth (Oxoid, UK).

FASTA files of sequence data for these strains, which were generated externally to this project, identified the *P. aeruginosa* as strain SE5369. *S. aureus* was identified as GHA11. *K. pneumoniae* was identified as ABFPV. All of these identifications represent best matches after BLAST search of sequence files.

2.2 | Preparation of media

The preparation processes for media used throughout the study are described in the following subsections.

2.2.1 | Non-specialist media

Mueller-Hinton agar (MHA) (Oxoid, UK) was prepared by adding 38g of MHA powder to 1L of distilled water. This was then sterilised by autoclave at 121°C for 15 minutes. Molten agar was cooled to around 50°C before pouring into non-vented, 90x16mm petri dishes (VWR international, USA) in proximity to a flame. Agar was left to set overnight before storing, inverted, at 4°C.

Lysogeny broth (LB) (Oxoid, UK) was prepared by adding 21g LB powder to 1L of distilled water. Broth was then sterilised by autoclave at 121°C for 15 minutes. This was then allowed to cool and stored benchtop at room temperature.

2.2.2 | M9 broth

M9 broth was prepared by adding 10.5g of M9 broth powder (Sigma, UK) to 1L of distilled water. This was then sterilised by autoclave at 121°C for 15 minutes. A 1M solution of $MgSO_4$ (VWR international, USA) was prepared in distilled water. 20mL of 20% glucose (Sigma, UK) and 2mL of 1M $MgSO_4$ were then sterilised by vacuum filtration and added to the autoclaved M9 broth to supplement.

2.2.3 | Minimal media for *S. aureus*

M9 broth required further supplementation to permit the growth of *S. aureus*. This was prepared in 1L distilled water with 10.5g M9 broth powder, 2mM $MgSO_4$, 0.1mM $CaCl_2$ (VWR international, USA), 1% glucose (Sigma, UK), 1% casein hydrolysate (Sigma, UK), 1mM Thiamine-HCl (Sigma, UK), and 0.05mM nicotinamide (Sigma, UK) (Reed *et al*., 2015). M9 broth powder was sterilised by autoclaving at 121°C for 15 minutes, other supplements were sterilised by vacuum filtration.

2.3 | Antimicrobial testing by disc diffusion assay

The antimicrobial sensitivity profile of each isolate was tested by disc diffusion assay. Overnight cultures of *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* were prepared by streaking onto Mueller-Hinton agar (Oxoid, UK) from glycerol stocks. These cultures were incubated for 18 hours aerobically at 37°C until single colonies were visible. Bacterial suspensions were prepared from these colonies in phosphate buffered saline (PBS) (SLS, UK) to the density of a 0.5 McFarland turbidity standard. Each suspension (100µL) was spread onto MH agar plates, to

which antimicrobial discs (Oxoid, UK) were then firmly applied. Selected antibiotics were: ampicillin-sulbactam (20µg); piperacillin-tazobactam (110µg); oxacillin (1µg); vancomycin (30µg); cefoxitin (30µg); meropenem (10µg); levofloxacin (5µg); ciprofloxacin (5µg); doxycycline (30µg); tetracycline (30µg); linezolid (10µg); trimethoprim-sulfamethoxazole (25µg); cefuroxime (30µg); penicillin G (1iu); amoxicillin-clavulanic acid (3µg); cefotaxime (5µg); clarithromycin (15µg); and cefpodoxime (10µg).

Plates were then incubated aerobically at 37°C within 15 minutes of disc application for 18 hours, in accordance with EUCAST guidelines (Matuschek *et al*., 2014). Growth was checked for uniformity, visible lawns of bacterial growth with uniform circular inhibition surrounding the antibiotic-impregnated disc. Plates were read and measured by eye using a ruler, rounding to the nearest millimetre, and performed in triplicate with average measurement of zone of inhibition taken. These readings were compared against EUCAST sensitivity breakpoint tables to determine sensitivity profiles, graded as susceptible or resistant (European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0, 2020).

2.4 | Cytokine effects on bacterial growth *in vitro*

The simple effects of the cytokines TNFα, IL-6 and IL-1β on planktonic and biofilm growth of VAP isolates was tested *in vitro*. This was performed by adding different concentrations of these cytokines to broth cultures of *S. aureus, K. pneumoniae*, and *P. aeruginosa*.

2.4.1 | Changes in planktonic growth

Overnight broth cultures of *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* were prepared in minimal media. M9 minimal medium broth (Sigma, USA) was used for *K. pneumoniae* and *P. aeruginosa*. A modified M9 broth was used to support growth of *S. aureus* (Reed *et al*., 2015). M9 minimal medium contains glucose as the single carbon source (added separately after filter sterilisation), and was selected to reduce the potential for interactions between the cytokines and the nutrient composition of richer media (Ehrenberg *et al*., 2013). For example, if ionic charge carried on cytokine molecules would result in attachment to components of media.

Broth cultures were washed and resuspended in M9 broth (modified for *S. aureus*), and then diluted to an optical density at 600nm (OD_{600}) of 0.1. Cytokines (Peprotech, UK) were reconstituted in sdH_2O to 100µg/mL. These were diluted to working concentrations representing physiologically normal, elevated, and very elevated concentrations of cytokines in VAP, shown in Table 2.1 (Hellyer *et al*., 2015). Physiological normal concentrations aim to reflect those encountered in a state of health. Elevated and very elevated concentrations reflect those under different levels of inflammation. The concentrations selected are not fully representative of those encountered in the human host, due to variations in immune response between individuals. They have been selected to represent a manageable panel approximate values for the high-throughput nature of the methods used throughout this project.

Each concentration of cytokine was added to broth cultures of bacterial isolates, in triplicate, in a 96-well plate. These were then incubated for 18 hours aerobically at 37°C.

Table 2.1: Concentrations of TNFα, IL-6, and IL-1β used for study of cytokine effects on bacteria *in vitro*.			
Cytokine	Physiologically normal	Elevated	Very elevated
TNFα	30pg/mL	300pg/mL	3000pg/mL
IL-6	100pg/mL	500pg/mL	1000pg/mL
IL-1β	30pg/mL	300pg/mL	3000pg/mL

Following incubation, negative control wells were checked for absence of growth, and the OD_{600} of planktonic bacterial growth was recorded using a FLUOstar omega microplate reader (BMG Labtech, UK). This measurement indicated the density of the planktonic phase which were used for growth comparisons between different cytokine concentrations.

2.4.2 | Changes in biofilm growth

Cytokine-induced changes in biofilm formation were investigated by crystal violet staining. 96-well plates (VWR international, USA) used for study of cytokine effects on *in vitro* planktonic growth were retained following measurement of planktonic phase. Crystal violet biofilm assays were performed on these plates to determine changes in biofilm formation with the addition of differing concentrations of cytokines IL-6, IL-1β and TNFα (O'Toole, 2011).

Following measurement of OD_{600}, the planktonic phase of 96-well plates from the *in vitro* planktonic growth assay was aspirated and discarded. Following which plates were washed four times in PBS to remove loosely adhering cells and M9 broth. 0.1% crystal violet (125µL) (Sigma, USA) was added to each well in all 96-

well plates. Plates were then incubated at room temperature for 10 minutes, to allow for uptake of stain by biofilm material adhering to the walls of the test wells. Following incubation, plates were washed again in PBS, repeated four times. Plates were then vigorously blotted onto paper towels to remove excess stain, PBS, and loosely adhering bacteria. Plates were left inverted to dry overnight, and then stored at room temperature until reading.

Prior to reading, crystal violet stain was solubilised in 95% ethanol (200µL). Crystal violet absorbance was read at 570nm using a FLUOstar omega microplate reader (BMG Labtech, UK). The mean absorbance of negative control wells was averaged and subtracted from each well in the plate. These wells were not inoculated with bacteria during the *in vitro* planktonic growth assay, and thus were expected to contain no biofilm. Relative biofilm forming units (BFU), the biofilm productivity of each culture on a per plankton basis, was calculated using the following equation:

$$Relative\ biofilm\ forming\ units = \frac{\text{Crystal violet absorbance (nm)}}{\text{Planktonic } OD_{600}}$$

2.5 | Cytokine effects on bacterial sensitivity by modified broth microdilution assay

2.5.1 | Changes in *Pseudomonas aeruginosa* sensitivity

The effects of the cytokines TNFα, IL-6 and IL-1β on antimicrobial sensitivity of *P. aeruginosa* were investigated using a modified broth microdilution assay. These cytokines have all been demonstrated to have potential effects on bacterial growth (Meduri *et al*., 1999). The assay was performed in M9 minimal medium broth.

The antibiotics selected were levofloxacin (LVX) (Alfa Aesar, USA), meropenem (MEM) (Sigma, USA), and piperacillin-tazobactam (TZP) (Sigma, USA). These antibiotics were selected as disc diffusion assay had demonstrated that all bacterial isolates were sensitive to them. This panel of antibiotics was later expanded to include imipenem (IPM) (Sigma, USA). 15mL stock solutions of these antibiotics were prepared to a concentration of 1.28mg mL^{-1} in M9 broth. TZP was prepared in an 8:1 ratio of piperacillin to tazobactam. Imipenem stocks were prepared to the same concentration of 1.28mg/mL but in a smaller volume of 5mL M9 broth, as they store poorly long term.

Antibiotic stock solutions were diluted to working concentrations and transferred to a 96-well flat-bottomed microtiter plate (VWR international, USA). Serial twofold dilutions were prepared in accordance with the assay template (Figure 2.1). The highest concentrations tested for each antibiotic were as follows: 8µg/mL LVX; 32µg/mL TZP; 2µg/mL MEM; and 4µg/mL IPM. These concentrations were informed by EUCAST minimum inhibitory concentration (MIC) breakpoint tables.

Cytokine aliquots were prepared to three times the concentrations in Table 2.1, as they were threefold diluted when added to test wells containing antibiotic and inoculum.

A 1.5 x 10^6 CFU/mL inoculum of *P. aeruginosa* was prepared using an overnight culture, which was suspended and diluted to a 0.5 McFarland turbidity standard, and then diluting in M9 broth by a factor of 100. When added to the 96-well plate this would be threefold diluted upon mixing with the previously added cytokine and antibiotic, to an inoculum of approximately 5x10^5 CFU/mL. Antibiotic dilutions, cytokine and inoculum were combined in equal parts (33µL) in the 96-well plate, to

a total volume of 99µL per well. Each concentration of cytokine was tested in duplicate according to the assay template. 99µL of sterile M9 broth was used as a negative control, and 33µL of *P. aeruginosa* inoculum in 66µL of M9 broth was used as a positive control. The first column of the 96-well plate contained no antibiotic and served as a cytokine-only control. If bacterial growth was found to be suppressed in this column, it would thus indicate some antimicrobial property of the added cytokine, or potential spill-over of antibiotic into these wells.

Plates were incubated under constant agitation at 100rpm aerobically at 37°C for 18 hours, using a Stuart shaking incubator SI500 (SLS, UK). Following incubation, the OD_{600} of planktonic bacterial growth was recorded using a FLUOstar omega microplate reader (BMG Labtech, UK). Readings of planktonic OD could then be used to determine bacterial MICs, as well as to investigate cytokine induced changes in planktonic growth. Plates were retained after reading for investigation of cytokine effects on *P. aeruginosa* biofilm formation, by crystal violet absorbance assay.

	No Antibiotic	Serial twofold antibiotic dilutions →								Negative	Positive
No cytokine											
Normal cytokine											
Elevated cytokine											
V. elevated cytokine											

Figure 2.1: Template for modified broth microdilution assay. This was used for investigation of cytokine effects on antimicrobial sensitivity of bacteria. Each cytokine concentration was tested in duplicate.

The negative control wells were checked for contamination by eye and by raw optical density readings. Wells contaminated with *P. aeruginosa* would appear cloudy and display the characteristic green pigmentation of this species. Assuming no contamination, the mean optical density of the negative control wells was subtracted from each well in the plate. If contaminated, the experiment was terminated and repeated. MICs were identified by determining wells at the lowest antibiotic concentrations displaying bacterial growth. The MICs was then compared between different cytokines and concentrations of those cytokines used. The measurements of optical density taken in those wells containing bacteria were also then used to study effects of cytokines on bacterial growth, in different concentrations of sublethal antibiotic. Further to this, the effects of

sublethal antibiotic were then measured, using the crystal violet biofilm staining assay described prior.

2.5.2 | Changes in *Staphylococcus aureus* sensitivity

Cytokine-mediated changes in *S. aureus* sensitivity were measured using the same method used for *P. aeruginosa*. However, the cytokine-antibiotic combination used was informed by the results of the *P. aeruginosa* study. As such, only meropenem was selected as the antibiotic, and only the effects of TNFα were investigated. TNFα was used at the same concentrations as for *P. aeruginosa*, according to Table 2.1.

The minimal broth media also differed in composition. The preparation of minimal media for *S. aureus* is outlined prior, in accordance with Reed *et al.* (2015). Following setup and incubation of test plates, planktonic and crystal violet absorbance readings were taken

2.6 | Influence of TNFα on adaptation of *P. aeruginosa* to meropenem *in vitro*

The effects of different concentrations of TNFα on the rate of adaptation of *P. aeruginosa*, *S. aureus*, and *K. pneumoniae* to meropenem was tested by disc diffusion over a series of passages. TNFα was prior observed to modify *P. aeruginosa* sensitivity to meropenem, as described later in Chapter 3, and the aim of this experiment was to see if there was impact on rate of adaptation.

Frozen cytokine stocks (Peprotech, UK) were diluted to physiologically normal, elevated, and very elevated working concentrations, as for prior susceptibility testing. Working concentrations were added in triplicate to the wells of two 6-well plates (Sarstedt, Germany). Three wells in one plate contained no cytokine to serve as cytokine free controls. MH agar was prepared as prior described, and after sufficient cooling time so as not to denature the TNFα, 2mL was added to each well in the 6-well plates. These agar beds incorporating different TNFα concentrations were then allowed to set.

Overnight cultures of bacterial isolates were suspended to a 0.5 MacFarland turbidity standard in PBS (SLS, UK). Each agar bed was then inoculated using a sterile cotton swab, dipped in the suspension, swabbed in three directions to ensure complete surface coverage. 10µg meropenem discs (Oxoid, UK) were then firmly applied to the surface of each agar bed. Meropenem discs were placed towards the edge of each well, as a precaution against large inhibition zones covering the entire surface of the well. Plates were then incubated aerobically at 37°C for 18 hours, in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (Matuschek *et al*., 2014). Growth was checked for uniformity, visible colonies of bacterial growth with uniform circular inhibition surrounding the meropenem disc. Each zone of inhibition was read and measured to the nearest millimetre. As discs had been offset to the edges of each well, measurements were taken of the radii and doubled. Mean averages of each triplicate set of wells were then calculated.

Colonies from the very edge of the zones of inhibition were then passaged onto new agar beds incorporating TNFα, prepared as before, and the experiment was repeated. Sub-passages were repeated until complete adaptation or until 15 sub-

passages had been performed. Frozen stocks of each passage were collected and retained at -80°C for further investigation if required.

2.7 | *Galleria mellonella* virulence assay

Virulence of VAP isolates was determined by infecting larvae of the greater wax moth, *Galleria mellonella* (Latimer *et al*., 2012). This was performed to establish the suitability of this model for investigation of virulence in VAP isolates.

G. mellonella larvae (Livefoods Direct Inc., Sheffield, UK) in the fifth instar stage were taken from storage at 4°C and incubated at 37°C for 30 minutes prior to inoculation. 16 larvae were allocated to each of five inoculation groups: *S. aureus*; *K. pneumoniae*; *P. aeruginosa*; mock inoculation (PBS); and inoculation negative. Overnight cultures of *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* were washed twice in PBS to remove metabolic products potentially toxic to *G. mellonella* larvae (Jønsson *et al*., 2017). Cultures were then diluted in PBS to an optical density of 0.1 at 600nm, corresponding to approximately $1x10^8$ colony forming units per millilitre (CFU/mL) confirmed by viable count on Mueller-Hinton agar.

5µL of each inoculum was injected into each larva using a 10µL Hamilton syringe and 27-gauge hypodermic needles (Sigma, USA), corresponding to an inoculum around $5x10^5$ CFU/mL. Injection was performed via the most common infection route, intrahaemocoelic injection though the last left pro-leg (Tsai *et al*., 2016). The PBS group were injected with 5µL of sterile PBS, to check larval death was a true effect of infection and not due to trauma from injection. The negative control group were not injected.

Larvae were incubated at 37°C in plastic petri dishes (VWR international, USA) and observed every 24 hours. The number of surviving larvae was recorded. The progression of bacterial infection could be tracked by the gradual melanisation of the larvae, dead larvae appear black in colour and are unresponsive to touch (Latimer *et al*., 2012). The experiment was terminated following two cumulative deaths in either the PBS or negative control group. Remaining surviving larvae were frozen at -80°C to kill them and then all larvae were disposed of by decontamination with chemgene.

2.7.1 | Adaptation of *G. mellonella* assay for *P. aeruginosa*

Due to the virulent nature of *P. aeruginosa* in *G. mellonella*, inoculation with an equivalent number of cells to *S. aureus* and *K. pneumoniae* was unsuitable. In the literature, *P. aeruginosa* is cited as having an LD50 in *G. mellonella* as low as 10 cells (Jander *et al*., 2000). Modifications to the methodology were explored for the investigation of *P. aeruginosa* virulence.

P. aeruginosa was diluted to give final inoculum doses of 1 CFU; 5 CFU; 10 CFU; and 50 CFU. These were injected into *G. mellonella* and survival was checked every 24 hours until total larval death or 2 cumulative deaths in either control group.

The virulence of the VAP isolate of *P. aeruginosa* was also recorded alongside the reference strain PA01. Timepoints at which surviving larvae were recorded were modified. Following inoculation with *P. aeruginosa* and incubation at 37°C, larvae were checked every 30 minutes, as opposed to once daily. The experiment was terminated following the death of all 16 larvae in the *P. aeruginosa* group and

PA01 group or following two cumulative deaths in the PBS or negative control group. Larvae were disposed of as before, by freezing and decontamination with chemgene.

2.8 | TNFα impact on adaptation of *S. aureus* to meropenem in an *in vivo* insect model

In vitro adaptation of *S. aureus* to meropenem, and the effects of TNFα on this, were adapted to an *in vivo* insect model, using *G. mellonella*. These larvae have been used in the past for testing efficacy of antimicrobials, as well as for screening novel antimicrobial compounds (Thomas *et al*., 2013; Cools *et al*., 2019; Cutuli *et al*., 2019). To our knowledge, *G. mellonella* have yet to be utilised as a model for *in vivo* adaptation of bacteria to an antimicrobial compound.

2.8.1 | *G. mellonella* dose responses to TNFα, meropenem, and *S. aureus*

To design the method for an *in vivo* adaptation, the dosages of TNFα, meropenem and *S. aureus* tolerable to the larvae of *G. mellonella* required identification. The MIC of meropenem against *S. aureus in vitro* was prior determined to be between 0.0625μg/mL and 0.125μg/mL. Groups of 10 larvae in the fifth instar stage were taken from storage at 4°C and inoculated with meropenem (5μL). The concentrations of meropenem were in doubling dilutions ranging from 1μg/g to 0.0625μg/g of total larval mass (~230mg). Inoculation was via the last left pro-leg, consistent with previous methods. The range of meropenem concentrations was selected to identify not just the maximum tolerable dose of meropenem, but also as the MIC against *S. aureus* may differ when *in vivo*.

For TNFα, larvae were inoculated with 5µg of suspensions, made to a final concentration of 30pg/g, 300pg/g, and 3000pg/g of total larval mass. These concentrations are in line with those used in prior methods, to represent increasing physiological concentrations of TNFα.

Separate groups of larvae were also injected with a range of *S. aureus* dilutions. Prior results demonstrated the time to reach 50% mortality with a *S. aureus* suspension of 5-8 x 10^5 was 8 days. As passages would be daily, a greater dosage of *S. aureus* could potentially be used, if required to adapt in the presence of meropenem. Larvae were split into groups of 10 and inoculated with suspensions with OD_{600} of 0.1; 0.2; 0.4; 0.6; 0.8; and 1.0 (5µL). In CFU/mL, this relates to doses of 5-8 x 10^5; 1-1.6 x 10^6; 2-3.2 x 10^6; 3-4.8 x 10^6; 4-6.4 x 10^6; and 5-8 x 10^6, respectively. Handling of larvae and inoculation site was consistent with previous methods.

Once each test group was inoculated with corresponding antibiotic, cytokine, or bacteria, larvae were incubated in plastic petri dishes at 37°C and survival was tracked daily at 24h intervals. A mock inoculation group (PBS) and inoculation free group were included, to control for death due to trauma or otherwise. Readings were terminated following 2 cumulative deaths in either control group. Following termination of experiment, larvae were disposed by freezing at -80°C and then bathing in chemgene.

2.8.2 | Passage of *S. aureus* in *G. mellonella* haemolymph

G. mellonella in the fifth instar stage were taken from storage and incubated at 37°C for 30 minutes prior to inoculation, as before. Groups of 10 larvae were

selected for each test condition: inoculation negative; mock inoculation (PBS); TNFα-free; 30pg/g TNFα; 300pg/g TNFα; 3000pg/g TNFα. Larvae were selected with a body mass of 207-253mg (230mg ± 10%). Overnight culture of *S. aureus* was prepared to a density of 5-8 x 10^5 CFU/g.

Each larva was injected with 5µL of their respective concentration of TNFα, via the last left pro-leg. Following this, all larvae minus controls were injected with *S. aureus* (5µL) via the same route. Larvae were then incubated for 2 hours at 37°C to allow the bacteria time to establish infection. Then, all test larvae were inoculated with 0.25µg/g meropenem (5µL). All larvae were then incubated for a further 22 hours at 37°C.

Following incubation larvae were collected and frozen at -80°C. The bottom 2-3mm of each larva was then removed aseptically with dissecting scissors, which were dipped in ethanol and flamed to sterilise between larvae. Only live and visibly non-melanised larvae were selected for this process, to avoid reinjection of potentially necrotic larval tissues that could influence rate of death. Each larva was placed into a sterile 1.5mL Eppendorf tube, and haemolymph was left to drain for 30 minutes at 4°C. Larval remains were then removed and bathed in chemgene prior to disposal. Collected haemolymph was centrifuged at 8000G for 1 minute.

The inoculation process was then repeated on healthy larvae as before, but with collected haemolymph used in place of a *S. aureus* suspension. Following inoculation with TNFα and haemolymph containing *S. aureus*, larvae were incubated for 2 hours at 37°C. Larvae were then injected with 0.25µg/g meropenem (5µL) before subsequent incubation at 37°C for 22 hours. Passages

were repeated 15 times, until total larval death in test groups, or until 2 deaths were seen in a control group.

Remaining haemolymph (5µL) was spread onto the surface of MH agar set into 6 well plates. A 10µg meropenem impregnated disc was then firmly placed onto the surface of the agar. All plates were incubated aerobically at 37° C for 18 hours. Spread plates with meropenem discs were used to track *S. aureus* adaptation to meropenem throughout the experiment.

2.9 | Statistical analysis

GraphPad Prism 9 was used for the analysis of data. Graphs were prepared using GraphPad Prism 9. Results are presented as means ± standard deviation. GraphPad Prism 9 was used for all statistical testing, using one-way and two-way analysis of variance (ANOVA) with Tukey correction for multiple comparisons. Results were considered significant at $p < 0.05$.

Chapter 3 | Impact of HMI on growth and biofilm production in VAP isolates

3.1 | Introduction

There is a current lack of understanding around the role of HMI in VAP. There is evidence that sensing of cytokines by bacteria can result in enhancements in growth. However, it remains less certain the effect of human inflammatory cytokines may have on the expression of other phenotypic traits of bacteria relevant in VAP. Factors such as AMR, expression of virulence factors, and formation of biofilms all influence patient outcomes, and are possibly regulated by the host immune response to infection in VAP.

A paucity of evidence exists suggesting how bacteria may respond to sensing host immune mediators. From a panel of IL-10, IL-12, IL-8, TNFα, and IFNγ (3µg/mL), Mahdavi *et al.* (2013) demonstrated TNFα and IL-8 binding to surface pili of *Neisseria meningitidis*. This was followed by dose-dependent upregulation of genes coding for adhesion, metabolism, binding proteins, cell envelope, survival, and amino acid synthesis. All of these genes are assumed to regulate virulence under inflammatory stimulus. The authors also concluded that changes in surface proteins and surface structure suggest extensive remodelling of the cell membrane when exposed to cytokine. Mahdavi *et al.* (2013) also described *in vivo* TNFα DNA binding activity, the mechanisms of which could potentially explain changes in bacterial phenotype, if delineated. This study did not include IL-1β or IL-6 as part of their cytokine panel, which along with TNFα are shown to be significantly elevated in nonsurvivors of ARDS patients with VAP, compared to survivors (Meduri *et al*., 1995).

Further to this, prior studies have been discussed in which proinflammatory cytokines may influence the growth and proliferation of bacteria. Porat *et al.* (1991) and Denis *et al.* (1991) both describe proliferation of virulent *E. coli* following binding to IL-1β (1, 10, 100ng/mL), IL-2 (10, 20, 100µg/mL), and GM-CSF (100µg/mL). This increase in growth was observed in both nutritionally rich and minimal growth mediums, brain heart infusion broth and RPMI respectively. It is therefore unlikely that IL-1β modulates bacterial growth through action as a nutrient. Interestingly, Porat *et al.* found no growth modulating effect of TNFα in virulent *Escherichia coli*. This may suggest that certain cytokine-bacteria interactions have different underlying mechanisms. TNFα has been described to have these effects in other species.

Meduri *et al.* had performed similar studies. In 1999, they demonstrated that proliferation of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* increase in a cytokine dose-dependent manner. The three cytokines utilised by Meduri *et al.* were IL-1β, IL-6, and TNFα (10pg, 50pg, 100pg, 500pg, 1ng, 10ng/mL). These are also the cytokines selected for use in our study. In minimal media (RPMI), they found *S. aureus* growth enhanced in IL-1β, *P. aeruginosa* growth enhanced in IL-6, and *Acinetobacter* spp. growth upregulated in IL-1β. In a richer medium, *S. aureus* growth was enhanced in all three tested cytokines (Meduri *et al*., 1999). The mechanisms of action in this paper were merely speculated at, however. At the time, it was suggested that cytokines may be internalised and used as growth factors, either whole or following cleavage into smaller active fragments. As we know now, it may be the ability of cytokine to act as modulators of transcription (Mahdavi *et al*., 2013).

There is a similar small evidence base for the role of anti-inflammatory cytokines in promoting growth of bacteria. IL-4 was demonstrated to promote *S. aureus* growth, as IL-4 knockout mice have reduced frequency of *S. aureus* septic arthritis (Hultgren *et al*., 1998). Hultgren *et al.* also demonstrated reduced intracellular killing of *S. aureus* by macrophages exposed to IL-4. IL-4 knockout mice had reduced bacterial loads in joints, the liver, and the kidneys. There is some debate around a mechanism, whether IL-4 directly enhances growth of *S. aureus* or decreases its clearance, or both.

IL-10 was demonstrated to enhance growth of *Legionella pneumophila* within monocytes 100-fold (Park and Skerrett, 1996). Furthermore, IL-10 blocked protective effects of IFN-γ and TNFα. IL-1 receptor antagonist (IL-1ra) was demonstrated to increase extracellular *S. aureus* growth in a dose-dependent manner (Kanangat *et al*., 2001). Meduri *et al.* (1999) had already demonstrated *S. aureus* growth enhancement by IL-1β, and this utilisation of the anti-inflammatory component may represent the ability of bacteria to respond and utilise their microenvironment depending on stage of infection.

There is, therefore, a small but dated evidence base for cytokines impacting bacterial growth, and virulence. Together, these findings suggest that upregulated or unregulated inflammatory responses may enhance growth or virulence of bacteria in disease, to the patient's detriment. These early findings have, to our knowledge, seen little follow up in the years since publication but represent a potentially interesting area for improving our understanding of HMI in VAP. Following up these studies and expanding them to look at other key determinants in VAP, e.g. biofilm production may begin to reveal a clearer picture of VAP

pathogenesis. Do the effects of host immune mediators on pathogenic bacteria extend beyond changes in growth and virulence?

3.1.1 | Chapter aims

This chapter outlines the findings of early *in vitro* investigations into whether proinflammatory cytokines modulate important characteristics of commonly implicated VAP pathogens. Changes in planktonic growth and changes in relative biofilm formation were both investigated. Cytokine induced changes in planktonic growth has already been established in literature, and this work has been repeated with our VAP isolates, using the proinflammatory cytokines IL-1β, IL-6, and TNFα. Due to conditions within the VAP patient where antibiotic doses fall throughout a course of use, these characteristics were explored against a backdrop of subinhibitory antibiotics. Those selected for this purpose are commonly prescribed in VAP (Arthur et al., 2016).

IL-1β and TNFα were selected for use in this study due to their importance in initiating all immune responses to both infectious and non-infectious insults. These cytokines are highly synergistic in effect, with much overlap in the cytokine cascades triggered by their induction (Oppenheim et al., 1989). It is this omnipresence that makes them logical choices for inclusion in this study.

IL-6 is considered a key cytokine in the response to infection. It is diverse in function, such as induction of acute phase proteins, which are critical to the defence against bacterial pathogens (Gauldie et al., 1987; Hochepied et al., 2003; Simons et al., 2014). It also has numerous functions in active immunity, as a growth factor for B cells, as well as inducing other cytokines such as IL-4, IL-17,

and IL-21 through stimulation of T helper cells (Dienz and Rincon, 2009). The pleiotropic effects in immune response to bacterial infection is the reason for its inclusion in this study.

This *in vitro* work was carried out as its simple and high-throughput nature allowed for selection of which candidate inflammatory cytokines, antimicrobials, and bacterial strains could be adapted to more complex and human analogous models.

The aim of this chapter was to investigate the capability of selected proinflammatory cytokines to change total planktonic and biofilm growth of VAP isolates of *S. aureus, Klebsiella pneumonia*, and *P. aeruginosa*. This was investigated with and without subinhibitory concentrations of piperacillin-tazobactam, levofloxacin, meropenem, and imipenem. These antibiotics were selected based on clinical use, after demonstration of their efficacy against the isolates used. To address this aim, the following research questions were formulated. Can proinflammatory cytokines IL-1β, IL-6, and TNFα modify the growth of *S. aureus, K. pneumoniae*, and *P. aeruginosa* in planktonic phase or biofilm phase? What role does the addition of subinhibitory antibiotic have in changing planktonic or biofilm growth with proinflammatory cytokine?

3.2 | Antimicrobial susceptibility patterns

Antimicrobial susceptibilities of *S. aureus, P. aeruginosa,* and *K. pneumoniae* were determined in reference to the EUCAST breakpoints for testing by disc diffusion (European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0, 2020).

S. aureus was found to be sensitive to each of: ampicillin sulbactam (SAM); piperacillin-tazobactam (TZP); oxacillin (OXA); cefoxitin (FOX); meropenem (MEM); levofloxacin (LVX); ciprofloxacin (CIP); linezolid (LZD); trimethoprim-sulfamethoxazole (SXT); cefuroxime (CXM); cefotaxime (CTX); clarithromycin (CLR); and cefpodoxime (CPD) (Table 3.1). *S. aureus* was found to be resistant to each of: doxycycline (DOX); tetracycline (TET); penicillin G (BPC); and amoxicillin-clavulanic acid (AMC). As stated by EUCAST, susceptibility of *S. aureus* to vancomycin (VAN) cannot be determined using only disc diffusion (European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0, 2020).

P. aeruginosa was sensitive to each of: TZP; MEM; LVX; and CIP and resistance to each of: SAM; OXA; VAN; FOX; DOX; TET; LZD; SXT; CXM; BPC; AMC; CTX; CLR; and CPD (Table 3.2).

K. pneumoniae was sensitive to each of: TZP; FOX; MEM; LVX; CIP; SXT; CXM; CTX; and CPD. It was resistant to each of: SAM; OXA; VAN; DOX; TET; LZD; BPC; AMC; and CLR (Table 3.3).

S. aureus was shown to be the most susceptible species to the range of tested antibiotics. *P. aeruginosa* was found to be the most resistant. The most effective antibiotic in all cases was MEM, which gave average zone diameters of 40.67mm (*S. aureus*), 37.33mm (*K. pneumoniae*), and 33.33mm (*P. aeruginosa*). The only antibiotics to which all three VAP isolates were susceptible were TZP, MEM, LVX, and CIP. TZP, MEM, and LVX were selected for use in the cytokine modified MIC assays.

Table 3.1: Antimicrobial susceptibility of *S. aureus* VAP strain to 19 antibiotics. Values ± SEM.

Antibiotic	Antimicrobial disc content (µg)	Average zone diameter (mm) n=3	Susceptibility - sensitive (S) or resistant (R)
SAM	20	27.00 ± 0.58	S
TZP	110	35.33 ± 0.88	S
OXA	1	26.67 ± 0.33	S
VAN	30	20.33 ± 0.33	†
FOX	30	28.67 ± 0.33	S
MEM	10	40.67 ± 0.66	S
LVX	5	31.33 ± 0.33	S
CIP	5	26.67 ± 0.33	S
DOX	30	13.00 ± 1.0	R
TET	30	6.33 ± 3.18	R
LZD	10	34.67 ± 0.88	S
SXT	25	35.33 ± 0.66	S
CXM	30	35.00 ± 0.58	S
BPC	1iu*	20.00 ± 0	R
AMC	3	22.33 ± 0.88	R
CTX	5	21.00 ± 1.0	S
CLR	15	31.67 ± 0.33	S
CPD	10	25.33 ± 0.88	S

Diameter of zones of inhibition reported from 3 replicates and compared to EUCAST susceptibility breakpoints to determine if *S. aureus* was sensitive (S) or resistant (R). SAM – ampicillin-sulbactam; TZP – piperacillin-tazobactam; OXA – oxacillin; VAN – vancomycin; FOX – cefoxitin; MEM – meropenem; LVX – levofloxacin; CIP – ciprofloxacin; DOX – doxycycline; TET – tetracycline; LZD – linezolid; SXT – trimethoprim-sulfamethoxazole; CXM – cefuroxime; BPC – penicillin G; AMC – amoxicillin-clavulanic acid; CTX – cefotaxime; CLR – clarithromycin; CPD – cefpodoxime

*Penicillin G is measured in international units, 1iu = 0.6µg

†VAN resistance in *S. aureus* cannot be accurately determined by disc diffusion alone, according to EUCAST guidelines

Table 3.2: Antimicrobial susceptibility of *P. aeruginosa* VAP strain to 19 antibiotics. Values ± SEM.

Antibiotic	Antimicrobial disc content (µg)	Average zone diameter (mm) n=3	Susceptibility - sensitive (S) or resistant (R)
SAM	20	0.00 ± 0	R
TZP	110	32.67 ± 0.88	S
OXA	1	0.00 ± 0	R
VAN	30	0.00 ± 0	R
FOX	30	0.00 ± 0	R
MEM	10	37.33 ± 1.45	S
LVX	5	31.67 ± 0.66	S
CIP	5	36.33 ± 0.88	S
DOX	30	0.00 ± 0	R
TET	30	0.00 ± 0	R
LZD	10	0.00 ± 0	R
SXT	25	0.00 ± 0	R
CXM	30	0.00 ± 0	R
BPC	1iu*	0.00 ± 0	R
AMC	3	0.00 ± 0	R
CTX	5	11.67 ± 0.88	R
CLR	15	0.00 ± 0	R
CPD	10	0.00 ± 0	R

Diameter of zones of inhibition reported from 3 replicates and compared to EUCAST susceptibility breakpoints to determine if *P. aeruginosa* was sensitive (S) or resistant (R).

SAM – ampicillin-sulbactam; TZP – piperacillin-tazobactam; OXA – oxacillin; VAN – vancomycin; FOX – cefoxitin; MEM – meropenem; LVX – levofloxacin; CIP – ciprofloxacin; DOX – doxycycline; TET – tetracycline; LZD – linezolid; SXT – trimethoprim-sulfamethoxazole; CXM – cefuroxime; BPC – penicillin G; AMC – amoxicillin-clavulanic acid; CTX – cefotaxime; CLR – clarithromycin; CPD – cefpodoxime

Table 3.3: Antimicrobial susceptibility of *K. pneumoniae* VAP strain to 19 antibiotics. Values ± SEM.

Antibiotic	Antimicrobial disc content (µg)	Average zone diameter (mm) n=3	Susceptibility - sensitive (S) or resistant (R)
SAM	20	0.00 ± 0	R
TZP	110	25.67 ± 2.66	S
OXA	1	0.00 ± 0	R
VAN	30	0.00 ± 0	R
FOX	30	25.67 ± 2.73	S
MEM	10	33.33 ± 0.88	S
LVX	5	32.00 ± 0.58	S
CIP	5	35.67 ± 1.33	S
DOX	30	15.33 ± 0.88	R
TET	30	18.67 ± 1.86	R
LZD	10	0.00 ± 0	R
SXT	25	28.33 ± 0.33	S
CXM	30	24.33 ± 1.2	S
BPC	1iu*	0.00 ± 0	R
AMC	3	0.00 ± 0	R
CTX	5	31.67 ± 0.33	S
CLR	15	9.33 ± 0.66	R
CPD	10	31.00 ± 0.58	S

Diameter of zones of inhibition reported from 3 replicates and compared to EUCAST susceptibility breakpoints to determine if *K. pneumoniae* was sensitive (S) or resistant (R).

SAM – ampicillin-sulbactam; TZP – piperacillin-tazobactam; OXA – oxacillin; VAN – vancomycin; FOX – cefoxitin; MEM – meropenem; LVX – levofloxacin; CIP – ciprofloxacin; DOX – doxycycline; TET – tetracycline; LZD – linezolid; SXT – trimethoprim-sulfamethoxazole; CXM – cefuroxime; BPC – penicillin G; AMC – amoxicillin-clavulanic acid; CTX – cefotaxime; CLR – clarithromycin; CPD – cefpodoxime

3.3 | Cytokine-induced changes in planktonic growth of VAP isolates

Investigation of cytokine-induced changes in planktonic growth of *S. aureus, K. pneumoniae*, and *P. aeruginosa* were performed *in vitro* through addition of TNFα, IL-1β, and IL-6, at concentrations in accordance with those in Table 2.1. These effects were also investigated in *P. aeruginosa* with the further addition of subinhibitory concentrations of antibiotic, as planktonic growth measurements were taken from plates used for modified broth microdilution.

3.3.1 | Cytokine-induced changes in planktonic growth *in vitro*

The mean OD_{600} of *S. aureus* planktonic growth ranged from 0.35 to 0.47 with IL-1β; 0.3 to 0.35 with IL-6; and 0.35 to 0.49 with TNFα (Figure 3.1). There was no significant change in OD_{600} of *S. aureus* planktonic growth when cultured with IL-1β at any concentration tested [$F(3, 8) = 0.3821$, $p = 0.7688$], IL-6 [$F(3, 8) = 0.2856$, $p = 0.8346$], or TNFα [$F(3, 8) = 1.693$, $p = 0.2450$].

The mean OD_{600} of *K. pneumoniae* planktonic growth ranged from 1.61 to 1.71 with IL-1β; 1.56 to 1.81 with IL-6; and 1.52 to 1.77 with TNFα. There is a significant decrease in OD_{600} of *K. pneumoniae* planktonic growth between 500pg/mL and 1000pg/mL IL-6, a difference of 14% [$F(3, 8) = 5.420$, $p = 0.0250$]. There are also significant differences in OD_{600} of *K. pneumoniae* planktonic growth between 30pg/mL and 3000pg/mL TNFα (14%), and 300pg/mL and 3000pg/mL TNFα (11%) [$F(3, 8) = 8.078$, $p = 0.0084$]. There was no significant change in OD_{600} of *K. pneumoniae* planktonic growth when cultured with any concentration of IL-1β [$F(3, 8) = 0.7887$, $p = 0.5333$].

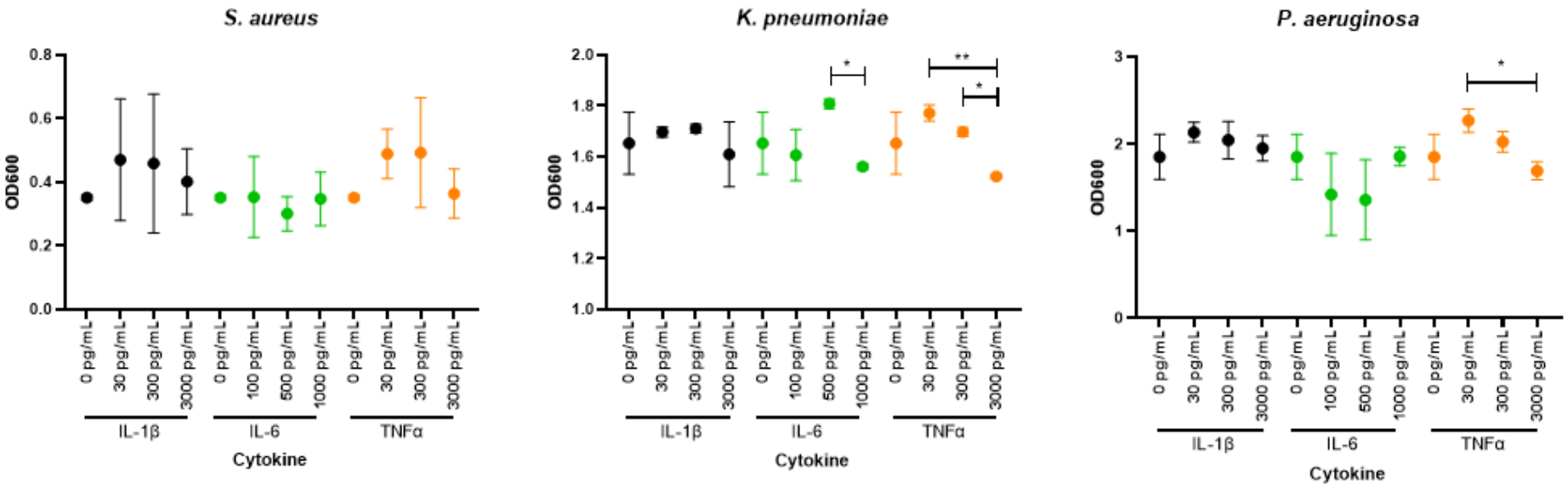


Figure 3.1: Growth of *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* in the presence of IL-1β, IL-6, and TNFα. There were significant changes in optical density at 600nm (OD_{600}) of *K. pneumoniae* and *P. aeruginosa* with the addition of TNFα ($p = 0.0084, 0.0139$). However, there was no significant change in OD_{600} compared to 0pg/mL TNFα. There were also significant changes in OD_{600} of *K. pneumoniae* between the addition of 500pg/mL and 1000pg/mL IL-6 ($p = 0.0222$), but no significant difference compared to no IL-6. There were no IL-1β-mediated changes in OD_{600} in any of *S. aureus* ($p = 0.7688$), *K. pneumoniae* ($p = 0.5333$), or *P. aeruginosa* ($p = 0.3661$). Results of multiple comparisons with Tukey method. Growth without cytokine measurement repeated within each dataset for ease of comparison between each cytokine. **n=3 technical, 3 biological** ±SD

The mean OD_{600} of *P. aeruginosa* planktonic growth ranged from 1.85 to 2.13 with IL-1β; 1.36 to 1.86 with IL-6; and 1.69 to 2.27 with TNFα. There is a significant decrease in OD_{600} of *P. aeruginosa* planktonic growth between 30pg/mL and 3000pg/mL TNFα, a difference of 26% [$F(3, 8) = 6.751$, $p = 0.0139$]. There was no significant change in OD_{600} of *P. aeruginosa* planktonic growth with IL-1β [$F(3, 8) = 1.212$, $p = 0.3661$], or IL-6 [$F(3, 8) = 1.718$, $p = 0.2402$] at any concentration tested.

For each bacterial species, a single OD_{600} measurement of planktonic growth was taken with no cytokine and repeated across each cytokine dataset. For *S. aureus* this measurement was 0.35. For *K. pneumoniae* this was 1.65. For *P. aeruginosa* this was 1.85.

3.3.2 | Cytokine-induced changes in planktonic growth of *P. aeruginosa* with subinhibitory antibiotic

All subinhibitory antibiotic concentrations presented represent those tested that fell above the MIC of modified broth microdilution assay. As such, the number of concentrations tested varies slightly between different antibiotics.

There were significant differences in OD_{600} of *P. aeruginosa* with the addition of TNFα in subinhibitory TZP (Figure 3.2a). In 0.125μg/mL TZP, the OD_{600} was significantly increased with 3000pg/mL TNFα compared to TNFα-free and 30pg/mL TNFα, increases of 41.7% and 48.5% respectively [$F(3, 16) = 4.698$, $p = 0.0155$]. In 0.25μg/mL TZP, the OD_{600} was also significantly increased with 3000pg/mL TNFα compared to TNFα-free and 30pg/mL TNFα, increases of 39.5% and 40.3% respectively [$F(3, 16) = 7.382$, $p = 0.0025$]. In 0.5μg/mL TNFα the

OD_{600} was significantly increased with 3000pg/mL TNFα compared to 30pg/mL TNFα and 300pg/mL TNFα, increases of 57.3% and 40.7% respectively [F (3, 16) = 6.377, p = 0.0048].

There were significant differences in OD_{600} of *P. aeruginosa* with the addition of TNFα in subinhibitory MEM (Figure 3.2b). In 0.0078125µg/mL MEM, the OD_{600} was significantly increased with 3000pg/mL TNFα compared to TNFα-free and 30pg/mL TNFα, increases of 51.8% and 37% respectively [F (3, 16) = 6.562, p = 0.0042]. There was no significant change in OD_{600} with 0.015625µg/mL MEM with addition of TNFα [F (3, 16) = 2.613, p = 0.0871].

There were no significant differences in OD_{600} of *P. aeruginosa* in subinhibitory LVX at any concentration of TNFα tested (Figure 3.2c). There was large variability between replicate measurements of OD_{600} in 0.03125µg/mL LVX, and no significant difference between TNFα concentrations [F (3, 8) = 0.09199, p = 0.9624]. This was similar in 0.0625µg/mL LVX [F (3, 8) = 0.5144, p = 0.6837]. There was little variability in OD_{600} of *P. aeruginosa* in 0.125µg/mL MEM, ranging from 0.05 to 0.06 with no significant differences between TNFα concentration [F (3, 8) = 0.1905, p = 0.8999].

There were significant differences in OD_{600} of *P. aeruginosa* in the presence of TNFα in subinhibitory IPM (Figure 3.2d). In 0.015625µg/mL IPM, the lowest concentration tested, the OD_{600} was significantly increased with 3000pg/mL TNFα compared to TNFα-free and 30pg/mL TNFα, increases of 57.7% and 49.9% respectively. [F (3, 8) = 13.94, p = 0.0015]. There was however no significant change in OD_{600} with 0.03125µg/mL IPM [F (3, 8) = 0.4882, p = 0.7], or 0.0625µg/mL IPM [F (3, 8) = 2.157, p = 0.1712].

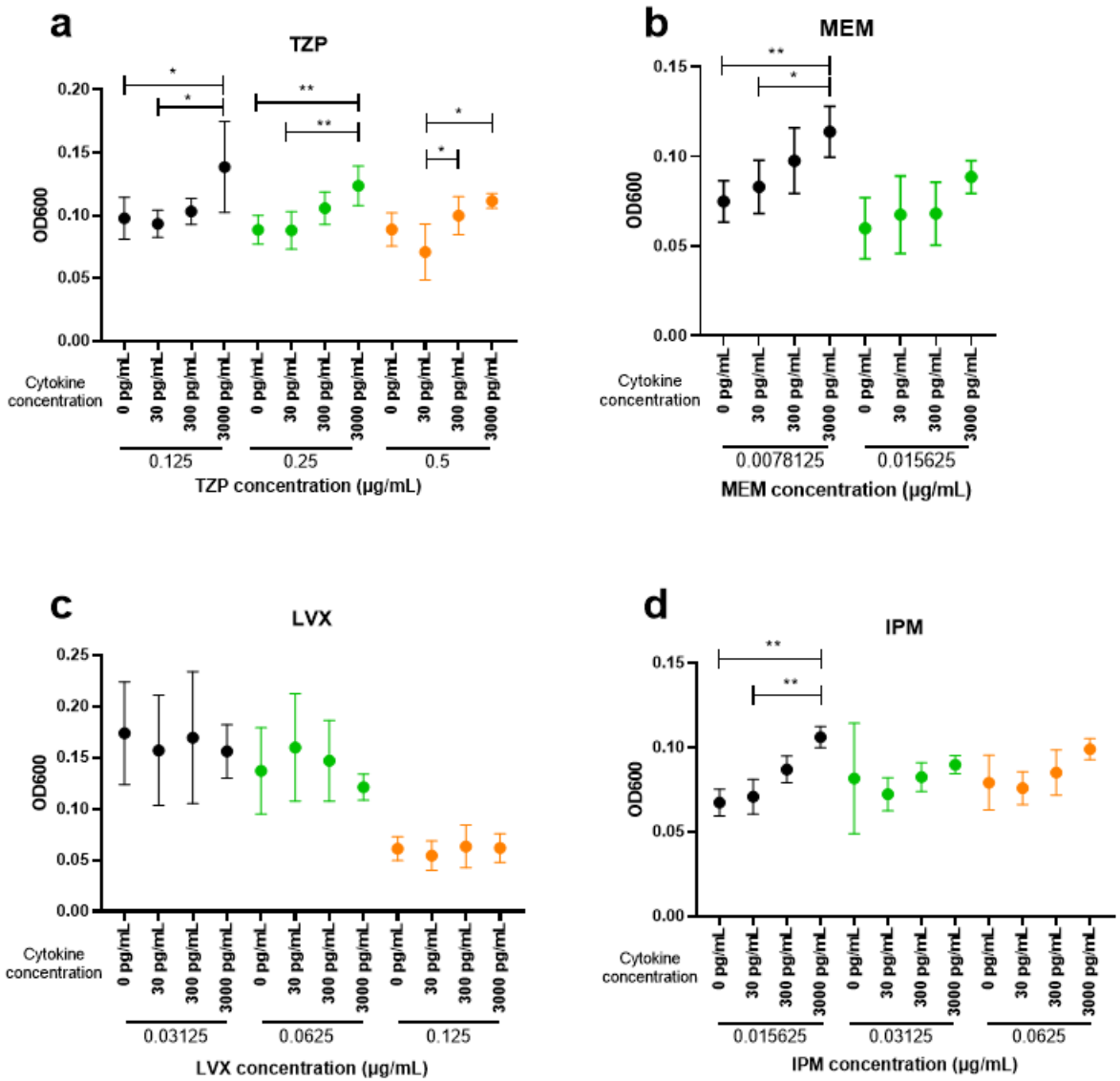


Figure 3.2: Effect of TNFα on planktonic growth of *P. aeruginosa* with sublethal antibiotic. Significant increases in optical density of planktonic culture at 600nm were seen with 3000pg/mL of TNFα, compared to TNFα-free or 30pg/mL. This phenomenon was present with TZP (a), MEM (b), and IPM (d). (TZP p = 0.0155, 0.0025, 0.0048; MEM p = 0.0042; IPM p = 0.0015). Significant increases were seen in all concentrations of TZP, whilst only in the lowest concentrations of MEM and IPM. There were no significant changes in optical density of *P. aeruginosa* with the addition of TNFα in the presence of LVX (c) (p = 0.9624)

a/b **n=2 technical, 5 biological** ±SD c/d **n=2 technical, 3 biological** ±SD

TZP – piperacillin-tazobactam; MEM – meropenem; LVX – levofloxacin; IPM – imipenem

There was no significant change in the OD_{600} of *P. aeruginosa* planktonic growth at any IL-1β concentration tested in 0.125μg/mL TZP [F (3, 15) = 1.510, p = 0.2524]; in 0.25μg/mL TZP [F (3, 16) = 1.630, p = 0.2219]; or in 0.5μg/mL TZP [F (3, 15) = 2.037, p = 0.1519] (Figure 3.3a).

There were no significant changes in the OD_{600} of *P. aeruginosa* planktonic growth at any IL-1β concentration tested in 0.0078125μg/mL MEM [F (3, 16) = 3.036, p = 0.0596]; or in 0.015625μg/mL MEM [F (3, 16) = 1.350, p = 0.2937] (Figure 3.3b).

There were no significant changes in the OD_{600} of *P. aeruginosa* planktonic growth at any IL-1β concentration tested in 0.03125μg/mL LVX [F (3, 8) = 0.4879, p = 0.7002]; in 0.0625μg/mL LVX [F (3, 8) = 0.1683, p = 0.9148]; or in 0.125μg/mL LVX [F (3, 8) = 0.9316, p = 0.4689] (Figure 3.3c).

There were no significant changes in the OD_{600} of *P. aeruginosa* planktonic growth at any IL-1β concentration tested in 0.015625μg/mL IPM [F (3, 8) = 3.570, p = 0.0666]; in 0.03125μg/mL IPM [F (3, 8) = 0.5929, p = 0.6370]; or in 0.0625μg/mL IPM [F (3, 8) = 2.280, p = 0.1563] (Figure 3.3d).

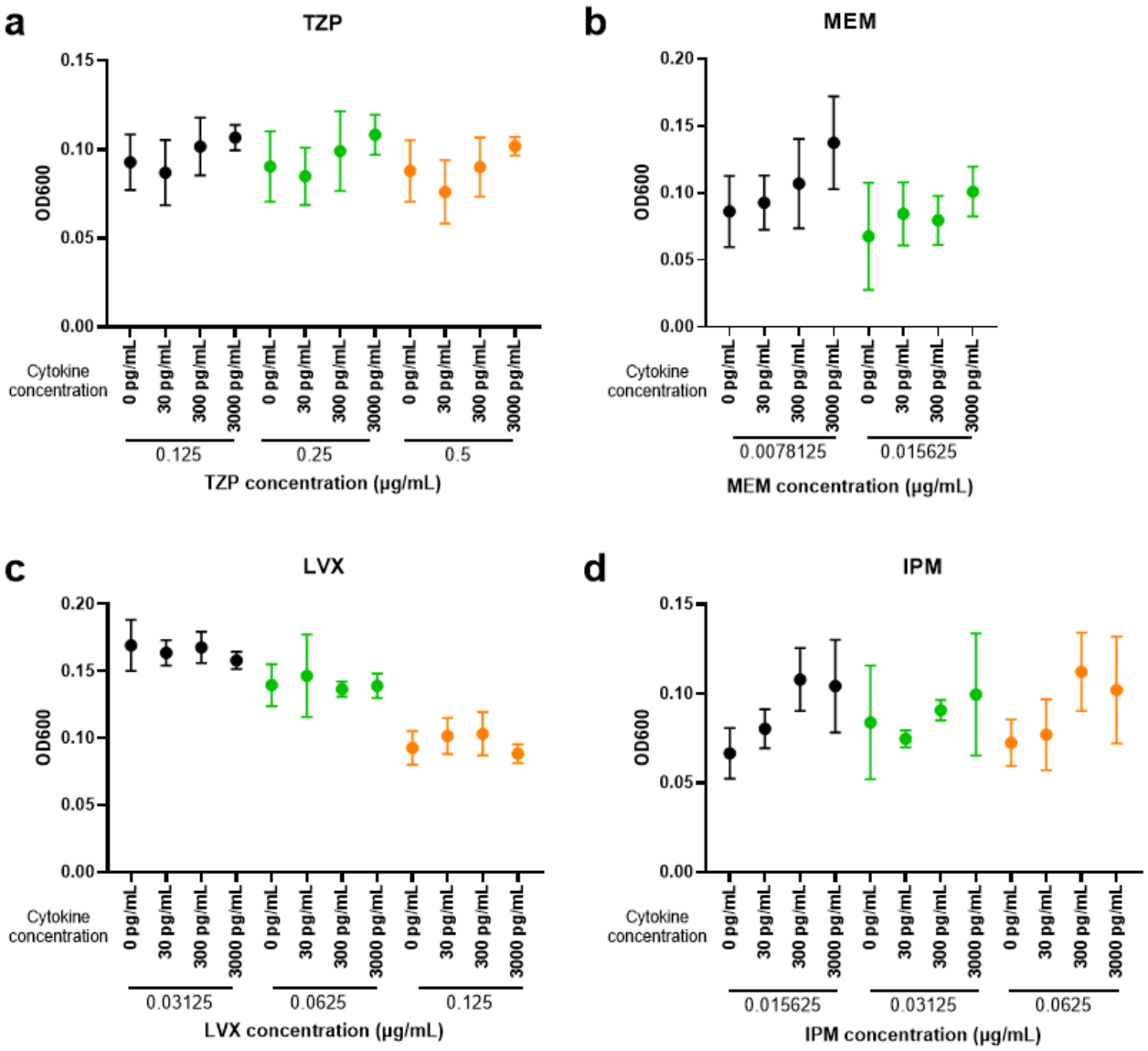


Figure 3.3: Effect of IL-1β on planktonic growth of *P. aeruginosa* with sublethal antibiotic. There were no significant changes in optical density of planktonic culture with the addition of IL-1β, regardless of concentration of sublethal antibiotic. Results of multiple comparisons with Tukey method.

a/b **n=2 technical, 5 biological** ±SD

c/d **n=2 technical, 3 biological** ±SD

TZP – piperacillin-tazobactam; MEM – meropenem; LVX – levofloxacin; IPM – imipenem

There were no significant changes in the OD_{600} of *P. aeruginosa* planktonic growth at any IL-6 concentration tested in 0.125μg/mL TZP [F (3, 16) = 1.547, p = 0.2410]; in 0.25μg/mL TZP [F (3, 16) = 1.278, p = 0.3157]; or in 0.5μg/mL TZP [F (3, 16) = 2.407, p = 0.1052] (Figure 3.4a).

There were no significant changes in the OD_{600} of *P. aeruginosa* planktonic growth at any IL-6 concentration tested in 0.0078125μg/mL MEM [F (3, 16) = 0.9944, p = 0.4206]; or in 0.015625μg/mL MEM [F (3, 16) = 0.5768, p = 0.6386] (Figure 3.4b).

There were no significant changes in the OD_{600} of *P. aeruginosa* planktonic growth at any IL-6 concentration tested in 0.03125μg/mL LVX [F (3, 8) = 1.394, p = 0.3135]; in 0.0625μg/mL LVX [F (3, 8) = 3.104, p = 0.0890]; or in 0.125μg/mL LVX [F (3, 8) = 2.250, p = 0.1598] (Figure 3.4c).

There were no significant changes in the OD_{600} of *P. aeruginosa* planktonic growth at any IL-6 concentration tested in 0.015625μg/mL IPM [F (3, 8) = 1.699, p = 0.2439]; in 0.03125μg/mL IPM [F (3, 8) = 1.938, p = 0.2020]; or in 0.0625μg/mL IPM [F (3, 8) = 1.095, p =0.4055] (Figure 3.4d).

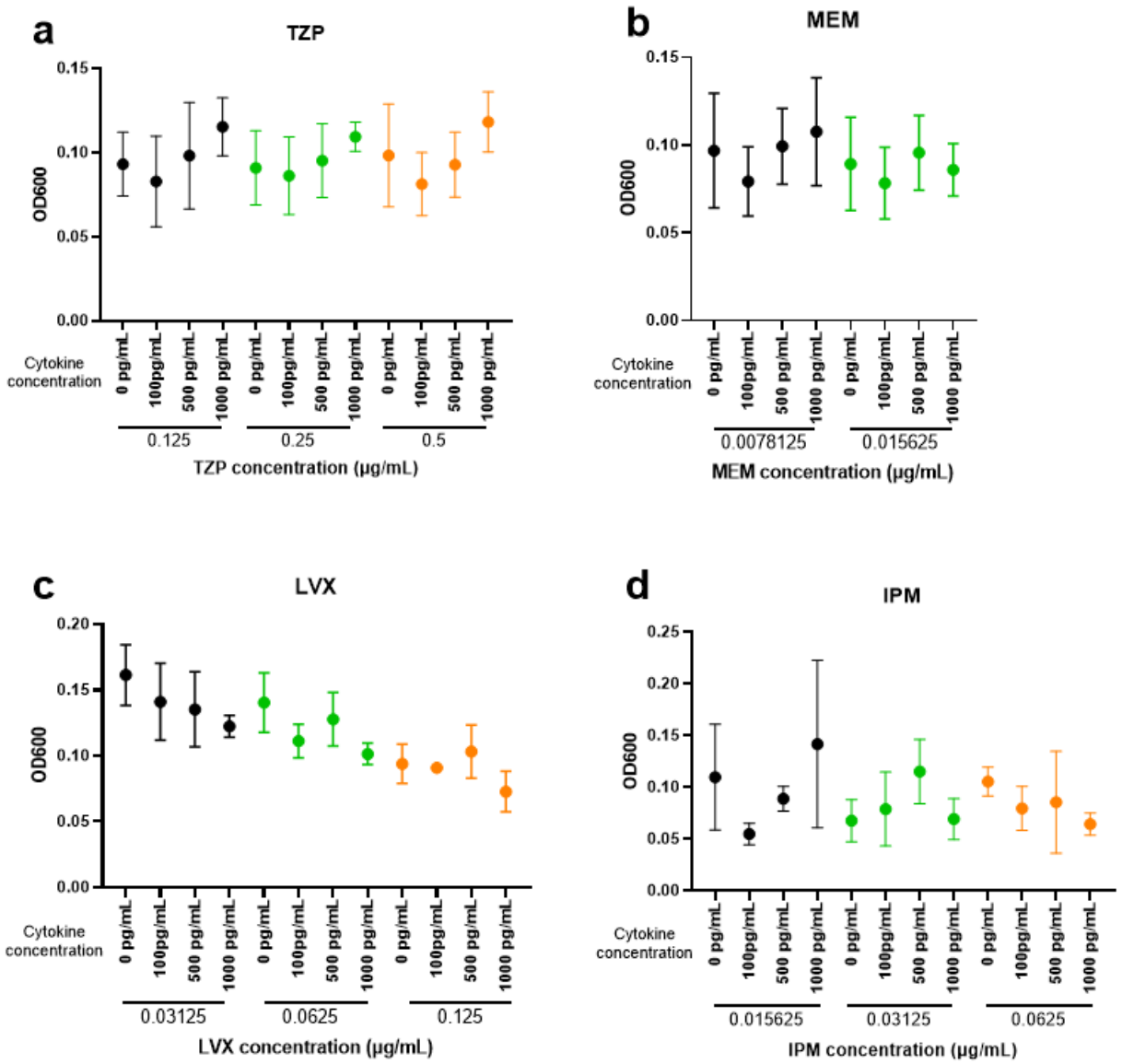


Figure 3.4: Effect of IL-6 on planktonic growth of *P. aeruginosa* with sublethal antibiotic. There were no significant changes in optical density of planktonic culture with the addition of IL-6, regardless of concentration of sublethal antibiotic. Results of multiple comparisons with Tukey method.

a/b **n=2 technical, 5 biological** ±SD

c/d **n=2 technical, 3 biological** ±SD

TZP – piperacillin-tazobactam; MEM – meropenem; LVX – levofloxacin; IPM – imipenem

3.3.3 | Cytokine-induced changes in planktonic growth of *S. aureus* with subinhibitory meropenem

For investigation of *S. aureus,* the specific interaction between TNFα, subinhibitory meropenem, and planktonic growth was investigated. This decision was informed by observations in *P. aeruginosa*, and in work by Meduri *et al.* (1999, 2002).

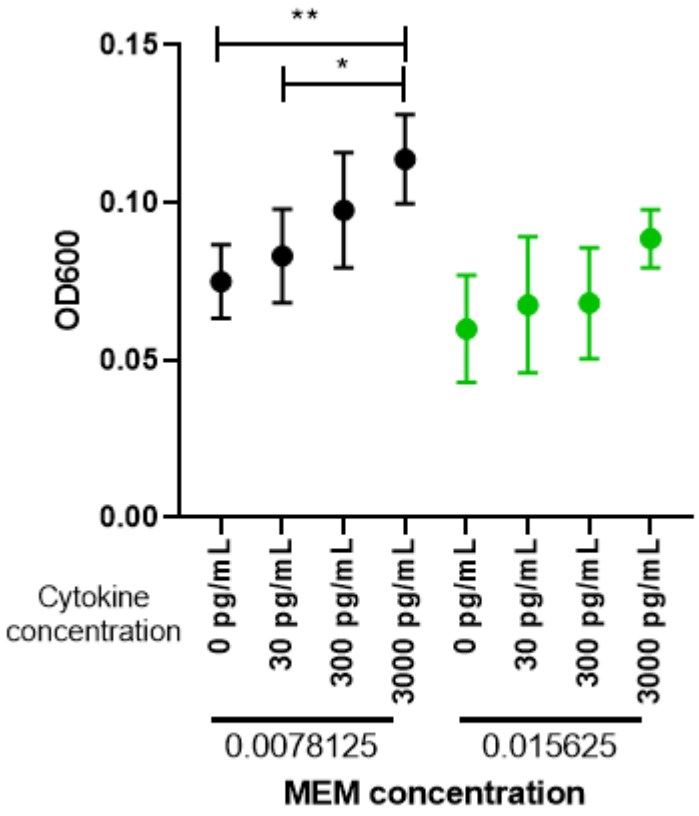


Figure 3.5: Effect of TNFα on planktonic growth of *S. aureus* with sublethal MEM. Significant increases in the optical density of planktonic culture were seen with 3000pg/mL TNFα, compared to TNFα-free and 30pg/mL TNFα ($p = 0.0042$), at the lowest concentration of sublethal MEM. Results of multiple comparisons with Tukey method.

n=3 technical, 3 biological ± SD

MEM - meropenem

There were significant changes in OD_{600} of planktonic growth with the addition of TNFα in subinhibitory MEM (Figure 3.5). In 0.0078125μg/mL MEM, the OD_{600} was significantly increased with the addition of 3000pg/mL TNFα, compared to TNFα-free and 30pg/mL TNFα [F (3, 16) = 6.562, p = 0.0042]. From 0.0749 and 0.083 respectively, to 0.1137. There was no significant TNFα-mediated change in OD_{600} in 0.0125625μg/mL MEM [F (3, 16) = 2.613, p = 0.0871]. There was elevation with 3000pg/mL TNFα, to 0.0885 from an average of 0.06516, but this was within error.

3.4 | Cytokine-induced changes in relative biofilm formation of VAP isolates

Results of crystal violet staining of *P. aeruginosa* biofilms were normalised to the planktonic phase using OD of planktonic growth. Relative biofilm formation represents the biofilm productivity of a bacterial culture without considering the size of the culture i.e. on a per-plankton basis. Without normalisation it is impossible to distinguish increased biofilm mass because of upregulated production from there simply being more bacterial cells in the culture.

3.4.1 | Cytokine-induced changes in relative biofilm formation *in vitro*

The mean relative BFU production of *S. aureus* ranged from 1.00 to 1.99 with IL-1β; 1.00 to 1.59 with IL-6; and 1.00 to 1.94 with TNFα (Figure 3.6). There was no significant change in relative BFU in *S. aureus* when cultured with any concentration of IL-1β [F (3, 8) = 2.046, p = 0.1860]; IL-6 [F (3, 8) = 1.385, p = 0.3159]; or TNFα [F (3, 8) = 0.9851, p = 0.4470].

The mean relative BFU production of *K. pneumoniae* ranged from 0.31 to 0.46 with IL-1β; 0.31 to 0.38 with IL-6; and 0.31 to 0.44 with TNFα. There was a significant interaction between IL-6 and *K. pneumoniae* relative BFU according to ANOVA [F (3, 8) = 4.275, p = 0.0446], however the results of multiple comparisons by Tukey method did not find any significance between means of columns. There was also a significant increase in *K. pneumoniae* BFU with 30pg/mL, 300pg/mL and 3000pg/mL TNFα compared to TNFα-free; by 24.3%, 39.8%, and 43.9% respectively [F (3, 8) = 16.86, p = 0.0008]. There was no significant change in *K. pneumoniae* BFU with any concentration of IL-1β [F (3, 8) = 2.847, p = 0.1053].

The mean relative BFU production of *P. aeruginosa* ranged from 0.24 to 0.27 with IL-1β; 0.24 to 0.33 with IL-6; and 0.24 to 0.34 with TNFα. There was no significant change in *P. aeruginosa* BFU when cultured with any concentration of IL-1β [F (3, 8) = 0.3215, p = 0.8099]; IL-6 [F (3, 8) = 1.196, p = 0.3712]; or TNFα [F (3, 8) = 3.144, p = 0.0867].

For each bacterial species, a single measurement of relative biofilm formation was taken with no cytokine and repeated across each cytokine dataset for ease of comparison. For *S. aureus* this measurement was 1.00. For *K. pneumoniae* this was 0.31. For *P. aeruginosa* this was 0.24.

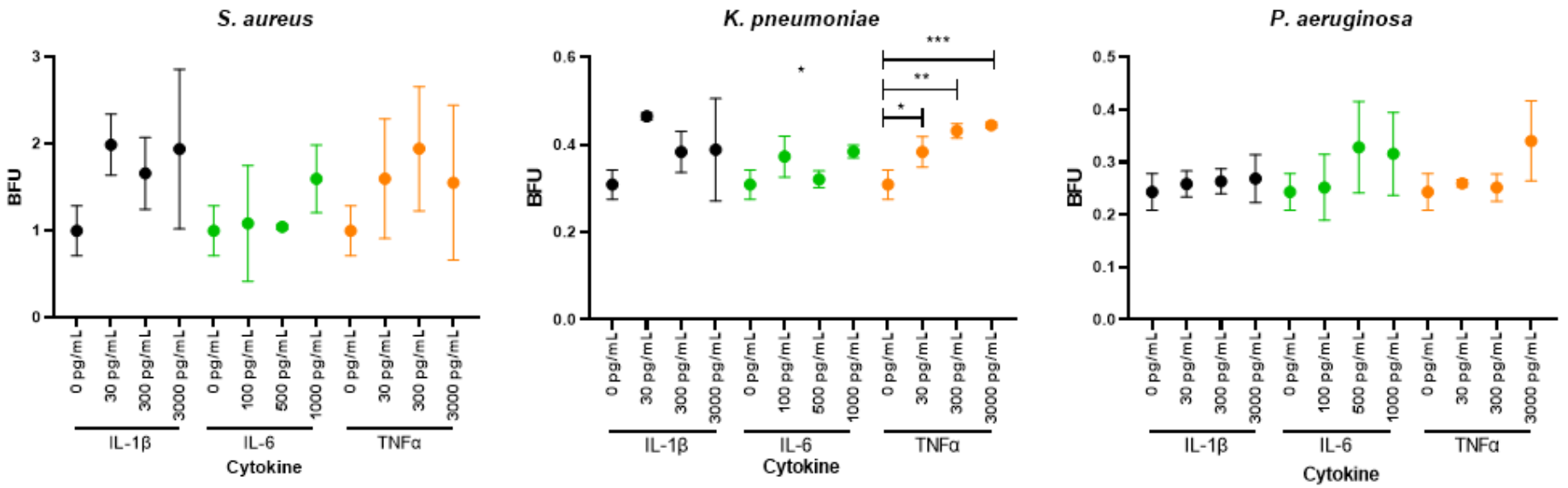


Figure 3.6: Biofilm forming units (BFU) of *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* in the presence of IL-1β, IL-6, and TNFα. There were significant dose-dependent changes in BFU of *K. pneumoniae* with the addition of TNFα (p = 0.0008). There was also significant interaction between IL-6 and BFU of *K. pneumoniae*, but none of the multiple comparisons passed the threshold for significance (p = 0.0446). There were no other IL-1β or IL-6 -mediated changes in BFU in any of *S. aureus* (IL-1β p = 0.1860; IL-6 p = 0.3159), *K. pneumoniae* (IL-1β p = 0.1053), or *P. aeruginosa* (IL-1β p = 0.8099; IL-6 p = 0.3712). Furthermore, there were no TNFα-mediated changes in BFU in *S. aureus* (p = 0.4470) or *P. aeruginosa* (p = 0.0867). Results of multiple comparisons with Tukey method. Biofilm formation without cytokine measurement repeated within each dataset for ease of comparison between each cytokine. **n=3 technical, 3 biological** ±SD

3.4.2 | Cytokine-induced changes in relative biofilm formation of *P. aeruginosa* with subinhibitory antibiotic

There was no significant change in relative BFU of *P. aeruginosa* at any TNFα concentration tested in 0.125μg/mL TZP [F (3, 16) = 0.8051, p = 0.5092]; in 0.25μg/mL TZP [F (3, 16) = 0.07745, p = 0.9713]; or in 0.5μg/mL TZP [F (3, 16) = 0.7496, p = 0.5384] (Figure 3.7a).

There was no significant change in relative BFU of *P. aeruginosa* at any TNFα concentration tested in 0.0078125μg/mL MEM [F (3, 16) = 0.1972, p = 0.8968]; or in 0.015625μg/mL MEM [F (3, 16) = 0.6776, p = 0.5784] (Figure 3.7b).

There was no significant change in relative BFU of *P. aeruginosa* at any TNFα concentration tested in 0.03125μg/mL LVX [F (3, 8) = 0.7969, p = 0.5293]; in 0.0625μg/mL LVX [F (3, 8) = 1.341, p = 0.3279]; or in 0.125μg/mL LVX [F (3, 8) = 0.9045, p = 0.4804] (Figure 3.7c).

There was no significant change in relative BFU of *P. aeruginosa* at any TNFα concentration tested in 0.015625μg/mL IPM [F (3, 8) = 0.2798, p = 0.8386]; in 0.03125μg/mL IPM [F (3, 8) = 1.158, p = 0.3839]; or in 0.0625μg/mL IPM [F (3, 8) = 1.539, p = 0.2779] (Figure 3.7d).

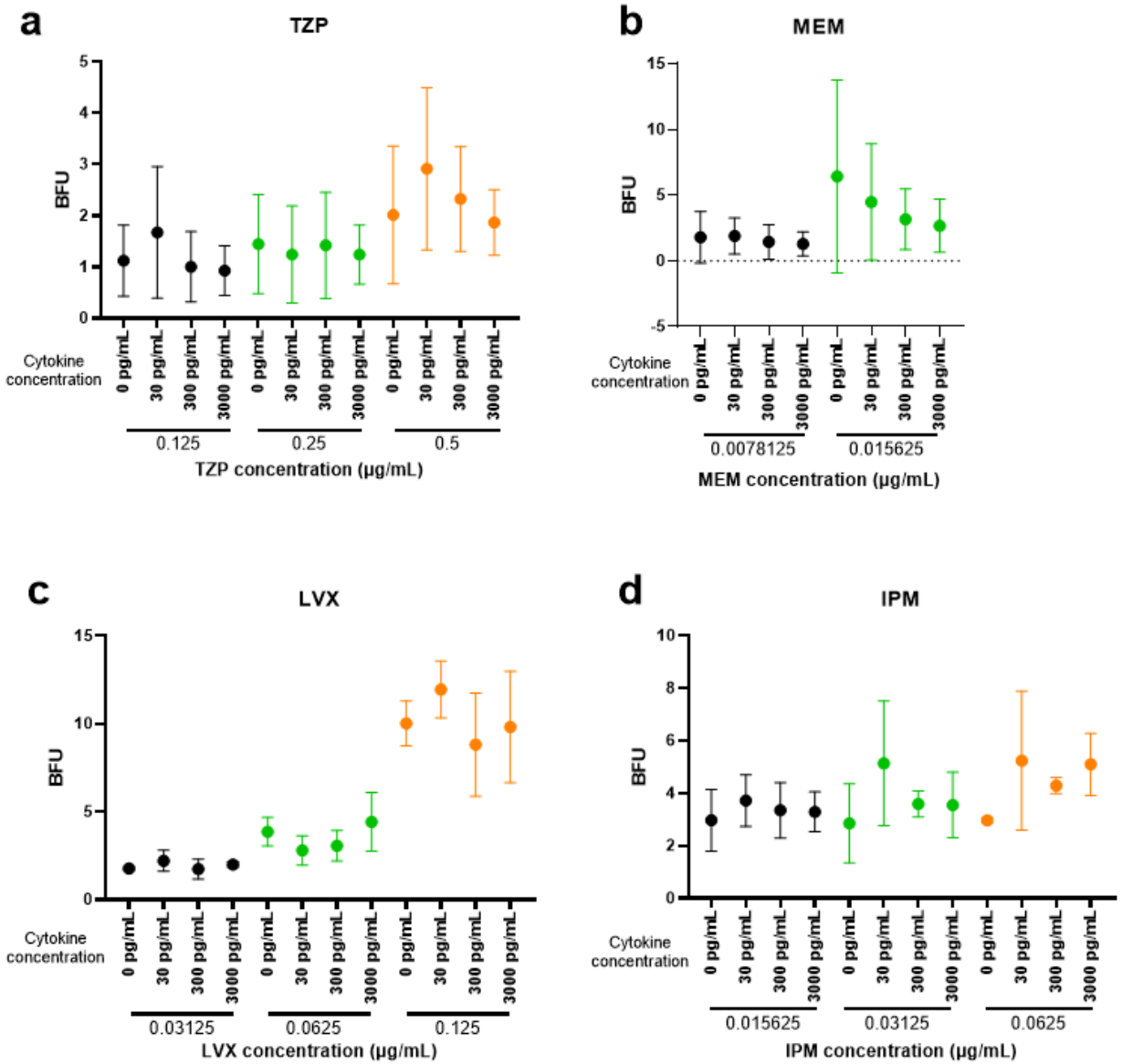


Figure 3.7d: Effect of TNFα on biofilm formation of *P. aeruginosa* with sublethal antibiotic. There were no significant changes in biofilm production of *P. aeruginosa* with the addition of TNFα, regardless of concentration of sublethal antibiotic. Results of multiple comparisons with Tukey method.

a/b **n=2 technical, 5 biological** ±SD

c/d **n=2 technical, 3 biological** ±SD

TZP – piperacillin-tazobactam; MEM – meropenem; LVX – levofloxacin; IPM – imipenem

There was no significant change in relative BFU of *P. aeruginosa* at any IL-1β concentration tested in 0.125μg/mL TZP [F (3, 16) = 0.02712, p = 0.9937]; in 0.25μg/mL TZP [F (3, 16) = 0.1332, p = 0.9388]; or in 0.5μg/mL TZP [F (3, 16) = 0.2273, p = 0.8760] (Figure 3.8a).

There is no significant change in relative BFU of *P. aeruginosa* at any IL-1β concentration tested in 0.0078125μg/mL MEM [F (3, 16) = 0.5127, p = 0.6793]; or in 0.015625μg/mL MEM [F (3, 16) = 0.4177, p = 0.7427] (Figure 3.8b).

There is no significant change in relative BFU of *P. aeruginosa* at any IL-1β concentration tested in 0.03125μg/mL LVX [F (3, 8) = 0.1989, p = 0.8943]; in 0.0625μg/mL LVX [F (3, 8) = 0.4066, p = 0.7525]; or in 0.125μg/mL LVX [F (3, 8) = 0.8444, p = 0.5071] (Figure 3.8c).

There is no significant change in relative BFU of *P. aeruginosa* at any IL-1β concentration tested in 0.015625μg/mL IPM [F (3, 8) = 0.2441, p = 0.8633]; in 0.03125μg/mL IPM [F (3, 8) = 3.348, p = 0.0763]; or in 0.0625μg/mL IPM [F (3, 8) = 1.232, p = 0.3599] (Figure 3.8d).

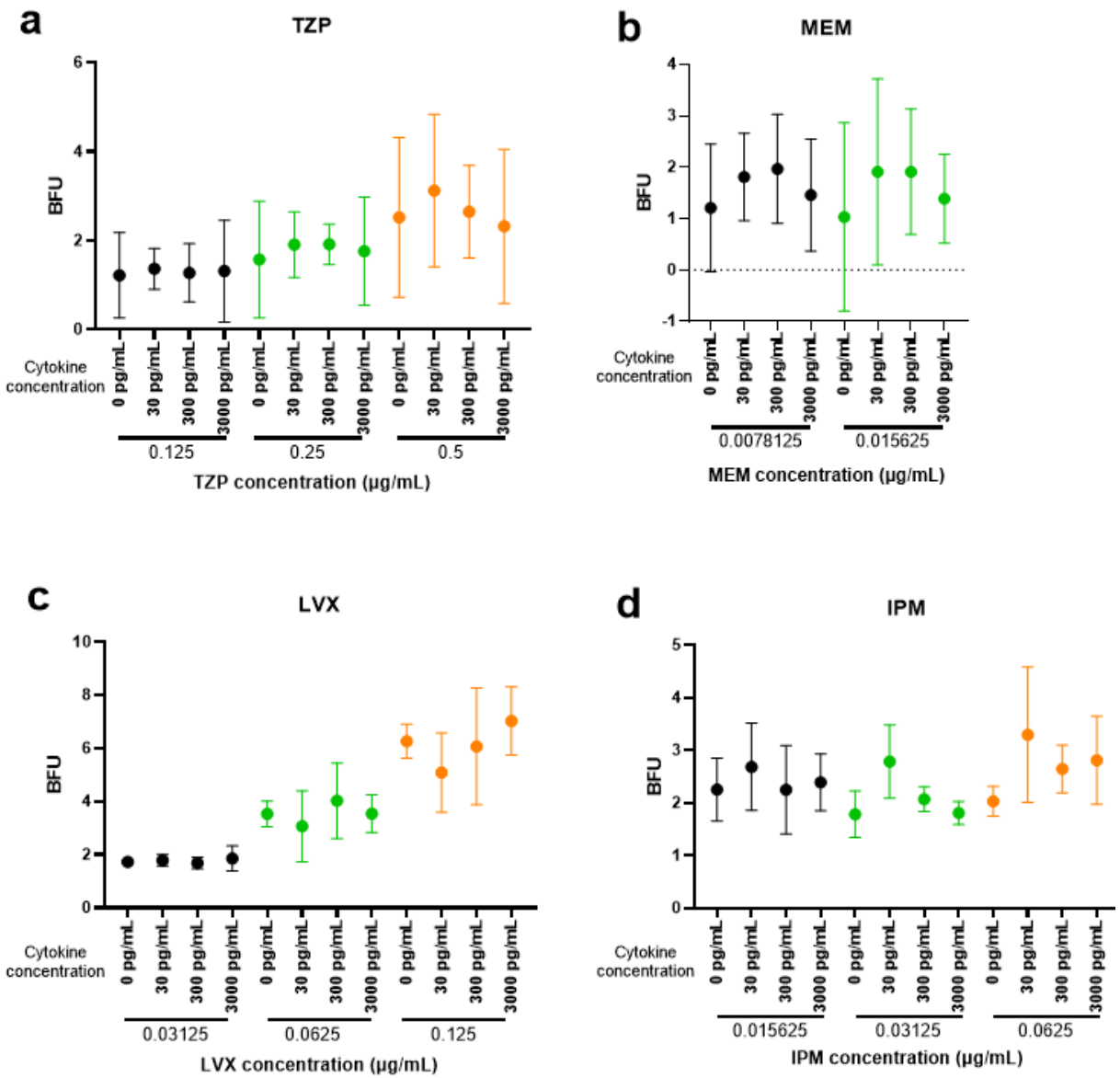


Figure 3.8: Effect of IL-1β on biofilm formation of *P. aeruginosa* with sublethal antibiotic. There were no significant changes in biofilm production of *P. aeruginosa* with the addition of IL-1β, regardless of concentration of sublethal antibiotic. Results of multiple comparisons with Tukey method.

a/b **n=2 technical, 5 biological** ±SD

c/d **n=2 technical, 3 biological** ±SD

TZP – piperacillin-tazobactam; MEM – meropenem; LVX – levofloxacin; IPM – imipenem

There was no significant change in relative BFU of *P. aeruginosa* at any IL-6 concentration tested in 0.125μg/mL TZP [F (3, 16) = 0.7573, p = 0.5342]; in 0.25μg/mL TZP [F (3, 16) = 0.5153, p = 0.6776]; or in 0.5μg/mL TZP [F (3, 16) = 0.9306, p = 0.4487] (Figure 3.9a).

There was no significant change in relative BFU of *P. aeruginosa* at any IL-6 concentration tested in 0.0078125μg/mL MEM [F (3, 16) = 0.1366, p = 0.9367]; or in 0.015625μg/mL MEM [F (3, 16) = 0.8551, p = 0.4843] (Figure 3.9b).

There was no significant change in relative BFU of *P. aeruginosa* at any IL-6 concentration tested in 0.03125μg/mL LVX [F (3, 8) = 1.831, p = 0.2195]; in 0.0625μg/mL LVX [F (3, 8) = 1.884, p = 0.2107]; or in 0.125μg/mL LVX [F (3, 8) = 0.1468, p = 0.9289] (Figure 3.9c).

There was no significant change in relative BFU of *P. aeruginosa* at any IL-6 concentration tested in 0.015625μg/mL IPM [F (3, 8) = 2.669, p = 0.1187]; in 0.03125μg/mL IPM [F (3, 8) = 0.5973, p = 0.6344]; or in 0.0625μg/mL IPM [F (3, 8) = 1.073, p = 0.4135] (Figure 3.9d).

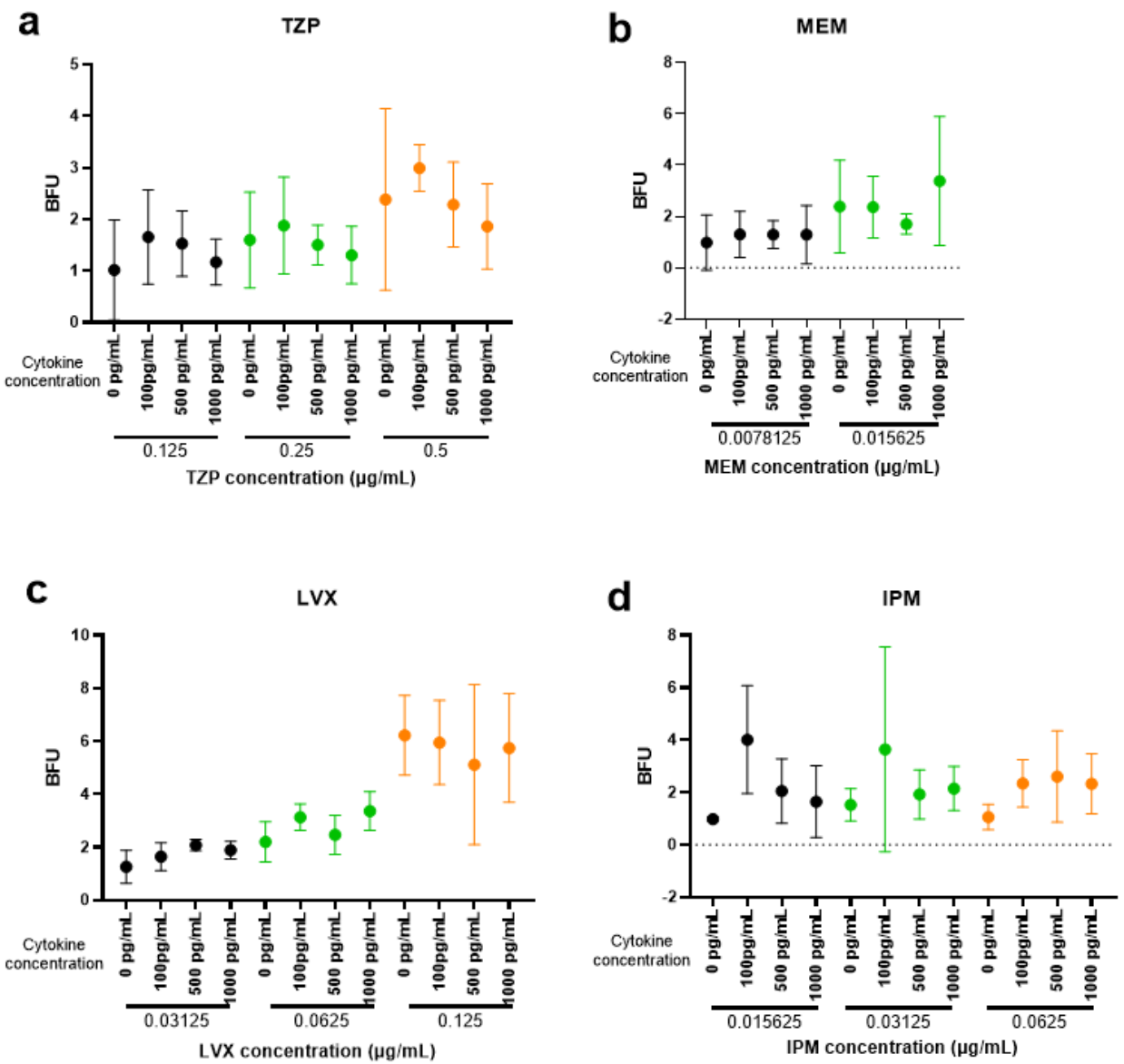


Figure 3.9: Effect of IL-6 on biofilm formation of *P. aeruginosa* with sublethal antibiotic. There were no significant changes in biofilm production of *P. aeruginosa* with the addition of IL-6, regardless of concentration of sublethal antibiotic. Results of multiple comparisons with Tukey method.

a/b **n=2 technical, 5 biological** ±SD
c/d **n=2 technical, 3 biological** ±SD

TZP – piperacillin-tazobactam; MEM – meropenem; LVX – levofloxacin; IPM – imipenem

3.5 | Summary of key chapter data

These results demonstrate a potential relationship between TNFα and increased *in vitro* planktonic growth of *S. aureus, K. pneumoniae* and *P. aeruginosa*. Furthermore, they demonstrate that TNFα increases *in vitro* biofilm production in *K. pneumoniae*. There was variability in growth enhancement with and without subinhibitory antibiotic, depending on species.

S. aureus growth was significantly enhanced with 3000pg/mL TNFα, but only when exposed to 0.0078125µg/mL meropenem. *P. aeruginosa* growth was significantly enhanced *in vitro*, but with contradictory results between groups with and without subinhibitory antibiotic. Enhancement of *P. aeruginosa* growth was dose-dependent with addition of subinhibitory antibiotic, but not dose-dependent without. *K. pneumoniae* growth was significantly enhanced with 30pg/mL and 300pg/mL TNFα compared to 3000pg/mL TNFα. Additionally, *K. pneumoniae* growth was significantly enhanced by 500pg/mL IL-6 compared to 1000pg/mL IL-6. This was the only enhancement of growth observed across all species that was not mediated by TNFα.

The other major finding of this chapter was in cytokine-induced changes in biofilm formation. Relative biofilm formation of *K. pneumoniae* was significantly enhanced *in vitro* at all tested concentrations of TNFα compared to TNFα-free. This relationship appeared dose-dependent, with greater significance compared to TNFα-free control with increased concentration of TNFα applied to culture.

There was no significant TNFα-mediated enhancement in the biofilm growth of *S. aureus* or *P. aeruginosa* observed in this study, despite enhancements of

planktonic growth. This suggests that cytokine-mediated enhancements of growth in these species are specific for cultures in the planktonic phase.

3.6 | Chapter discussion

This chapter indicates a potential importance of the pressure exerted by subinhibitory meropenem in enhancing TNFα-mediated growth of *S. aureus*. *S. aureus* enhancement by IL-1β or IL-6 with subinhibitory meropenem was not measured. Results from *P. aeruginosa* had confirmed an interest in the specific relationship between TNFα, meropenem, and enhanced *in vitro* growth.

Findings in measurements of *P. aeruginosa* growth with subinhibitory antibiotic were in line with previous publications, which report that cytokine-mediated enhancements of bacterial growth are dose-dependent phenomena (Denis *et al*., 1991; Porat *et al*., 1991; Meduri *et al*., 1999).

Growth enhancements of *K. pneumoniae* do not follow the dose-dependent trend reported previously in the literature. If the results observed in *K. pneumoniae* remain applicable *in vivo*, colonisation with this species could be most problematic in patients with a lower or inadequate inflammatory response to this species. This isn't entirely unexpected, as lower TNFα or IL-6 response would naturally lead to reduced immune recruitment, but it may especially potentiate *K. pneumoniae* infection. Despite this observation, *K. pneumoniae* investigation was not followed up in subinhibitory antibiotic. This may represent a future avenue of investigation for delineating a role for IL-6 in changing bacterial growth. Meduri *et al.* (1999) have already demonstrated a role for IL-6 in enhancing the growth of *P.*

aeruginosa. TNFα was selected for experimental focus based on the significant changes in growth it exerted across multiple species.

The observed changes in bacterial growth in this chapter somewhat contradict the observations made by Meduri *et al*. in 1999. In their study, *S. aureus* growth was enhanced by the presence of IL-1β, whereas no changes were observed with the presence of TNFα. The key differences in findings between the results are that TNFα did not enhance *S. aureus* growth until subinhibitory meropenem was included in the culture. Additionally, many of the significant increases to planktonic growth mediated by TNFα were observed in *P. aeruginosa*, which was not included in the study by Meduri *et al.* (1999). It is unclear why our results did not demonstrate any effect of IL-1β on planktonic growth of any species. While the highest concentration of IL-1β used by Meduri *et al.* was 10000pg/mL, compared to 3000pg/mL used in this study, maximal response was measured at 1000pg/mL. This may explain why this study saw no effect related to IL-1β, as this 1000pg/mL "sweetspot" may have been missed by the concentrations used in this study's methods. This does not explain the complete absence of any dose-dependent response, however.

There were a few notable differences in methodology between our study and previous investigations. Previously, enhancements in growth have been measured by culturing bacteria with cytokine in a minimal medium (RPMI) followed by dilution and plating onto LB agar to be counted as CFU/mL (Meduri *et al*., 1999; Kanangat *et al*., 2001). In this study, following culture in M9 minimal broth with cytokine, the optical density of planktonic culture was measured at 600nm. By culturing a sample from broth culture onto solid culture, an additional incubation step is added. Furthermore, the cytokine stimulus that may have growth modifying

effects is removed from the microenvironment during this step. Whether this may result in reduced growth after transfer to solid culture is unknown, however. This is avoided by measuring OD_{600}, which calculates a measurement between detected and emitted light through a microbial sample. This measurement is an estimation of culture density, as dead cells and particulate matter will also scatter light and potentially increase the reading (Eppendorf, 2015).

One potential expansion for experiments measuring cytokine enhancement of growth would be to change the sampling times, and measure OD_{600} at more than just an end point time following 18 hours incubation. This is the methodology followed by previous studies, and it allows for measurement of cytokine-mediated changes in growth rate, in addition to total growth once the stationary phase is reached. This is where another significant deviation in this study's findings from those of Meduri *et al.* (1999) is apparent. In their study, Meduri *et al*. found that growth in the stationary phase was not changed with the addition of cytokines. The significant changes reported in their study reflect changes during the log phase, at a timepoint of 6 hours, and thus represent a change in growth rate and speed of reaching the stationary phase, rather than an increase in total growth. This may explain some of the differences highlighted between the datasets i.e. responses to TNFα, and absence of a response to IL-6 in *P. aeruginosa*.

The enhancement of *K. pneumoniae* biofilm formation is interesting when considered in context of the results for planktonic growth. Exposure to increasing concentrations of TNFα appeared to have a detrimental effect on overall planktonic growth of *K. pneumoniae*. It is possible that sensing of TNFα by *K. pneumoniae* is responsible for a shift to biofilm phenotype and a consequent reduction in the density of planktonic culture. This may be a consequence of long-

term evolution of the bacteria alongside the human immune response, with *K. pneumoniae* adapting to respond to immune challenge. The capability of the bacterial biofilm to attenuate the actions of the inflammatory response is well documented (Thurlow *et al*., 2011; Roilides *et al*., 2015).

Alternatively, this result may suggest that responsiveness to TNFα in *K. pneumoniae* is a consequence of products selectively produced under biofilm conditions, which are subsequently targeted by TNFα uptake. This would explain not just the upregulation of *K. pneumoniae* biofilm formation when exposed to TNFα; it may also explain the significant decrease in planktonic growth observed with increasing TNFα concentrations. Leukocytes have been demonstrated to adhere to and penetrate biofilms under appropriate conditions (Leid *et al*., 2002). However, in these cases phagocytosis has been shown to be diminished, without impairment of cytokine response. It is possible that the event of leukocyte penetration may result in significant enhancement of biofilm growth.

A study by Di Domenico *et al*. (2018) observed significant enhancement of *S. aureus* biofilms following culture with IL-1β and IFNγ. The concentrations of IL-1β and IFNγ used in this study were 10ng/mL, 20ng/mL, and 50ng/mL, with significant enhancement of *S. aureus* biofilms seen at 50ng/mL IL-1β, and 20 and 50ng/mL IFNγ (Di Domenico *et al*., 2018). These far exceed the maximum concentrations of IL-1β utilised in this chapter's experiments, in which the highest concentration of IL-1β used was 3000pg/mL and suggests that widening the tested cytokine concentrations may be appropriate. However, Di Domenico *et al.* did not give a reasoning for the concentrations used in the study in their methods, and given their research relates to atopic dermatitis these concentrations may not be appropriate for investigation of pathology of the lungs.

Only *P. aeruginosa* was tested with subinhibitory antibiotic. Based on biofilm findings in *K. pneumoniae*, it may be sensible to follow up under these conditions. Antibiotic dosages that fail to reach inhibitory concentrations are known to induce biofilm formation in bacteria (Kaplan, 2011). In the case that a significant interaction was observed, perhaps in *K. pneumoniae* as part of a future investigation, the effect of subinhibitory antibiotic and cytokine would need to be differentiated. This could be controlled for simply by utilising subinhibitory antibiotic-only controls.

The capability of TNFα to increase the total planktonic growth of *S. aureus, K. pneumoniae*, and *P. aeruginosa* at the stationary phase *in vitro* has been demonstrated. Further to this, the capability of TNFα to increase the relative biofilm formation of *K. pneumoniae in vitro* has been shown. This supports and adds to the existing evidence of cytokines enhancing bacterial growth across numerous species, despite some contradictions regarding which cytokines exert these effects. TNFα emerges from these results as a particular cytokine of interest, and these results supplement and expand upon previous research in this area.

Where this matters in the context of VAP is in development of infection. *P. aeruginosa* has been demonstrated to induce lung injury in VAP through the induction of TNFα production (Yang *et al*., 2017b). If TNFα enhancement of *P. aeruginosa* growth translates to *in vivo* host models, then this cycle is potentially self-propagating. *P. aeruginosa* establishes itself in the lungs of a ventilated individual, induces TNFα production. This response, if highly elevated or inappropriate, may then induce increased *P. aeruginosa* growth and rapid lung damage. *P aeruginosa* is frequently cited as the most common infectious cause of

VAP (Ramírez-Estrada *et al*., 2016), and this potential propagation of its growth may explain its prevalence in patients. In those with very heightened inflammatory responses, either inappropriately or because of comorbidity, this may result in more severe infection. In ARDS patients, for example, TNFα levels exceed those seen in severe pneumonia patients (Bauer *et al*., 2000). As VAP is a common complication of MV for treatment of ARDS, this patient group may be one particularly at risk of severe *P. aeruginosa* infection, due to TNFα-mediated enhancement of *P. aeruginosa* in the lungs. While there is a notable anti-inflammatory response early in ARDS to limit the inflammatory response, the initial cytokine burst may still be an important trigger in establishing VAP (Park *et al*., 2001). Furthermore, while we didn't demonstrate cytokine mediated enhancement of *P. aeruginosa* biofilm formation, this may be a consequence of the cytokine concentrations tested being too narrow (Di Domenico *et al*., 2018). If an initial large proinflammatory response were to stimulate biofilm formation, this may also result in development of VAP, given the pivotal role of the biofilm in its pathogenesis. This would have consequences for treatment as well, given the recalcitrance of biofilms to antimicrobials. It may be pertinent to consider the inflammatory response seen in the comorbidities which necessitated MV in the first place, as this may provide some predictive value.

Adaptation of these methods to more relevant infection models such as *Galleria mellonella* or EVPL will add more credence to these findings. The results of these experiments considered cytokines individually and demonstrated cytokine-mediated enhancements in planktonic growth and biofilm formation. A logical follow up would be to consider the effect of cytokines in combination, or in the presence of their effectors. There are myriad other pro and anti-inflammatory

cytokines that could be incorporated for further investigation, for example IL-2, GM-CSF, IL-4, IL-10, and IL-1ra. All of which have demonstrated the capability to enhance growth and proliferation of bacteria (Denis *et al*., 1991; Porat *et al*., 1991; Park and Skerrett, 1996; Hultgren *et al*., 1998; Kanangat *et al*., 2001). In the acute phase response to CAP, there are sharp elevations of IL-1ra, IL-6, IL-8, and IL-10, and panels such as this could provide a useful starting point for designing these experiments (Endeman *et al*., 2011). Even before considering more authentic models or the inclusion of effector immune cells, there is likely value in testing combined cytokine panels and their effects on growth and biofilm formation.

Existing published data on cytokine concentrations in circulation and localised at site of infection in VAP patients is still quite limited. There is known variability in circulating cytokine concentrations between individuals, healthy or otherwise (Dibbs *et al*., 1999; Biancotto *et al*., 2013; Valaperti *et al*., 2019). Given that VAP is secondary to existing illnesses that necessitate MV, it may be prudent to determine changes in growth over wider or at more specific concentrations to reflect those seen in these conditions.

The impact of subinhibitory antibiotic on cytokine-induced enhancement of bacterial growth warrants investigation due to expected troughs in antimicrobial therapy throughout use (Begg *et al*., 1999). If these troughs in antimicrobial concentration fall to subinhibitory levels, then bacterial growth would be expected to increase, and in the presence of an intense inflammatory component could lead to a spike in bacterial load and subsequent lung damage in VAP. Use of more informed selections of antimicrobial concentrations, based on the lowest levels examined in patients, may be worth exploring in future studies. This could potentially be explored by continuous culture, in which the antibiotic concentration

could be controlled to peak and trough over time, with measurements of the culture density taken as this fluctuates (Hendon-Dunn *et al*., 2018; Tonoyan *et al*., 2019).

Proposed mechanisms for these observations have been discussed previously. However, the experiments in this chapter have done little to help delineate the cause of changes in bacterial growth or biofilm formation upon exposure to cytokines. Potential mechanisms discussed include direct stimulation of bacterial signalling by cytokines. This may be as a whole or when broken into smaller peptide fragments. The ability of peptide fragments to enhance *S. aureus* growth was demonstrated by Kanangat *et al.* (2001). Besides cytokines or peptide fragments acting as receptor ligands, there is also the possibility of them acting directly upon DNA as a growth or transcription factor. Previously discussed was the demonstration of TNFα DNA binding activity by Mahdavi *et al.* (2013). This DNA binding may result directly in enhancement of growth depending on the resulting effect on the bacterial genome. A proposed experiment not included in this project due to time constraints was to characterise the transcriptome changes of populations of our VAP isolates upon exposure to proinflammatory cytokines using RNA-Seq (Wang *et al*., 2009). By comparing between cytokine concentrations and controls, it would be possible to compare gene expression profiles. This method could identify which specific pathways are being activated by cytokines, at which concentrations these effects manifest, and the intensity of these effects in response to different concentrations. Assuming that the mechanism of action is due to changes in gene expression, by DNA binding, action of peptide fragments as transcriptions factors, or other. This series of

investigations is one of the first that would be performed if this project were to continue.

To conclude, TNFα has been demonstrated to enhance planktonic growth of *S. aureus*, *K. pneumoniae*, and *P. aeruginosa in vitro*. Enhanced *S. aureus* growth was only observed with exposure to subinhibitory meropenem. TNFα was also demonstrated to enhance relative biofilm formation of *K. pneumoniae in vitro*. These responses were dose dependent and support existing findings. To draw more clinically relevant conclusions, these findings should be investigated further *in vivo*, whilst also considering the use of cytokine combinations and the inclusion of immune effectors in future studies.

Chapter 4 | Impact of HMI on AMR in VAP

4.1 | Introduction

The previous chapter explored the relationship between HMI and bacterial growth and biofilm formation. There was an existing precedent for these phenomena in the literature, as changes in bacterial growth and virulence upon exposure to cytokine *in vitro* had been observed. It may be that exposure to cytokines could change other phenotypic traits in bacteria. However, little attention has been directed towards potential changes in AMR due to HMI.

Presently, treatment of VAP accounts for as much as 50% of antibiotic use in ICU. VAP is most frequently treated with broad-spectrum antibiotics, due to the variability in causative species, which exerts great selective pressure onto bacterial populations within ICU (Nora and Póvoa, 2017). Deaths as a result of AMR, not limited to VAP, are estimated to increase to 10 million per year by 2050 (O'Neill, 2016). Due to the high number of prescriptions associated with VAP, its prevalence is of clear detriment to attempts to control the development of AMR in intensive care.

Gram-negative bacilli are the principal cause of respiratory infections in ICU, including VAP (Luyt *et al*., 2018). These bacteria are frequently resistant to all standard first line antibiotics used for pneumonia in intensive care (Park, 2005a). This is not a new phenomenon, and imipenem, long considered an antibiotic of last resort, has been routinely used for treatment of VAP since the early 2000s (Montero *et al*., 2004). In many cases, carbapenems are preferred to other therapies, such as rifampicin and colistin, even when treating *Acinetobacter baumannii*, a species frequently carbapenem resistant, reaching 90% in some

areas of the world (World Health Organization, 2018). Considering widespread use of carbapenems for treatment of VAP, one particularly problematic group of pathogens are carbapenemase-producing Enterobacteriaceae. In a study of an Egyptian ICU, rates of resistance in *Klebsiella* spp. isolates were 70.2% for imipenem, and 64.9% for ertapenem (Azzab *et al*., 2016). A study in 2019 found the efficacy of colistin to be comparable to meropenem as an empirical therapy, but did not recommend its use over meropenem due to complications related to nephrotoxicity (Cisneros *et al*., 2019). The climate around empirical treatment of VAP has long been accustomed to the use of antibiotics frequently considered last resort.

Even with an effective antibiotic available, time taken for selection and prescription results in increased morbidity and mortality. It is currently necessary to start patients on broad-spectrum antibiotics and de-escalate following microbiological results (Hunter, 2006). The optimal time to administer empirical therapy is not clear; most available data are from animal models and not wholly representative, but earlier administration has been linked to better outcomes (Swanson and Wells, 2013). VAP is presently excluded from NICE prescribing criteria for hospital-acquired pneumonia, so better guidance for handling empirical therapy is necessary (NICE, 2019).

Selection of empirical therapy in VAP is made more complicated by a lack of standardised diagnostic criteria. In a comparison of VAP rates determined by different scoring systems in the UK (CPIS, HELICS, CDC-NHSN), 50.9% of MV patients were receiving antibiotics without the attending clinician believing there was VAP (Wallace *et al*., 2015). Such zealous antibiotic usage will only serve to

contribute to the already problematic AMR profile of microorganisms implicated in VAP.

With high prevalence of MDR in VAP, there is an increased risk of treatment failure. Estimations of treatment failure in VAP vary but have been estimated to range between 30% and 62% (A. Iannella and M. Luna, 2012). The cause of treatment failure is not always obvious to clinicians, but is frequently a result of resistance to empirical therapy (Domínguez *et al*., 2006).

With MDR already presenting a clear issue in the clinical management of VAP, the potential for high inflammatory responses to modify this further could present a significant problem to clinicians. In hypothetical cases where elevated cytokine levels increase resistance, it may result in treatment failures due to inadequate therapy. In such cases patient inflammatory responses and cytokine levels would possibly inform prescribing decisions. The inverse case would be high inflammation increasing sensitivity, or low inflammation increasing resistance. In these cases, treatment failure may be a problematic outcome in those with very weak inflammatory responses. Particularly as a weak inflammatory response may result in reduced clearance of infection even before considering intervention.

4.1.1 | Chapter aims

This results chapter encompasses *in vitro* investigations into cytokine-induced changes in antimicrobial sensitivity and adaptation. Existing literature has focussed largely on the ability of cytokines to modify bacterial growth, and in some cases virulence. There has been little to no research into cytokine-induced changes in sensitivity as a phenotypic trait. The effects of IL-1β, IL-6, and TNFα

on the MIC of TZP, MEM, LVX and IPM for *Pseudomonas aeruginosa* was tested by modified broth microdilution. The effects of TNFα on the MIC of MEM for *Staphylococcus aureus* was tested by the same method. TNFα-induced changes in rate of adaptation of *S. aureus, Klebsiella pneumonia* and *P. aeruginosa* to MEM was tracked over 15 passages. This was performed by standard disc diffusion assay and picking and passaging the colonies closest to the MEM disc.

The aim of this chapter was to investigate the influence of proinflammatory cytokines on antimicrobial sensitivity of *P. aeruginosa* and *S. aureus*, and rate of adaptation in *S. aureus, K. pneumoniae,* and *P. aeruginosa*. This *in vitro* work was performed to address an identified knowledge gap. The role of cytokines in influencing antimicrobial sensitivity is not currently understood.

To address this aim, the following research questions were formulated. Can proinflammatory cytokines IL-1β, IL-6, and TNFα modify the antimicrobial sensitivity of *P. aeruginosa* and *S. aureus*? Does TNFα alter the rate of adaptation of *S. aureus, K. pneumoniae*, and *P. aeruginosa* to meropenem?

4.2 | Cytokine-induced changes in minimum inhibitory concentration of antibiotics

Changes in antimicrobial sensitivity of VAP isolates were investigated by a modified broth microdilution assay. Broth microdilution assay measures MIC of different antimicrobial compounds against a subject bacterial culture. Concentrations of the proinflammatory cytokines IL-1β, IL-6, and TNFα, selected to reflect different circulatory concentrations, were added to this assay to investigate potential influences on MIC.

4.2.1 | *Pseudomonas aeruginosa*

MICs are averaged from 3 replicates, and thus exact values may fall between tested doubling dilutions.

The MIC of TZP for *P. aeruginosa* ranged between 1.85μg/mL and 3μg/mL (Figure 4.1a). The MIC of TZP was not significantly affected by the addition of any tested concentration of IL-1β [$F(3,36) = 1$, $p = 0.4040$], IL-6 [$F(3,36) = 1.267$, $p = 0.3004$], or TNFα [$F(3, 36) = 2.804$, $p = 0.0535$].

The MIC of MEM for *P. aeruginosa* ranged between 0.028125μg/mL and 0.0875μg/mL (Figure 4.1b). The MIC of MEM significantly increased at the $p<.05$ level with the addition of 3000pg/mL TNFα when compared to TNFα-free, 30pg/mL, and 300pg/mL, increases of 133.3%, 154.5%, and 194.7% respectively [$F(3, 36) = 11.06$, $p < 0.0001$]. The MIC of MEM was not significantly affected by the addition of any tested concentration of IL-1β [$F(3, 36) = 1.732$, $p = 0.1778$], or IL-6 [$F(3, 36) = 1.392$, $p = 0.2609$].

The MIC of LVX for *P. aeruginosa* was 0.25μg/mL for all tested cytokine concentrations and across all replicates (Figure 4.1c). Consequently, the samples all had a standard error of zero, and the MIC was unchanged with any tested concentration of cytokine.

The MIC of IPM for *P. aeruginosa* ranged between 0.229167μg/mL and 0.375μg/mL (Figure 4.1d). There was no deviation in measurement of MIC (0.25μg/mL) with the addition of any tested concentration of IL-1β or TNFα. As such these samples had a standard error of zero. The MIC was also not significantly affected by the addition of any tested concentration of IL-6 [$F(3, 20) = 0.4496$, $p = 0.7203$].

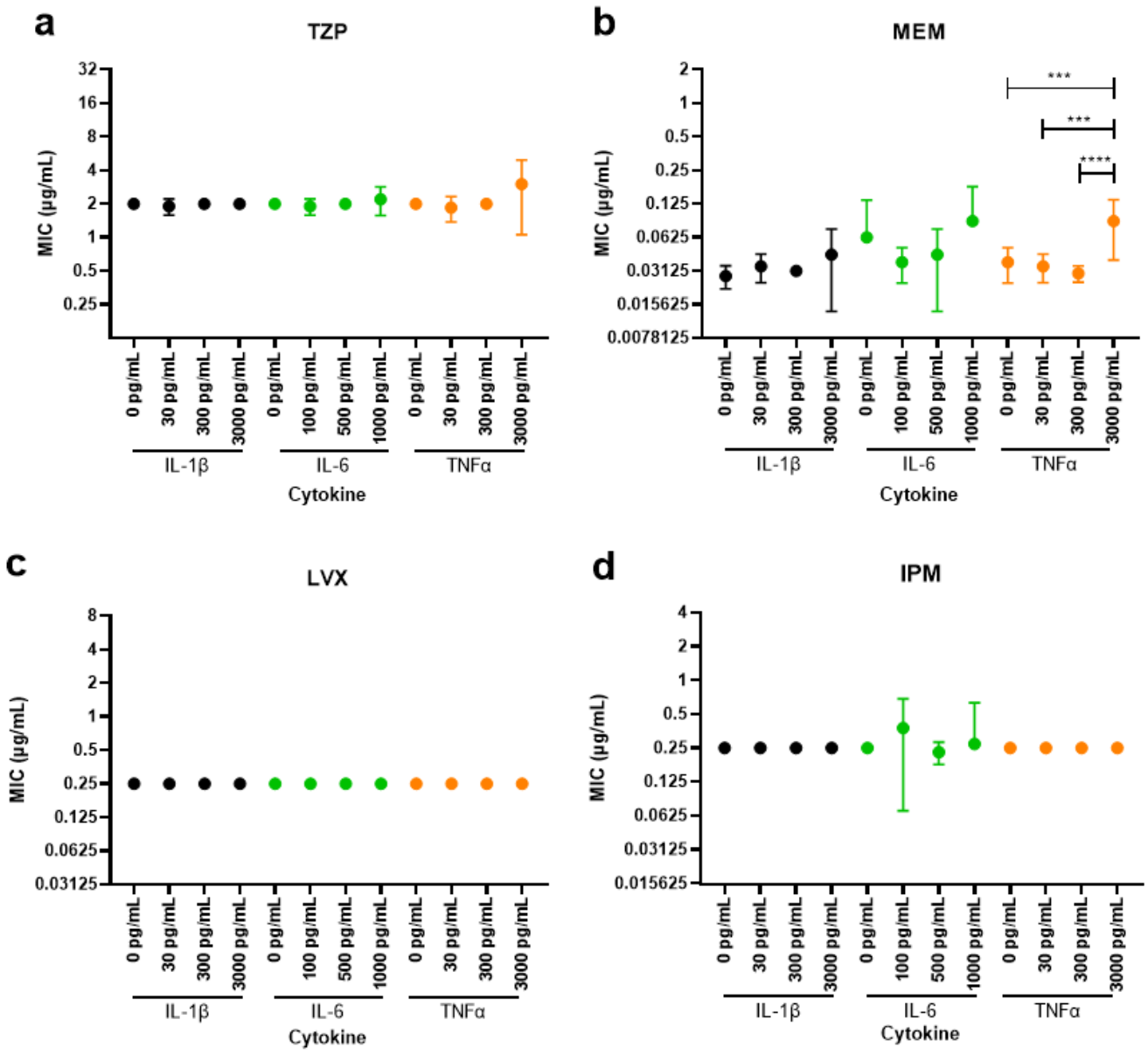


Figure 4.1: Changes in minimum inhibitory concentration (MIC) of antibiotics for *P. aeruginosa* in the presence of proinflammatory cytokines. A significant increase in the MIC of MEM (b) was observed with the addition of 3000pg/mL of TNFα ($p = 0.0006$ compared to 0pg/mL TNFα; $p = 0.0003$ compared to 30pg/mL; $p < 0.0001$ compared to 300pg/mL). Results of multiple comparisons with Tukey method. No significant changes in MIC of TZP, LVX or IPM were observe with the addition of IL-1β, IL-6, or TNFα.

a/b **n=2 technical, 5 biological** ±SD
c/d **n=2 technical, 3 biological** ±SD

TZP – piperacillin-tazobactam; MEM – meropenem; LVX – levofloxacin; IPM – imipenem

4.2.2 | *Staphylococcus aureus*

For *S. aureus,* only the specific potential for interaction between TNFα and meropenem was investigated. This was partly informed by results from *P. aeruginosa* MICs, growth, and biofilm formation, and partly from previously published literature. Both suggested a role for TNFα in modification of bacterial phenotype.

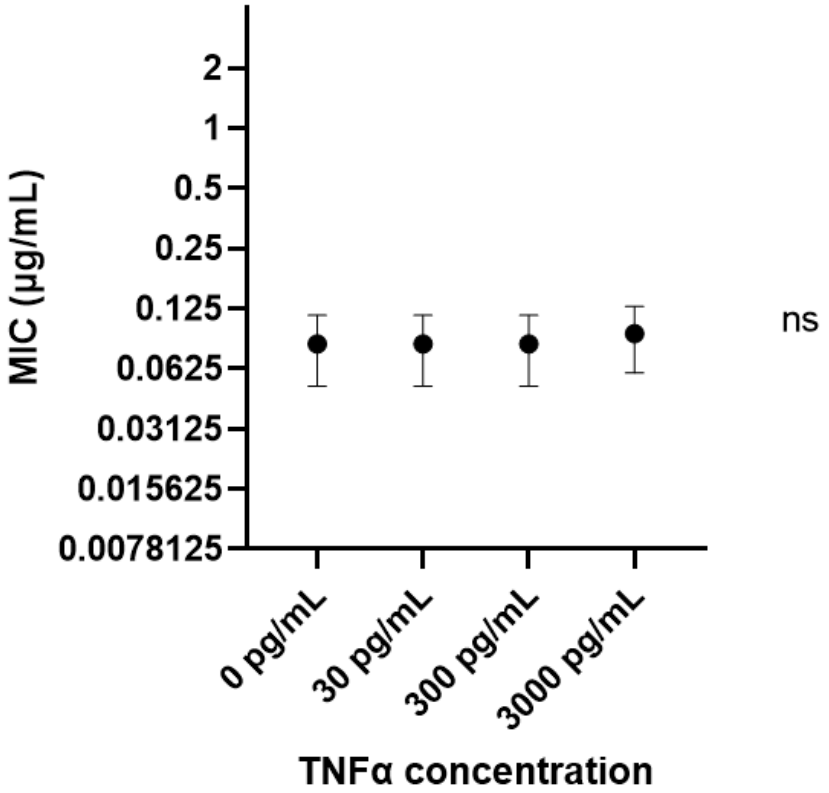


Figure 4.2: Effect of TNFα on MIC of MEM for *S. aureus*. The average MIC of MEM was 0.0859125µg/mL. There is no significant change in the MIC following the addition of TNFα (p = 0.9275). Results of multiple comparisons with Tukey method.

n=3 technical, 3 biological ±SD

MIC – minimum inhibitory concentration; MEM – meropenem

The MIC of MEM for *S. aureus* (Figure 4.2) ranged between 0.0833µg/mL and 0.09375µg/mL. The MIC of MEM was not significantly affected by the addition of

any tested concentration of TNFα [F (3, 20) = 0.1515, p = 0.9275]. This was unlike the result observed in *P. aeruginosa*, where TNFα significantly increased the MIC of MEM at very elevated concentrations (3000pg/mL).

4.3 | Influence of TNFα on adaptation rate of bacterial isolates to meropenem

TNFα influence on adaptation rates of bacteria to meropenem was measured by serial passage of bacteria onto MH agar incorporating TNFα. A 10µg meropenem disc was placed onto the agar surface, and a small sweep of bacteria (~5mm) growing closest to this disc was taken for each sub passage. Zone diameters were tracked over 15 passages and investigated for changes over time.

4.3.1 | *P. aeruginosa*

The adaptation of *P. aeruginosa* to meropenem was rapid (Figure 4.3). After one passage exposed to 10µg MEM, the zone diameter ranged between 33.3mm and 36mm across all plates. Following 3 passages exposed to 10µg MEM, the zone diameter of all concentrations had fallen to a range of 16mm to 18.66mm. The breakpoint for resistance of 18mm according to EUCAST (European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0, 2020). All test concentrations bar 300pg/mL (18.66mm) fell below this breakpoint by passage 3. There was a significant difference in zone diameter between TNFα-free and 3000pg/mL TNFα after two passages, a difference of 21.6% [p = 0.0143], but the rate at which clinical resistance was achieved did not differ significantly between TNFα concentrations.

A significant increase in zone diameter was observed, relative to TNFα-free, in the 30pg/mL, 300pg/mL, and 3000pg/mL TNFα groups at passage 6, increases of 83.3%, 125%, and 141.7% respectively [$p < 0.0001$]. However, the *P. aeruginosa* grown in this passage were taken from long-term frozen stocks stored at -80°C, as opposed to being picked directly from the previous passage. This was due to long-term closure of the lab which occurred between passages 5 and 6. This introduces potential extra variables related to cold shock or long-term storage. It is therefore impossible to determine if these differences in zone diameter reflect the effects of TNFα alone. There is a significant interaction between TNFα and rate of *P. aeruginosa* adaptation [$F (42, 120) = 1.993$, $p = 0.0020$], however the effect of cold shock on passage 6 may be responsible for this observation.

A spike in zone size is observed at passage 8. With zone diameters increasing from a mean of 14.66mm to 20.33mm. This spike is consistent between all TNFα concentrations however, with no significant differences in zone diameter. This increase in zone diameter took all measurements back above the resistance threshold of 18mm and into intermediate sensitivity, according to EUCAST. By passage 15, *P. aeruginosa* had completely adapted to MEM. All zone diameters measured 6mm, which is the diameter of the meropenem-impregnated disc set onto the agar.

The row factor, which in this case describes changes in the mean zone diameter between passages (i.e. that adaptation occurred over the 15 passages), was significant [$F (14, 120) = 156.6$, $p < 0.0001$].

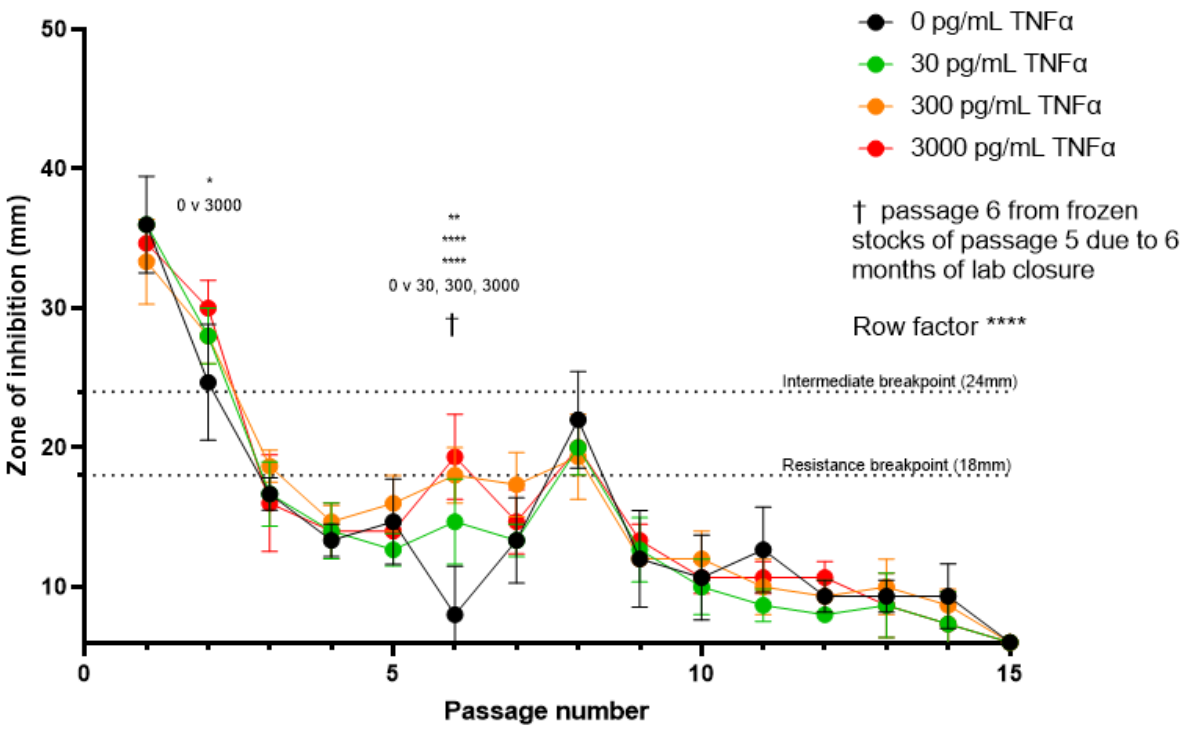


Figure 4.3: TNFα-mediated changes in rate of *P. aeruginosa* adaptation to 10µg meropenem (MEM). A significant increase in zone diameter was seen after passage 2 with 3000pg/mL TNFα compared to 0 pg/mL TNFα ($p = 0.0143$). Significant increases in zone diameter were seen after passage 6, with all concentrations of TNFα compared to TNF-free ($p < 0.0001$). However, *P. aeruginosa* at passage 6 were taken from frozen storage rather than picked directly from the previous passage, due to 6 months of lab closure †. Results of multiple comparisons with Tukey method. x intersects y at 6mm as this is the diameter of the antibiotic impregnated disc.

n=3 technical, 1 biological ±SD

4.3.2 | *S. aureus*

S. aureus did not adapt rapidly to meropenem (Figure 4.4). Over the first 9 passages, the mean zone diameter fell from 43.16mm to 33.33mm. There were significant differences in zone diameter between 30pg/mL, 300pg/mL, and 3000pg/mL TNFα after passage 4; 18.4% and 21% respectively [30pg/mL v 300pg/mL $p = 0.0256$; 30pg/mL v 3000pg/mL $p = 0.0077$]. A further significant difference in zone diameter between 30pg/mL and 300pg/mL TNFα was seen

after passage 6, a difference of 15.2% [p = 0.0256]. However, these significant changes in zone diameter reverted in both cases by the next passage [p = 0.6124, 0.1788; p = 0.6124]. Outside of these individual passages there is no significant interaction between any tested concentration of TNFα and rate of *S. aureus* adaptation [F (42, 120) = 1.079, p = 0.3659].

Unlike *P. aeruginosa*, there is no defined resistance breakpoint for *S. aureus* with meropenem. According to EUCAST breakpoint tables, meropenem sensitivity is inferred by cefoxitin, which was not used as part of this study (European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0, 2020).

There is a substantial drop in zone diameter between passages 9 and 10. There was a decrease in mean diameter from 33.33mm to 12.66mm. This drop is consistent between all concentrations of TNFα, with no significant differences between concentrations after passage 9 or passage 10. unlikelihood of all replicates of all concentrations adapting equivocally at the same passage outside factors were investigated. There was a change in meropenem discs used between passages 9 and 10, with those used after this passage being older 10μg MEM discs closer to their date of expiration. Frozen stocks from passage 5 were taken and adapted again through to passage 10 with these discs (Figure 4.5).

The row factor, which in this case describes changes in the mean zone diameter between passages, was significant [F (14, 120) = 242.2, p < 0.0001]. It can be concluded from this that *S. aureus* underwent some adaptation to MEM over the course of the experiment. However, this measurement may have been skewed by the sudden drop in average zone diameters between passages 9 and 10. Rate of

S. aureus adaptation had arrested after passage 2, and it is possible that the overall cytokine-mediated change in mean zone diameter would not have been significant without this drop.

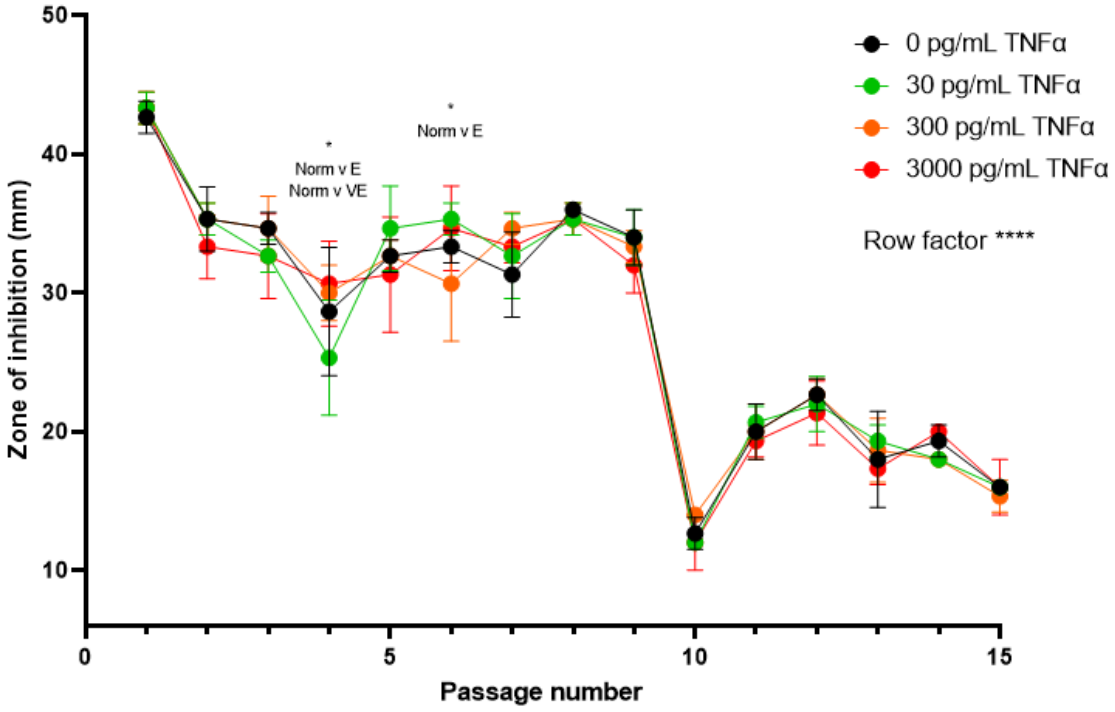


Figure 4.4: TNFα-mediated changes in rate of *S. aureus* adaptation to 10µg meropenem (MEM). A significant decrease in zone diameter was seen after passage 4 with 30pg/mL TNFα compared to 300pg/mL (p = 0.0256) and 3000pg/mL TNFα (p = 0.0077). After passage 6, the zone diameter with 30pg/mL was significantly increased compared to 300pg/mL (p = 0.0256). Results of multiple comparisons with Tukey method. There is a substantial drop in zone diameter after passage 10 across all TNFα concentrations, but there are no significant differences in zone measurements. This drop is suspected to be the result of older antibiotic discs closer to their date of expiration. x intersects y at 6mm as this is the diameter of the antibiotic impregnated disc.

n=3 technical, 1 biological ±SD

The average zone diameter between passage 5 and passage 10 when retesting older 10μg MEM discs fell from 32.88mm to 14.5mm. The rate of adaptation was gradual, without significant differences in zone diameter between TNFα concentrations [$F (15, 48) = 1.201$, $p = 0.3037$]. The adaptation rate contrasts with the newer 10μg MEM discs, where between passages 5 and 9, the adaptation of *S. aureus* had stalled. By passage 10 with the discs closer to expiration, the average zone diameter had fallen to 14.5mm, which mirrors the drop in zone diameter to 12.66mm seen after passage 10 when switching from newer discs. This difference in capability to arrest adaptation of *S. aureus* to MEM may explain the sudden, sharp, and consistent drop in zone diameter across all TNFα concentrations seen in Figure 4.4. The row factor, which here describes changes in mean zone diameter between passages, was significant [$F (5, 48) = 130.3$, $p < 0.0001$].

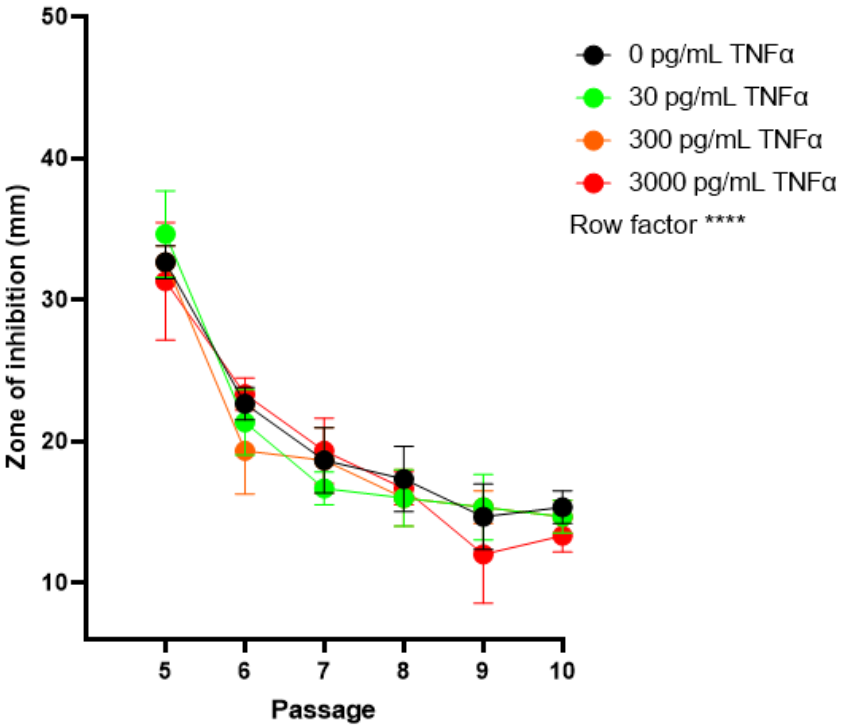


Figure 4.5: Passages 5 through 10 of *S. aureus* adaptation repeated using older 10µg meropenem (MEM) discs. Average zone diameter fell from 32.88mm to 14.5mm. There is no significant difference in zone diameter or rate of adaptation between TNFα concentrations (p = 0.3037). Results of multiple comparisons with Tukey method. x intersects y at 6mm as this is the diameter of the antibiotic impregnated disc.

n=3 technical, 1 biological ±SD

4.3.3 | *K. pneumoniae*

The rate of adaption of *K. pneumoniae* to meropenem was slow (Figure 4.6). At passage 8, the average zone diameter had fallen to a measurement of intermediate sensitivity, defined by a zone diameter breakpoint of 22mm (20.5mm). The zone diameter for 3000pg/mL TNFα (23.33mm) was significantly higher than TNFα-free (18mm) at passage 8, a difference of 29.6% [p = 0.0146]. Outside of this passage, there was no significant interaction between addition of

TNFα at any tested concentration and rate of adaptation [F (42, 120) = 1.051, p = 0.4069].

The row factor, which describes changes in the mean zone diameter between passages, was significant [F (14, 120) = 84.64, p < 0.0001]. It can be concluded that *K. pneumoniae* adaptation to MEM over the course of the experiment was significant.

As seen before in *S. aureus*, there is a marked drop in average zone diameter between passage 9 and passage 10, from 25.66mm to 13.83mm. This drop took the average zone diameter of *K. pneumoniae* from a measurement of clinical sensitivity to clinical resistance. However, in the remaining passages, the average zone diameter remained within the intermediate bracket. This drop in zone diameter, as for *S. aureus*, coincided with the usage of older 10µg MEM discs. As with *S. aureus*, frozen stocks of *K. pneumoniae* from passage 5 were taken and adapted again through to passage 10 with the older discs to investigate (Figure 4.7).

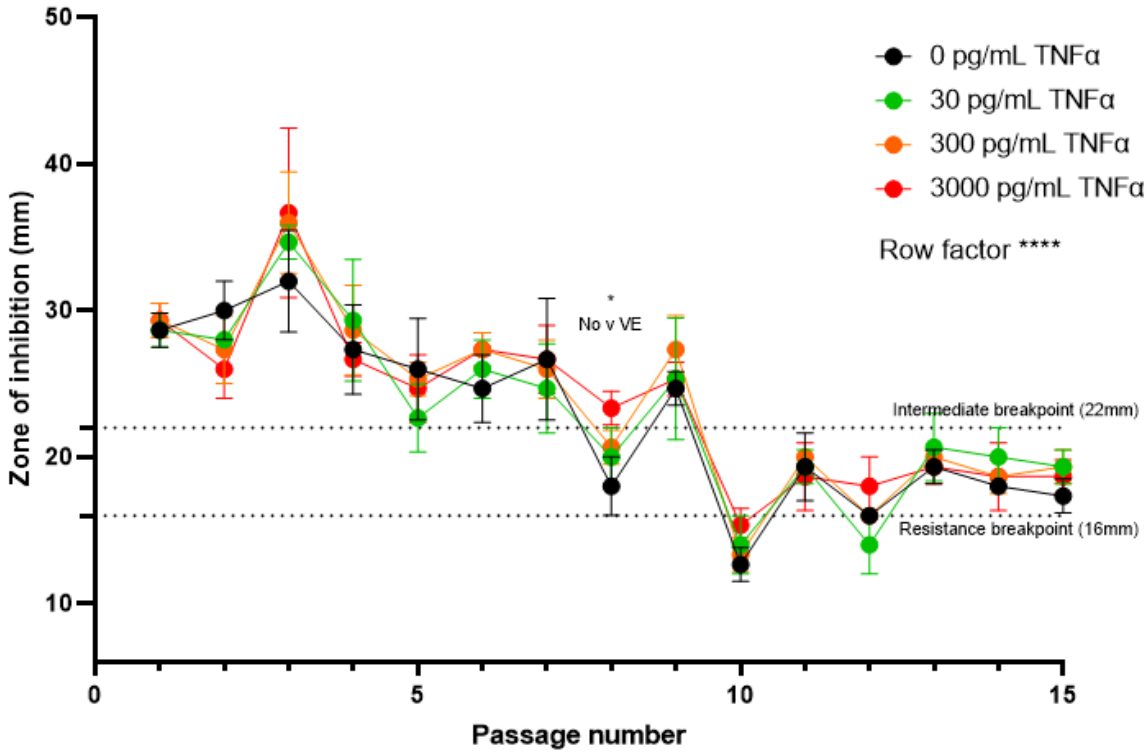


Figure 4.6: TNFα-mediated changes in rate of adaptation of *K. pneumoniae* to 10µg meropenem (MEM). A significant difference in zone diameter was seen after passage 8 with 3000pg/mL TNFα compared to TNFα-free (p = 0.0146). Results of multiple comparisons with Tukey method. There is a substantial drop in zone diameter after passage 10 across all TNFα concentrations, but there are no significant differences in zone measurements. This drop is suspected to be the result of older antibiotic discs closer to their date of expiration. x intersects y at 6mm as this is the diameter of the antibiotic impregnated disc.

n=3 technical, 1 biological ±SD

The rate of adaptation was sharp between passages 5 and 6 when retesting older 10µg MEM discs, where the average zone diameter fell from 24.66mm to 16mm. Rate of adaptation decreased following passage 6, with average zone diameter falling from 16mm to just 13.33mm by passage 10. There were no significant changes in rate of adaptation between TNFα concentrations [F (15, 48) = 0.9882, p = 0.4819]. These results contrast those with newer 10µg MEM discs, where

adaptation had stalled between passages 5 and 9. As in *S. aureus*, this difference in capability to arrest the adaptation of *K. pneumoniae* is a probable explanation for the sudden and sharp drop in zone diameter across all TNFα concentrations in Figure 4.6.

The row factor, which here describes changes in mean zone diameter between passages, was significant [F (5, 48) = 32.02, p < 0.0001].

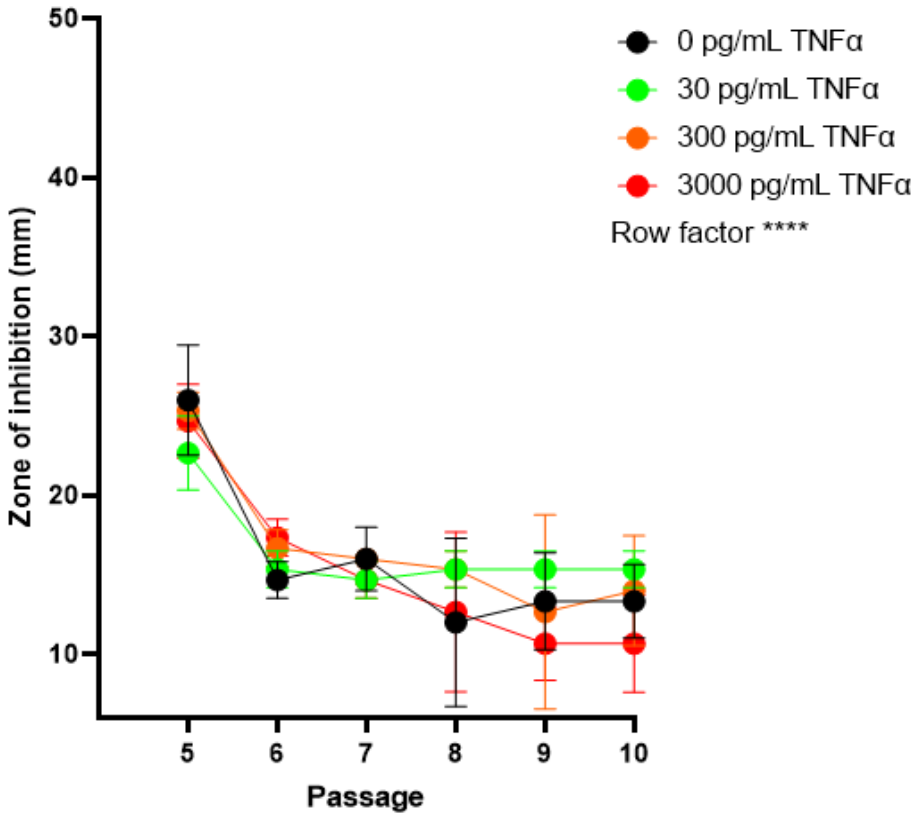


Figure 4.7: Passages 5 through 10 of *K. pneumoniae* adaptation repeated using older 10µg meropenem (MEM) discs. Average zone diameter fell from 24.66mm to 13.33mm. There is no significant difference in zone diameter or rate of adaptation between TNFα concentrations (p = 0.4819). Results of multiple comparisons with Tukey method. x intersects y at 6mm as this is the diameter of the antibiotic impregnated disc.

n=3 technical, 1 biological ±SD

4.4 | Summary of key chapter data

This chapter's first set of findings demonstrate a significant change in *P. aeruginosa* sensitivity to meropenem, mediated by TNFα. TNFα did not modify sensitivity of *P. aeruginosa* to piperacillin-tazobactam, levofloxacin, or imipenem.

In *P. aeruginosa*, 3000pg/mL TNFα significantly increased the MIC of meropenem [$p < 0.0001$] compared to all other concentrations tested. This interaction did not appear dose dependent. There were no significant differences between 0pg/mL,30pg/mL, and 300pg/mL TNFα.

In the chapter's latter results, there was little evidence that TNFα significantly increases or decreases rate of adaptation of *S. aureus, K. pneumoniae*, or *P. aeruginosa* to meropenem.

In *P. aeruginosa*, adaptation was rapid and consistent between each concentration of TNFα. TNFα was demonstrated to modify MIC of meropenem for *P. aeruginosa*, but it does not appear to have a significant effect on the rate at which resistance develops.

In *S. aureus*, the rate of adaptation was slow and largely consistent between each TNFα concentration. It is not possible to determine at which passage clinical resistance had developed, as EUCAST do not publish these figures for *S. aureus*. Instead, *S. aureus* resistance to meropenem is inferred by cefoxitin (European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0, 2020). The zone diameter of *S. aureus* incubated on 30pg/mL TNFα is significantly lowered at passage 4, but significantly elevated at passage 6. There is little consistency in these findings, and it is possible that the substantial drop in zone diameter after

passage 4 is erroneous, as it reverts by the following passage. It does not appear that any tested TNFα concentration significantly changes the rate of adaptation of *S. aureus* to meropenem.

In *K. pneumoniae*, adaptation was slow and with little significant difference between TNFα concentrations. At passage 8 there was a significant difference between 0pg/mL and 3000pg/mL. By the next passage however, there was no significant difference between TNFα concentrations, and average zone diameter had risen back above the threshold measurement of clinical sensitivity. It does not appear that any TNFα concentration tested significantly changes the rate of *K. pneumoniae* adaptation to meropenem.

4.5 | Chapter discussion

The relevance of these results in the context of VAP is in treatment. If an individual is subject to a strong inflammatory response, these results suggest a higher dosage of meropenem may be required for their treatment. It is likely that patients on MV in intensive care have elevated inflammatory responses. Serum concentrations of TNFα are highly elevated in ARDS patients compared to pneumonia patients and controls (Bauer *et al*., 2000). Severe trauma patients, i.e. those considered to have complications according to injury severity score, have significantly higher levels of circulating IL-6 and IL-1ra (Binkowska *et al*., 2018). There may be potential for elevated TNFα levels to be used as a biomarker to inform prescribing behaviours. There is already precedent for cytokine fingerprints in diagnostics, for infectious and non-infectious disease (Lippitz, 2013; Shen *et al*., 2018; Clifford *et al*., 2019). Such decisions depend on a better understanding of

the acute immune response to *P. aeruginosa* infection. At present, this is lacking, and there is a suggestion in murine models that MDR *P. aeruginosa* may induce a weaker cytokine response than a more sensitive strain (Gómez-Zorrilla *et al*., 2017). This suggests that MDR comes at a cost of reduced virulence, and that the strains inducing the strongest TNFα responses may be more sensitive to treatment anyway. These findings by Gómez-Zorilla (2017) were in murine models of intraperitoneal infection however and may not be the most reflective of the lung.

Alternatively, these results may suggest that alternatives to meropenem should be preferred. A recent study demonstrated the comparable efficacy of ceftazidime-avibactam to meropenem for Gram-negative pathogens (Torres *et al*., 2018). The widespread use of carbapenems in the treatment of VAP has already been discussed (Sections 1.5 and 4.1), and adoption of carbapenem-sparing strategies is prudent.

The lack of change in TNFα-mediated MIC of meropenem for *S. aureus* may suggest that the mechanism underlying the results in *P. aeruginosa* is specific to Gram-negative bacteria. These groups of bacteria differ considerably in their mechanisms of resistance (Woodford, 2005; Exner *et al*., 2017; Breijyeh *et al*., 2020). As a β-lactam, the mode of action of meropenem is through covalent binding to penicillin binding proteins in cell wall synthesis (Wiseman *et al*., 1995). Whilst meropenem is active against both Gram-positive and negative organisms, Gram-negative organisms can develop resistance through impermeability, a physiological difference between these groups (Zgurskaya *et al*., 2016). It may be through enhancement of a Gram-negative-specific resistance mechanism that TNFα increases MIC of meropenem for *P. aeruginosa*. Alternatively, TNFα-induced changes in MIC of antibiotics for Gram-positive organisms might simply

not apply to meropenem. This was the only antibiotic investigated in *S. aureus*, and expansion of the panel of antibiotics tested may show TNF-induced changes in MIC in other antibiotic classes.

These results provide useful initial *in vitro* data on a potential role of cytokines in mediating AMR. The adoption of relevant *in vivo* models to expand and continue this work would help to build a more concrete understanding of this potential interaction. As to our knowledge, this is not a phenomenon that has been demonstrated in literature. Considering the effects TNFα may have on the sensitivity of VAP pathogens to common antimicrobial treatments in an environment truer to that seen in VAP would be of benefit. If this could be demonstrated using *in vivo* mammalian models or patient samples, it could potentially begin to inform changes to current prescribing practices in intensive care. One way this could be modelled would be by inoculating mammalian host tissues i.e. ex vivo lungs, incubating in cytokine and antibiotic, and tracking acquisition of resistance by subculturing onto agar with antibiotic disks. This thesis began to explore cytokine-mediated changes in antibiotic adaptation rate *in vivo*, described in Chapter 5.

Rapid acquisition of *P. aeruginosa* resistance to meropenem in adaptation experiments is not surprising; the capability for carbapenem resistance in *P. aeruginosa* is well documented. Many strains acquire resistance through restricting diffusion of carbapenems into the cell, frequently through induced deficiency of the OprD porin (El Amin *et al*., 2005; Baumgart *et al*., 2010). This porin has been described as carbapenem-specific, and its localisation in the outer membrane means that if lost, permeability to carbapenems is severely reduced (Gutiérrez *et al*., 2007; Kao *et al*., 2016). Plasmid-mediated resistance is another

mechanism for the rapid acquisition of resistance. In this case, plasmids encoding resistance genes can be transferred between cells through conjugation (Poole, 2011). This occurs when a cell carrying the plasmid exists within the population, allowing it to spread the encoded resistance gene throughout. In this case, all *P. aeruginosa* used for adaptation were cultured from the same stock. Thus, unless plasmid carrying cells were introduced from an external source, it is unlikely these genetic elements would exist in the culture. If they did, *P. aeruginosa* should not expected to be sensitive to meropenem at passage 1.

In the future. given the phenomena observed after passage 6 in *P. aeruginosa* adaptation, it would be prudent to account for the potential effects of cold shock in cases were frozen stocks are used between passages. The understanding of the cold shock response in bacteria is not as great as the heat shock response. Exposure to cold inhibits protein synthesis outside those part of the cold shock response, by impairing movement of the DNA polymerase or the ribosome (Inouye and Phadtare, 2008). This may have knock on effects on resistance, as this could change the abundance of proteins governing resistance, or protein targets of antimicrobials. Accounting for this effect in our study may entail growing bacteria from stocks onto solid culture before picking for the next passage. This could arguably be considered an additional passage, with the removal of antimicrobial pressure and the potential changes in sensitivity this presents. It may be most appropriate to restart from passage 1 in cases where the experiment is paused for long periods.

There are a number of potential causes for sudden changes in zone diameter at particular passages, e.g. passage 8 in *P. aeruginosa*. Discs may not have been applied firmly onto the agar surface; the agar may have been poured too thinly on

these plates allowing for greater diffusion of meropenem throughout the media; the discs may have lower meropenem content than stated; or the plates may be poorly streaked (Troubleshooting Guide for Disk Diffusion Susceptibility Testing, 2016).

The failure of *S. aureus* to adapt rapidly to meropenem has precedence in literature. Carbapenem resistance is frequently encountered in MRSA, in which the *mecA* gene confers broad-spectrum β-lactam resistance (Chambers, 1999). MRSA is common in VAP, and is implicated in a significant number of Gram-positive cases (Chi *et al*., 2012). Based on the sensitivity of the *S. aureus* isolate used in this project to the β-lactams meropenem, cefuroxime, and cefotaxime, it is unlikely to carry *mecA*. This would go some way to explaining its slow development of meropenem resistance. This may represent a limitation in the experimental design, and investigation of the effects of cytokine on the rate of adaptation of other antibiotics may warrant further investigation. Especially when considering the potency of carbapenems and their stability against other common resistance determinants, such as extended-spectrum β-lactamases (Nicolau, 2008).

The sudden and stark drop in *S. aureus* zone diameter across all TNFα concentrations following passage 10 has already been subjected to scrutiny. It is likely based on the observed drop in average zone diameter that the older, alternative discs contained less active meropenem, despite both sets of discs used stating they contained 10μg. This explanation for a sudden surge in resistance is accounted for in disc diffusion troubleshooting guides (Troubleshooting Guide for Disk Diffusion Susceptibility Testing, 2016). It is exceptionally unlikely to be a consequence of the effects of TNFα, or a genuine

sporadic acquisition of meropenem resistance, as differences in zone diameter between all replicates with all TNFα concentrations remained insignificant, with minimal error. Beyond this drop in zone diameter, the differences between TNFα concentrations remained insignificant to termination of experiment. Stocks of passage 5 were used to repeat passages through to 10 with these alternative discs. Here, unlike in the initial experiment, the meropenem discs were incapable of arresting *S. aureus* adaptation, further suggesting their inadequacy. This drop in zone diameter between passages 9 and 10 is also seen in *K. pneumoniae*. This is believed to be the consequence of older, inadequate meropenem discs, as for *S. aureus*.

There is great precedent for *K. pneumoniae* resisting carbapenem antibiotics like meropenem. Clinically, carbapenem-resistant *K. pneumoniae* are extremely problematic. These bacteria are highly resistant to a majority of antibiotics, and are associated with high mortality rates, reportedly as high as 48% (Patel *et al*., 2008; Nordmann *et al*., 2009; Munoz-Price *et al*., 2013). Carbapenem-resistant *K. pneumoniae* are associated with resistant borne from plasmid-encoded carbapenemases. Carbapenem resistance rates have increased significantly since 2015, although rates in the UK are lower than averages from the EU and European economic area (European Centre for Disease Prevention and Control, 2019). Carbapenemase resistance has steadily increased worldwide due to the spread of carbapenemase genes OXA-48, and metallo-β-lactamases NDM-1 and VIM (Poirel *et al*., 2012; Dortet *et al*., 2014; Peirano *et al*., 2014). Given the sensitivity of the *K. pneumoniae* strain used in this study to the β-lactams meropenem, imipenem, cefuroxime, and cefotaxime, it can be inferred that it is not carbapenemase resistant. This would explain how it largely failed to adapt to

meropenem, and as for *S. aureus*, it may be prudent to investigate the effects of cytokine on the rate of adaptation of other antibiotics. The fact that elevated levels of TNFα don't incur increased adaptation or resistance to meropenem in *K. pneumoniae* may provide some relief to those dealing with these organisms in intensive care.

To conclude, TNFα has been demonstrated to decrease sensitivity of *P. aeruginosa* to meropenem *in vitro*, at high concentrations. No capability of TNFα to modify the MIC of meropenem for *S. aureus* or *K. pneumoniae* was documented. Furthermore, TNFα was not shown to significantly affect the rate of adaptation of *P. aeruginosa*, *S. aureus*, or *K. pneumoniae* to meropenem. Based on the lower intrinsic resistance of *S. aureus* and *K. pneumoniae* to carbapenem antibiotics, investigation into changes in sensitivity and adaptation rate with other antibiotic groups are worthy of consideration. meropenem is highly potent in its mode of action, and offers increased stability compared to non-carbapenem β-lactam antibiotics (Nicolau, 2008). Studies on changes in *S. aureus* and *K. pneumoniae* sensitivity incorporating more cytokines than just TNFα also warrant consideration, as the mode of action for TNFα changing sensitivity in *P. aeruginosa* may not be cross-applicable between species. These results require further application to *in vivo* models, to better understand their clinical relevance. If certain levels of inflammatory cytokine, or patterns in inflammatory cytokines can be correlated to changes in specific antimicrobial sensitivity profiles, it may improve selection of empirical treatments. This could result in improvements in patient outcomes, development of AMR, and frontline carbapenem use.

Chapter 5 | *In vivo* modelling of antibiotic adaptation

5.1 | Introduction

In Chapter 4, the capability of TNFα to modify bacterial adaptation to meropenem was investigated *in vitro*. The results of the previous chapters represent useful early data, but were produced under controlled *in vitro* conditions, and are not truly reflective of the disease state in VAP.

Galleria mellonella is the greater wax moth, and their larvae have been adopted as a useful model for investigating *in vivo* infection (Cotter *et al*., 2000). This model has been increasing in popularity compared to traditional mammalian models due to its compliance with the "3 Rs" rule in animal experimentation: reduce the number of animals used in the laboratory; refine protocols to reduce animal pain and discomfort; and replace animals for models without bioethical problems (Trevijano-Contador and Zaragoza, 2014). This model is inexpensive, simple to use, does not require specialist equipment, survives at a range of incubation temperatures, and is not ethically contentious (Aperis *et al*., 2007; Ramarao *et al*., 2012). *G. mellonella* can be utilised for the investigation of microbial virulence, host immune response to infection, and to investigate drug efficacy and toxicity.

The *G. mellonella* immune response is comprised of a cellular response and a humoral response (Figure 5.1) (Pereira *et al*., 2018). The primary component of the cellular immune response are haemocytes, phagocytic cells which produce antimicrobial compounds (Trevijano-Contador and Zaragoza, 2019). These cells are responsible for phagocytosing infectious cells, nodulation, encapsulation, and melanisation (Tojo *et al*., 2000; Satyavathi *et al*., 2014). Haemocytes differentiate

from prohemocytes, small basophilic cells with a large nucleus (Browne *et al*., 2013). The other classes of haemocytes include plasmatocytes, granular cells, coagulocytes, spherulocytes, and oenocytoids. Oenocytoids are haemocytes which contain cytoplasmic phenoloxidase, and are responsible for the melanisation of haemolymph during nodule formation (Altincicek *et al*., 2008).

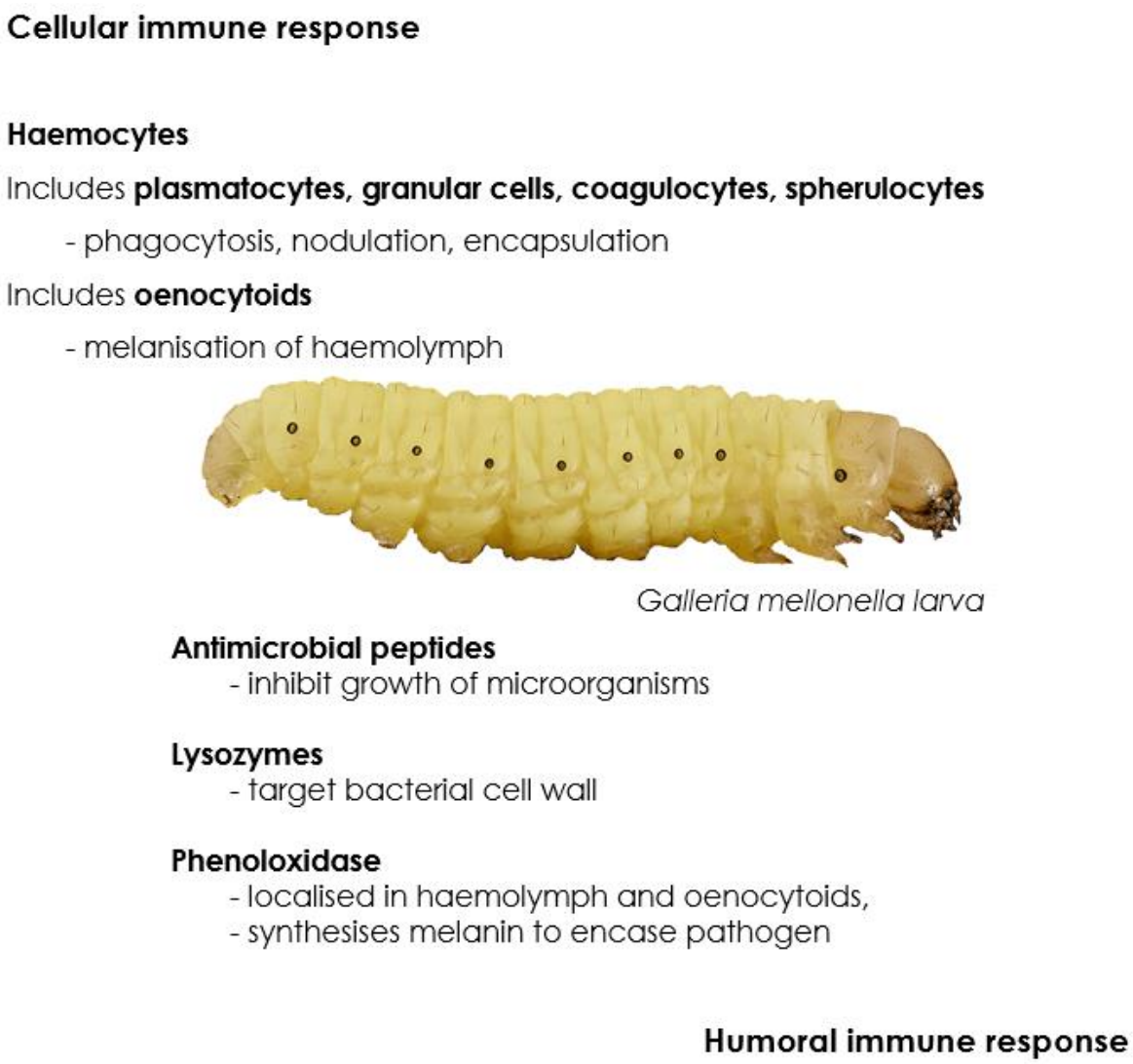


Figure 5.1: Components of the *Galleria mellonella* immune response. Cellular components (top) are governed by different classes of haemocyte, phagocytic cells which are sub-specialised into classes which secrete antimicrobial compounds. Humoral components (bottom) are soluble effectors, which have antimicrobial properties or support nodule formation and encasement of pathogens.

The humoral response comprises numerous soluble effector components. The major constituent of this response are antimicrobial peptides, which are released into the haemolymph from the fat body after contact with microorganisms (Zasloff, 2002). These peptides are responsible for inhibiting growth of microorganisms, including bacteria through inhibition of cell wall synthesis (Cytryńska *et al*., 2007; Kawaoka *et al*., 2008). Lysozymes are antimicrobial enzymes found in haemolymph of numerous insect orders, and damage bacterial cells through hydrolysis of β-1,4-glycosidic linkages (Lockey and Ourth, 1996; Vogel *et al*., 2011; Sowa-Jasiłek *et al*., 2014). The production of melanin in response to foreign matter results in accumulation of nodules, which encase microorganisms and prevent spreading (Ratcliffe *et al*., 1985; Götz, 1986). Phenoloxidase is localised in haemolymph and haemocytes and is activated by the detection of microbial cell walls (Söderhäll and Smith, 1986). Synthesis of melanin is catalysed by phenoloxidase, which oxidises phenols to produce quinones, which polymerise into melanin.

G. mellonella have been used as a successful infection model for studying numerous Gram-positive and Gram-negative bacteria. This model has been used to demonstrate the dose-dependent virulence of *Staphylococcus aureus* (Desbois and Coote, 2011). This study also demonstrated temperature-dependent virulence, with increasing temperatures up to 37°C resulting in greater larval death. Additionally, Desbois and Coote (2011) demonstrated the capability for this model to test drug efficacy, as survival was increased with the addition of daptomycin and vancomycin. *G. mellonella* have also been successfully employed for the targeted investigation of specific virulence factors, as SecDF knockout *S. aureus* were shown to be less virulence in *G. mellonella* (Quiblier *et al*., 2013).

Utilisation of *G. mellonella* as a model for *Klebsiella pneumonia* infection is more recent. This model was found to produce similar host responses to rodent pneumonia models, and survival rates are extremely variable depending on strain (Insua *et al*., 2013). *G. mellonella* have successfully been used for modelling HMI in *K. pneumoniae* infection. Lactate dehydrogenase was identified as a biomarker of cell damage and bacterial proliferation in *G. mellonella*, and levels of lactate dehydrogenase correlated with larvae survival (Wand *et al*., 2013). Use of *G. mellonella* for studying carbapenemase-producing *K. pneumoniae* is limited due to poor homogeneity with humans. In *G. mellonella*, these strains are reduced in virulence, which does not correlate to the disease severity observed clinically (McLaughlin *et al*., 2014).

G. mellonella have been used for the study of *Pseudomonas aeruginosa* for a long time, and numerous studies have explored the relationship between *P. aeruginosa* virulence factors and the *G. mellonella* immune response (Andrejko and Mizerska-Dudka, 2011; Mizerska-Dudka and Andrejko, 2014). This model has also been used to investigate the efficacy of antimicrobials for the treatment of *P. aeruginosa* infections (Hill *et al*., 2014). However, *P. aeruginosa* is extremely virulent in *G. mellonella*. In their study, Hill *et al.* (2014) demonstrated that a dosage as small as 25 CFU resulted in total larval death within 24 hours. In other publications, *P. aeruginosa* has a cited LD_{50} as low as 10 CFU (Jander *et al*., 2000). Many studies into *P. aeruginosa* have involved injecting with purified virulence factors of interest. Studies using *P. aeruginosa* are thusly limited in their duration.

Results of studies in larvae of *G. mellonella* largely correlate well to findings in mammalian models and human studies (Kavanagh and Sheehan, 2018). Recently, the genome of *G. mellonella* was sequenced for the first time. Access to

this will allow for homology between *G. mellonella* studies and those in human, murine, and other invertebrate or mammalian models to be better understood in the future (Lange *et al*., 2018). It is my belief that *G. mellonella* represent a robust and suitable model for further investigation of the findings of Chapters 3 and 4.

5.1.1 | Chapter aims and objectives

This results chapter describes numerous *in vivo* investigations utilising the *G. mellonella* virulence model. This model is well established and has been utilised for studies into virulence, immune responses, drug efficacy, and drug toxicity. However, to our knowledge, this model has never been used for the adaptation of bacteria to antimicrobials *in vivo*. The ability for TNFα to modify the adaptation of *S. aureus* to meropenem was tested in the haemolymph of *G. mellonella* larvae. This was passaged through different larvae, and development of resistance over generations was tracked through disc diffusion assay of haemolymph spread onto solid media.

The aim of this chapter was to apply the results of adaptation experiments in Chapter 4 to an *in vivo* model. To develop a method for this *in vivo* adaptation assay, some preliminary investigations were performed in *G. mellonella* larvae. The virulence of each of our isolates of *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* to *G. mellonella* was investigated. Extra scrutiny was given to *P. aeruginosa*, due to its highly virulent nature in *G. mellonella* larvae (Hill *et al*., 2014). Following this, the doses of TNFα and meropenem tolerated by *G. mellonella* were established. With the results of these investigations, it was

possible to devise a method for adaptation of bacteria *in vivo* in *G. mellonella* haemolymph.

To address this aim, the following research questions were formulated. How virulent are our VAP isolates of *S. aureus, K. pneumoniae,* and *P. aeruginosa* to larvae of *G. mellonella*? Is this model suitable for the investigation of our isolate of *P. aeruginosa*? What concentrations of TNFα, meropenem, and *S. aureus* are tolerated by *G. mellonella*? Does TNFα alter the rate of adaptation of *S. aureus* to meropenem *in vivo*, and how do these results compare to *in vitro* findings?

5.2 | *G. mellonella* waxworm virulence assay

Survival rates of *G. mellonella* following inoculation with 5µL of 0.1 OD_{600} *S. aureus, K. pneumoniae*, and *P. aeruginosa* VAP isolates are shown in Figure 5.2. *P. aeruginosa* was the most virulent species, with 0% of larvae surviving 1 day following inoculation. *K. pneumoniae* was less virulent, with 50% surviving to termination of experiment. There was an initial drop in larvae survival with *K. pneumoniae*, with 75% surviving following 2 days. However, death of larvae arrested at this percentage until day 7.

S. aureus was the least virulent species, with an initial slow rate of death, increasing closer to termination of experiment on day 8. By this point, 56.25% of larvae had survived, as rate of death increased. This demonstrates similar overall virulence between *S. aureus* and *K. pneumoniae* over the full duration of experiment.

The experiment was terminated on day 8 following cumulative deaths in the PBS control group, causing the surviving percentage of larvae to reach the threshold of 87.5% survival (2 dead individuals).

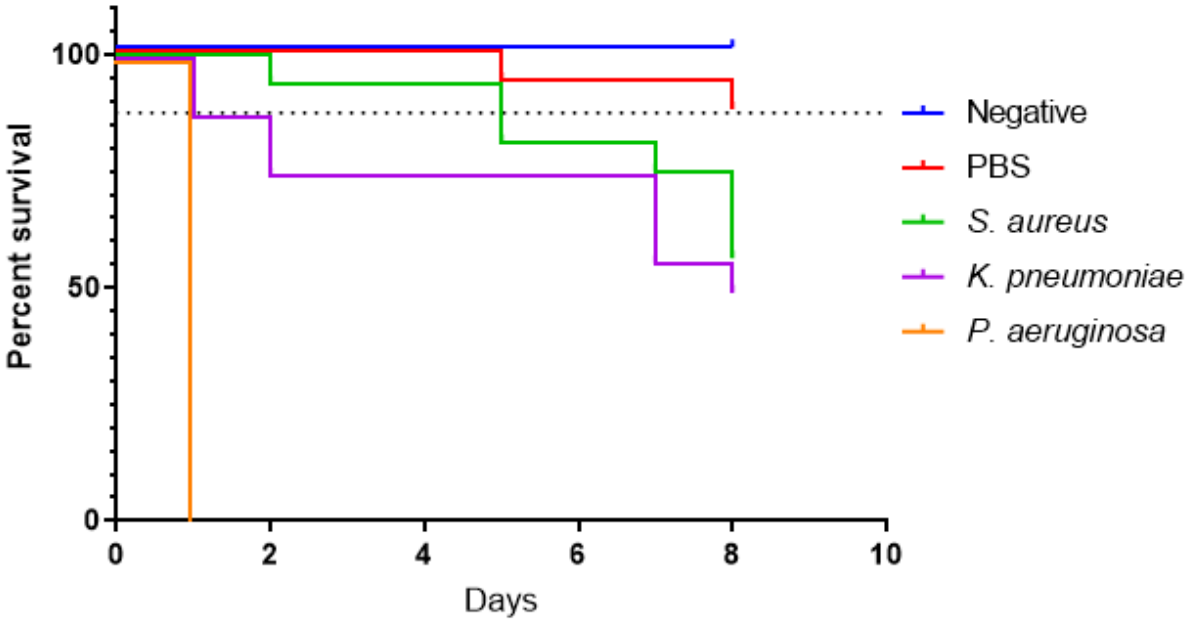


Figure 5.2: Survival of *G. mellonella* following infection with *S. aureus*, *K. pneumoniae*, or *P. aeruginosa*. Inoculation with *P. aeruginosa* resulted in 100% larval death within 1 day of infection. *S. aureus* and *K. pneumoniae* are less virulent by comparison. By termination of experiment on day 8, 56.25% of larvae inoculated with *S. aureus* and 50% of those with *K. pneumoniae* had survived. Experiment terminated on day 8 due to PBS survival reaching threshold of 87.5% (2 larval deaths). The dotted line is the threshold for experiment termination following cumulative deaths in negative control groups. Negative group were not inoculated at all, PBS group is mock inoculum control.

16 subjects per group, 1 biological replicate

PBS – phosphate buffered saline

Data sets offset slightly to prevent overlapping and improve readability

5.2.1 | *G. mellonella* waxworm virulence assay investigation of *P. aeruginosa*

To try and compensate for the high virulence of *P. aeruginosa* in *G. mellonella*, different methodologies were used. One method looked at the viability of inoculating larvae with a smaller dosage of *P. aeruginosa* (Figure 5.3). However, after one day following inoculation, 100% of larvae inoculated with 5, 10 and 50 CFU *P. aeruginosa* had perished. In the group inoculated with 1 CFU, 50% had survived following one day. The usage of such low doses of *P. aeruginosa* were not deemed to be a viable method however, as it cannot be ensured that the larva has been infected with any *P. aeruginosa* cells.

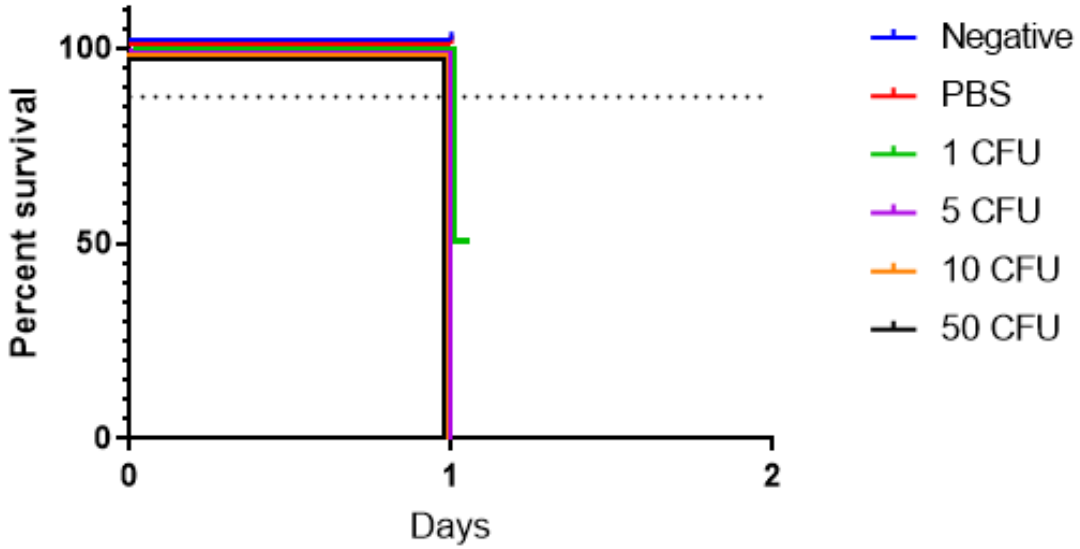


Figure 5.3: Survival of *G. mellonella* infected with different doses of *P. aeruginosa*. Experiment terminated after 1 day as all groups except 1 CFU were at 0% survival, and 1 CFU of *P. aeruginosa* was not deemed a realistic inoculum for future experiments.

16 subjects per group, 1 biological replicate

CFU – colony forming units

Data sets offset slightly to prevent overlapping and improve readability

The other methodology used changed the timepoints for measuring survival to half hourly intervals (Figure 5.4). In this experiment, PA01 was included to allow for comparison of the VAP *P. aeruginosa* isolate to a reference strain. At termination, 6.25% (1 larva) remained alive in the PA01 group, compared to 93.75% in the VAP group. It can be concluded that the VAP isolate of *P. aeruginosa* was markedly less virulent than PA01 from this result. 14 of the larvae in the PA01 group died between 7.30 hours and 8 hours. From these results, the virulence of the VAP isolate cannot be determined, the remainder of the larval population perishes at an undetermined point between 10 and 24 hours.

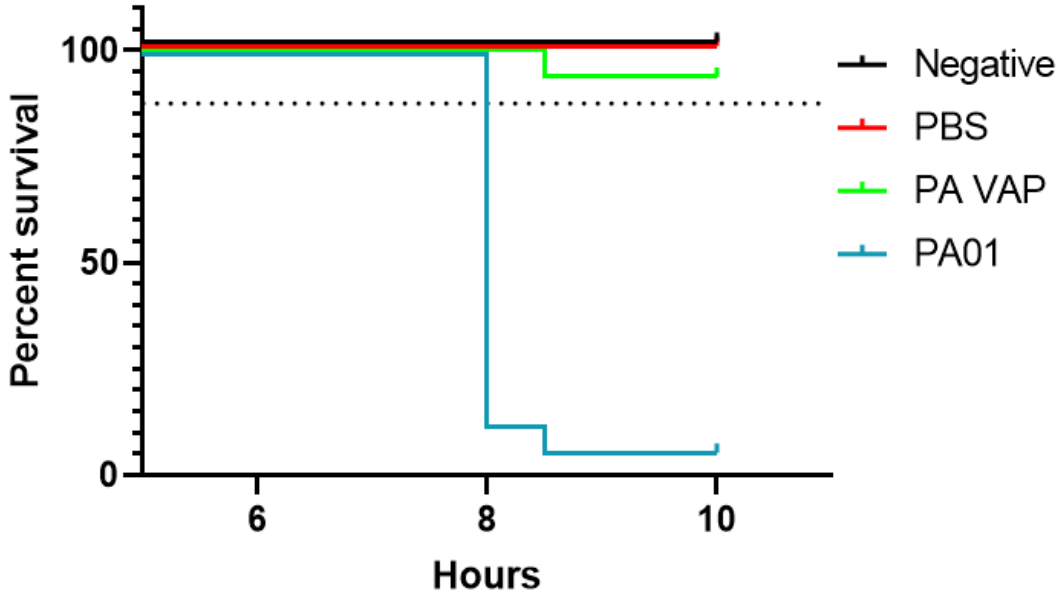


Figure 5.4: Survival of *G. mellonella* following infection with a VAP isolate of *P. aeruginosa* (PA VAP) and PA01. Survival was checked every 30 minutes following infection. Inoculation with PA01 resulted in rapid death of larvae 8 hours after injection, with only 12.5% surviving at this time. At termination of experiment, 6.25% of larvae infected with PA01 survived, whilst 93.75% of those infected with PA VAP survived.

16 subjects per group, 1 biological replicate

Data sets offset slightly to prevent overlapping and improve readability

5.3 | TNFα impact on adaptation rate of *S. aureus* to meropenem in an *in vivo* insect model

Prior to setting up passages of *S. aureus* in the haemolymph of *G. mellonella*, the concentrations of TNFα, MEM, and *S. aureus* tolerable to the larvae were established. This enabled the selection of appropriate concentrations to allow for daily passaging of *G. mellonella* haemolymph between larvae.

5.3.1 | *G. mellonella* dose responses to TNFα, meropenem, and *S. aureus*

G. mellonella generally tolerated injection with TNFα well (Figure 5.5). One day following injection with 30pg/g TNFα, 86.66% of larvae survived. 7 days following injection, this number had fallen to 76.66%. In those injected with 3000pg/g TNFα, 80% of larvae survived after 7 days. The survival rate of those injected with 300pg/g TNFα was 86.66% after 7 days. Probability of survival did not appear to be dosage dependent. As adaptation of *S. aureus* within *G. mellonella* would be daily, these concentrations of TNFα were concluded to be appropriate for use. The inoculation negative and PBS control groups did not cross the 80% survival threshold. This threshold is lower than in previous experiments due to the smaller groups of larvae.

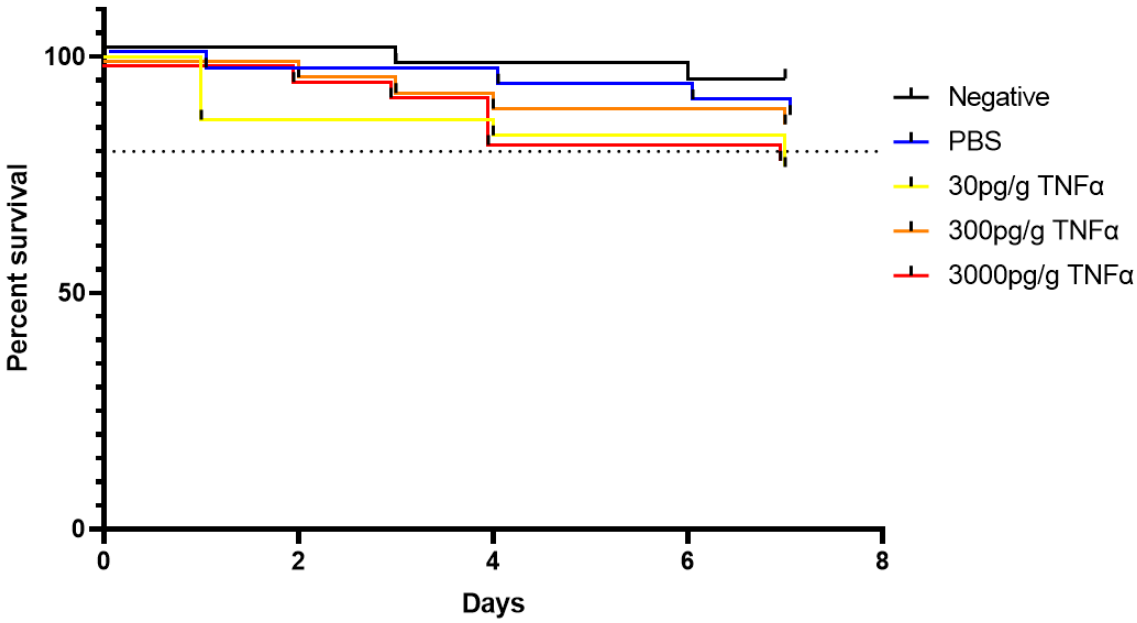


Figure 5.5: Survival proportions of *G. mellonella* inoculated with different concentrations of TNFα. TNFα was generally well tolerated by *G. mellonella* over a 7-day period. Survival of larvae does not appear to be dosage dependent, after 7 days the groups with lowest survival were 30pg/g TNFα with 76.66%, and 3000pg/g TNFα with 80%. The inoculation negative and PBS controls remained above the threshold survival percentage of 80%.

10 subjects per group, 3 biological replicates

PBS – phosphate buffered saline

Data sets offset slightly to prevent overlapping and improve readability

G. mellonella survival when injected with MEM appeared to be dose dependent (Figure 5.6). Injected with dosages 0.25µg/g, larval death after 7 days did not exceed that of the PBS control group. At termination of experiment, 86.66% of larvae injected with PBS had survived, while 90% of those injected with 0.25µg/g MEM; 93.33% of those injected with 0.125µg/g MEM; and 90% of those injected with 0.0325µg/g MEM had survived.

There were higher death rates at the greater tested concentrations of MEM. With 0.5μg/g MEM, 80% of larvae survived to termination of experiment on day 7. With 1μg/g, this number was 66.66%. Initial rate of death with the two highest concentrations was similar, but rate of death with 0.5μg/g MEM arrested at day 2, with 86.66% of larvae surviving until day 6. Rate of death remained steady with 1μg/g MEM throughout the duration.

As adaptation of *S. aureus* within *G. mellonella* would be daily, any tested concentration of MEM could be feasibly used. To ensure as high a survival rate after 1 day as possible, a lower concentration was preferred. This was with the caveat that lower concentrations of MEM may not be adequate to induce adaptation in *S. aureus*. Consequently, and following some preliminary experiments, 0.25μg/g was selected as an appropriate concentration for development of this model.

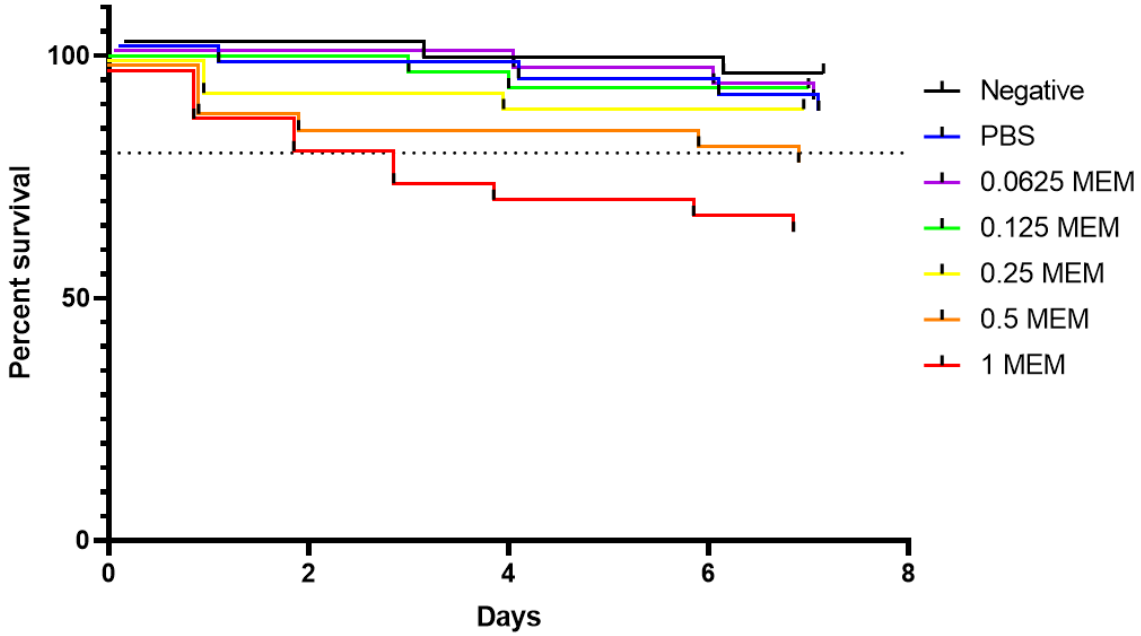


Figure 5.6: Survival proportions of *G. mellonella* inoculated with different concentrations of meropenem. *G. mellonella* survival appeared to be dosage dependant at higher concentrations of MEM. The lowest survival rate was in 1µg/g MEM, with 66.66% of larvae surviving after 7 days. The next lowest survival was in 0.5µg/g MEM, with 80% of larvae surviving after 7 days. In 0.25µg/g MEM, 90% of larvae survived 7 days, greater than the 86.66% surviving in the PBS control group. The inoculation negative and PBS controls remained above the threshold survival percentage of 80%.

10 subjects per group, 3 biological replicates

PBS – phosphate buffered saline; MEM – meropenem

Data sets offset slightly to prevent overlapping and improve readability

Survival of larvae inoculated with *S. aureus* was also dose dependent (Figure 5.7). Initial rate of death in all groups was steady. 2 days following inoculation, over 50% of individuals in the groups injected with 4-6.4 x 10^6 and 5-8 x10^6 CFU/mL *S. aureus* had died. In each other group, percentage survival had fallen no lower than 70% by this time.

At the termination of experiment on day 6, just 10% of individuals inoculated with 4-6.4 x 10^6 *S. aureus* had survived. 15% of those inoculated with 5-8 x 10^6 *S. aureus* survived by this time. The number of dead was not so severe in the other groups. In those inoculated with 3-4.8 x 10^6 *S. aureus*, 40% survived to termination of experiment. 45% had survived from the 2-3.2 x 10^6 *S. aureus* group, and 50% had survived from the 1-1.6 x 10^6 *S. aureus* group.

In contrast to this, just 40% of individuals survived to termination of experiment in the group inoculated with 5-8 x 10^5 *S. aureus*. This is the only datapoint to go against the dose dependent trend established by the other groups. This also differs from the result seen before, in Figure 5.2, where 56.25% of larvae inoculated with 5µL of a 5-8 x 10^5 suspension of *S. aureus* had survived 8 days following injection.

Considering these results, and those in Figure 5.2, the lowest concentration of *S. aureus* (5-8 x 10^5 CFU/mL) was selected for adaptation to MEM in *G. mellonella*. This was based on the dose dependent trend observed, despite this concentration going against this trend in Figure 5.7. This was to attain as high a survival rate after one day as possible, but also to avoid having an inappropriately high bacterial load in the larvae, which may slow the rate of adaptation. Just 10% of larvae injected with this dosage of *S. aureus* had died following one day.

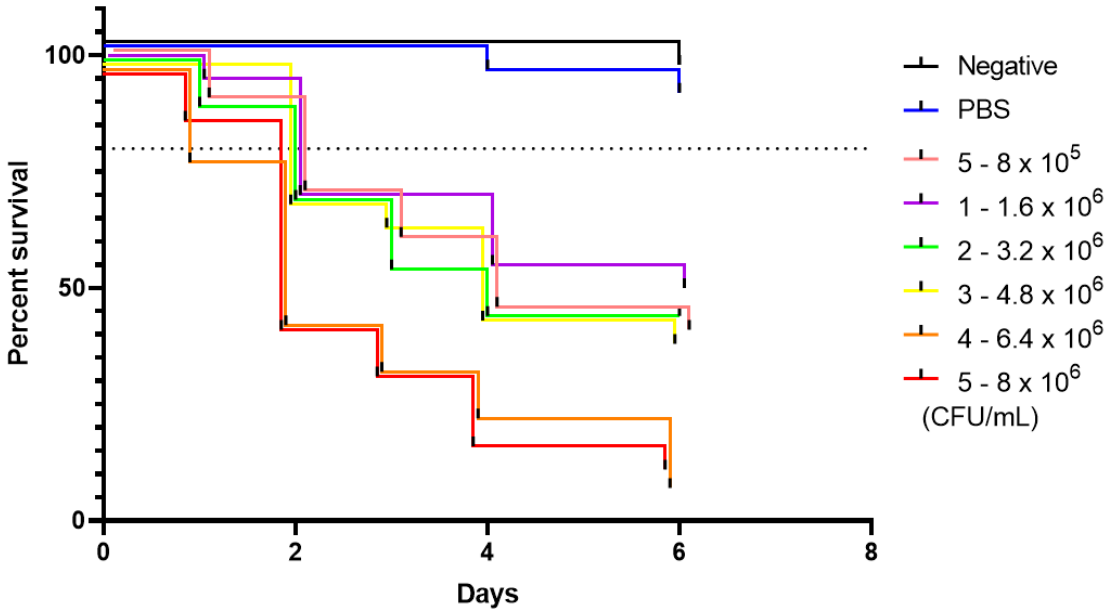


Figure 5.7: Survival proportions of *G. mellonella* infected with different dosages of *S. aureus*. Larval death after infection appeared to be dosage dependent. Death was most rapid with 5µL of OD_{600} 0.8 and 1.0 inocula. Following 6 days, just 10% of the 4 - 6.4x10^6 CFU/mL and 15% of the 5 – 8x10^6 CFU/mL groups had survived. 40% of the 3 - 4.8x10^6 CFU/mL group survived after 6 days. 45% of the 2 – 3.2x10^6 CFU/mL group survived after 6 days. 50% of the 1 – 1.6x10^6 CFU/mL group survived after 6 days. The 5 – 8x10^5 CFU/mL did not follow the trend, with just 40% surviving after 6 days incubation. The inoculation negative and PBS controls remained above the threshold survival percentage of 80%. **n=3**

10 subjects per group, 3 biological replicates

OD_{600} – optical density at 600nm; PBS – phosphate buffered saline, CFU – colony forming units

Data sets offset slightly to prevent overlapping and improve readability

5.3.2 | Adaptation of *S. aureus* to meropenem *in vivo*

S. aureus did not adapt rapidly to meropenem in *G. mellonella* haemolymph (Figure 5.8). Over the 5 successful passages, the mean zone diameter after spreading haemolymph onto MH agar had fallen from 32.45mm to 24.50mm. After passage 2, there were significant differences in zone diameter between TNFα-free and 30pg/g TNFα [$p = 0.0283$], and between 30pg/g TNFα and 3000pg/g TNFα [$p < 0.0001$]. At this point, the zone diameter with 30pg/g has fallen significantly below that measured with TNFα-free and 300pg/g TNFα, with an average diameter of 21mm. However, this is no longer significant by passage 3, where the average zone diameter with 30pg/g rising to 29mm, where there are no longer significant differences between concentrations.

After passage 4, the zone diameter with 300pg/g TNFα was significantly larger than with TNFα-free [$p = 0.0431$]. At this point, the zone diameter with 300pg/g is at its highest throughout the duration of experiment, at 33.6mm. This exceeds the measurement after just one passage, 31.4mm, suggesting that *S. aureus* had failed to adapt at all to meropenem with 300pg/g TNFα up to this passage.

Unlike the results seen *in vitro* in Chapter 4, the column factor, which here determines if there is a significant change between TNFα concentrations, was significant [$F (3, 180) = 3.827$, $p = 0.0109$]. This suggests that TNFα does influence the rate of adaptation of *S. aureus* to MEM *in vivo.* The row factor, which describes changes in the mean zone diameter between passages, was significant [$F (4, 180) = 10.56$, $p < 0.0001$].

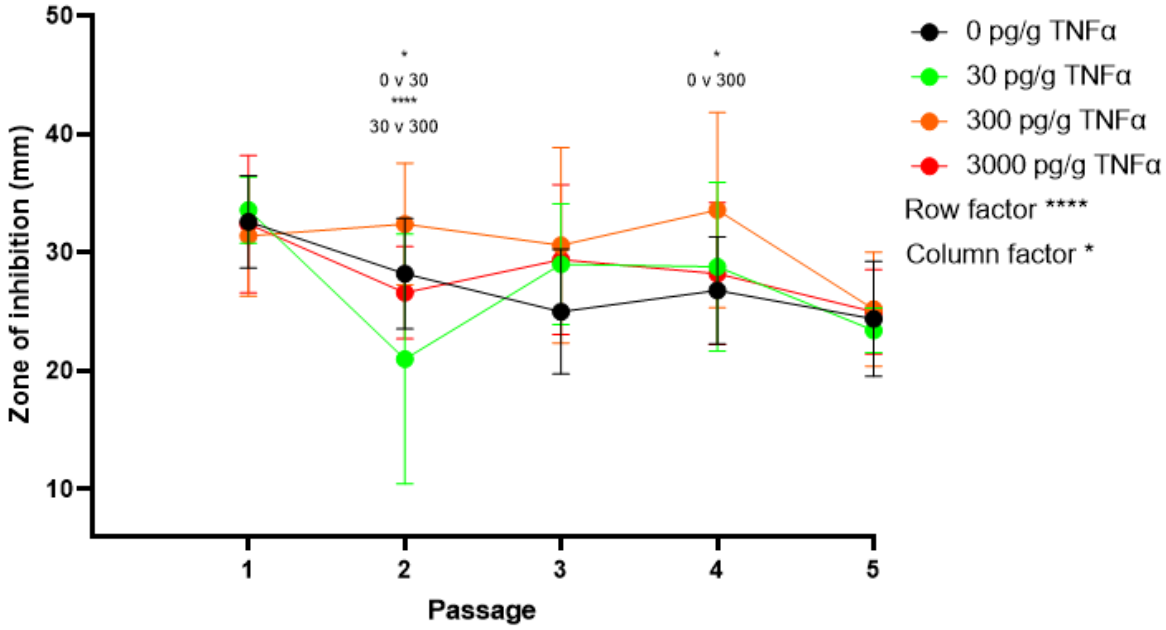


Figure 5.8: TNFα-mediated changes in rate of adaptation of *S. aureus* to 0.25µg/g MEM in *G. mellonella* haemolymph. Haemolymph from larvae was spread onto MH agar with a 10µg MEM disc between passages to track adaptation. There are significant changes in zone diameter after passage 2 between 0pg/g TNFα and 30pg/g TNFα ($p = 0.0283$), and between 30pg/g and 3000pg/g TNFα ($p < 0.0001$). After passage 4, the zone diameter with 300pg/g TNFα was significantly increased compared to 0 pg/g TNFα ($p = 0.0431$). Results of multiple comparisons with Tukey method. x intersects y at 6mm as this is the diameter of the antibiotic impregnated disc.

10 subjects per group, 1 biological replicate ±SD

MEM – meropenem

5.3.3 | Investigating brown colouration of MH agar during *in vivo* adaptation in haemolymph

An unexpected observation from *in vivo* adaptation experiments was the appearance of brown colouration on MH agar plates (Figure 5.9). We suspect this is caused by the action of active phenoloxidase in the haemolymph spread onto these plates. As outlined prior, phenoloxidase is activated following detection of

microorganisms, and produces quinones from phenols, which polymerise into melanin (Söderhäll and Smith, 1986; Altincicek *et al*., 2008).

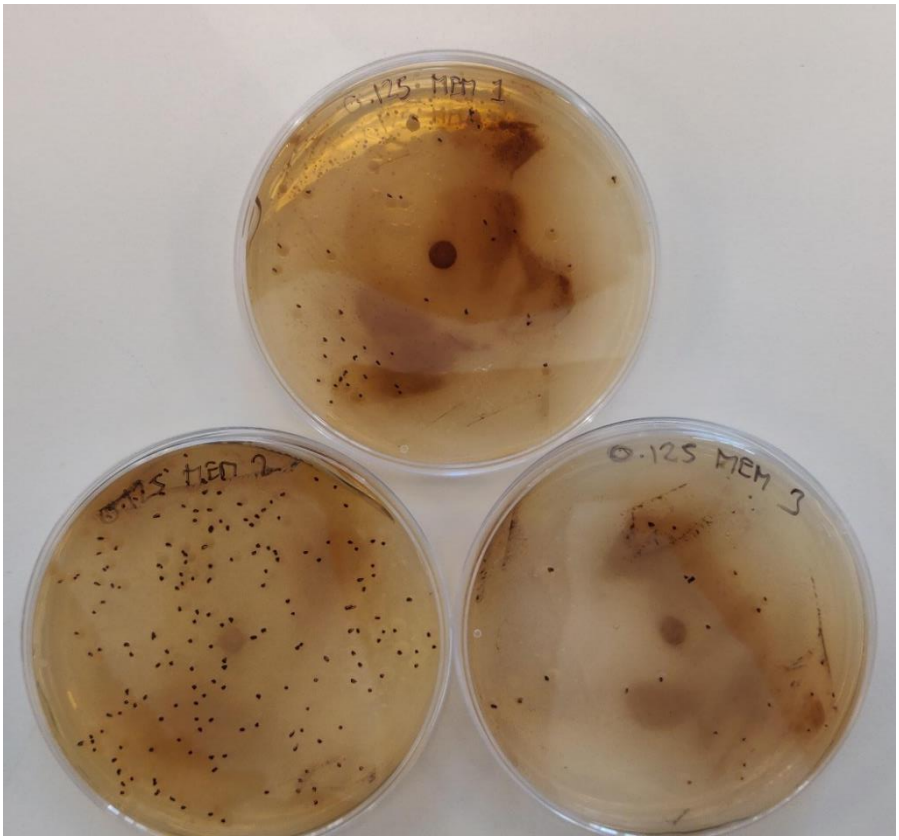


Figure 5.9: Brown colouration of Mueller-Hinton agar after extracted *Galleria mellonella* haemolymph (5µL) was spread onto it. This phenomenon is believed to be a consequence of active phenoloxidase in the haemolymph producing quinone from phenol sources in the agar, which polymerise into melanin.

One of the ingredients in MH agar is casein hydrolysate, which is a source of the amino acid tyrosine. Tyrosine possesses phenol functionality and is a precursor to natural phenols (Milić *et al*., 2008). This may be the source of phenol in MH agar producing this brown colouration. This change in media colour was not overly problematic but did add some difficulty to reading zone diameters. Mannitol salt was used as a potential alternative media, but the brown colouration in these plates was far more intense, possibly due to the pH indicator phenol red. It was

considerably harder to take readings from mannitol salt plates, so MH agar was selected for use.

5.4 | Summary of key chapter data

Initial virulence assay showed that VAP isolates of *S. aureus* and *K. pneumonia* resulted in 43.75% and 50% death after 8 days, respectively. *P. aeruginosa* exhibited its high virulence in *G. mellonella*, with 100% larval death after one day.

Attempts to further investigate the viability of this model for investigating virulence of the VAP isolate of *P. aeruginosa* were unsuccessful. Larvae injected with different dosages of bacteria corresponding to 5 CFU, 10 CFU, and 50 CFU all died within 24 hours. 50% of those injected with 1 CFU survived 24 hours, but at such low inoculum there is no guarantee larvae are even infected. Other larvae were checked at half-hourly timepoints and compared to PA01. The VAP isolate was less virulent than PA01, resulting in just 6.25% death at 10 hours, compared to 93.75% in the PA01 group. Whilst it can be concluded that PA01 is the more virulent strain, it cannot be concluded exactly how virulent the VAP isolate is.

G, mellonella injected with TNFα appeared to tolerate it well, up to a maximum concentration used of 3000pg/g. The group injected with 30pg/g TNFα suffered the sharpest decline in survival, with 13.34% dead after 24 hours. Any toxic effect of TNFα in *G. mellonella* larvae did not appear dose dependent.

Survival of *G. mellonella* injected with meropenem up to concentrations of 1μg/g appeared to be dose dependent. All concentrations were generally tolerated short term. After 24 hours, concentrations resulting in the greatest number of deaths were 0.5μg/g and 1μg/g, with 90% of larvae surviving. The concentration of

meropenem selected for *in vivo* adaptation was 0.25μg/g. This exceeds the MIC determined *in vitro*, but preliminary results from *in vivo* adaptation experiments demonstrated that 0.25μg/g meropenem was not preventing *S. aureus* growth. This was desirable as a concentration of meropenem strong enough to facilitate adaptation was necessary, without completely eradicating *S. aureus* infection.

S. aureus adaptation to meropenem *in vivo* was slow and inconsistent. After passage 2, the zone diameter with 30pg/g was significantly reduced. After passage 4, there was a significant difference in zone diameter between TNFα-free and 300pg/g TNFα. After passage 4, the measurement of zone diameter with 300pg/g had risen above the measurement taken after passage 1, suggesting a failure of this group to adapt to meropenem at all. There was a high margin of error amongst the results, suggesting more variability in *S. aureus* adaptation to meropenem *in vivo* than *in vitro*. There was a significant effect between TNFα concentrations over the 5 *in vivo* passages. It appears that TNFα, especially 300pg/g, may reduce rate of *S. aureus* adaptation to meropenem *in vivo*. By passage 5 zone diameters between groups had largely converged, suggesting that any effect of TNFα on adaptation is on rate rather than endpoint sensitivity.

5.5 | Chapter discussion

This chapter demonstrates the modification of the *G. mellonella* infection model to develop an *in vivo* antibiotic adaptation model. To develop this, numerous parameters required deciding upon; what bacterial species to use, and what infectious dose with which to infect the larvae; what concentrations of TNFα and

MEM to use, and what concentrations are tolerated by the larvae. And based on these results, what timeframe to use for the experiment.

The exceptional virulence of *P. aeruginosa* observed is in line with prior publications which demonstrate the very low infectious doses of *P. aeruginosa* required to kill *G. mellonella* (Jander *et al*., 2000; Hill *et al*., 2014). For deciding on a bacterium to use for *in vivo* adaptation however, this chapter's findings are illustrative enough of the unsuitability of *P. aeruginosa* to disqualify its use. Larvae would not survive the full incubation period of 24 hours between passages.

As outlined previously, modelling *K. pneumoniae* infection in *G. mellonella* can yield mixed results, as the virulence and rate of larval death is subject to high variability (Insua *et al*., 2013).

The concentrations of TNFα used during this Chapter's experiments are acceptably well tolerated for the purpose of daily passage of bacteria through different larvae. *G. mellonella* have TNFα-like molecules of their own. These were demonstrated in 1999 by binding of polyclonal anti-TNFα antibodies binding to *G. mellonella* hemocytes (Wittwer *et al*., 1999). Whether these are entirely homologous in function to human recombinant TNFα or would influence the results of experiment was not considered, however.

In studies of larval tolerance of meropenem, larvae were injected with antibiotic doses based on total weight. This matches antibiotic dosage guidance in humans, but also reflects the injection of larvae in previous publications, where dosage was considered in mg/kg (Thomas *et al*., 2013). No issues with the injection of *G. mellonella* with meropenem were expected. The use of this model for the investigation of antimicrobial efficacy and drug discovery is very well established

for a number of different therapeutics and bacterial species (Peleg *et al*., 2009; Hill *et al*., 2014; Krezdorn *et al*., 2014; Yang *et al*., 2017a; Cools *et al*., 2019). *In vivo* there is far greater complexity in interactions between meropenem, host, and *S. aureus* (Delacher, 2000). *S. aureus* is likely to undergo changes in exposure to meropenem *in vivo* over time, for example due to differences in tissue absorption. This is a possible explanation for the continued growth of *S. aureus* injected alongside a concentration of meropenem greater than the MIC determined *in vitro*.

The results of *in vivo* adaptation suggest that there is potential value in continued development of methods of this nature. There is already deviation from results taken *in vitro*, where no significant change on rate of *S. aureus* adaptation to meropenem was observed. This change could be a consequence of adapting the method to a host or could potentially be explained by a change in the concentration of meropenem used for adaptation. Larvae were injected with 0.25μg/g meropenem, whilst the disc affixed to MH agar plates contained 10μg meropenem. If the microenvironment of the *G. mellonella* larva is contributing to this difference in adaptation rate, the cause of this is unknown. It may be through interference from the host's own cytokine response. Very few insect cytokines have been investigated, and most have only been categories in *Drosophila*. However, one example cytokine found in Lepidoptera (i.e. moths and butterflies) is growth-blocking peptide (Matsumoto *et al*., 2012). This peptide has been demonstrated to be involved in stress response of moth larvae (Matsumura *et al*., 2018). Insect cytokines are poorly understood and may not act in a homologous manner to those in mammals, so it is difficult to conclude what influence they may have on this experiment if any.

As part of the immune response in *G. mellonella*, plasmatocytes and granulocytes act to begin nodulation (Walters, 1970). These begin following the release of adhesion proteins by granulocytes, which trap bacteria into microaggregates with other granular cells (Ratcliffe and Gagen, 1976). The nodule is formed after involvement of the plasmatocytes, which isolate and encase the pathogen to prevent further damage to the larva (Ratcliffe and Gagen, 1977). The capability of meropenem to penetrate these nodules and reach the bacteria encased is unknown. Similarly, it is unknown to what degree meropenem remains localised in the haemolymph of *G. mellonella* following injection, and thus access to all injected bacteria may be prevented. Bacterial infection of *G. mellonella* is systemic, however histopathology of infected larvae shows distinct loss of tissue structure (Kay *et al*., 2019). This may allow meropenem to access bacteria not localised to haemolymph, but only in infections that have progressed to this stage. Given each passage took place after 24 hours in this method, it is unclear whether bacteria localised in larvae tissues could represent a reservoir in which no adaptation can occur. How antibiotics localise within the larvae could be determined using histological sections of infected larva and fluorophore-antibiotic conjugates, use of which were subject to recent review (Miao *et al*., 2020). This could be expanded further to colocalization of two probes, by adding a fluorescent conjugate to the bacteria of interest. This would allow the observer to look at areas in which meropenem and bacteria are both present, and quantify overlap in distribution between the two (Dunn *et al*., 2011).

This i*n vivo* adaptation experiment investigated the effect of TNFα on *S. aureus* adaptation to meropenem. This may not have been the most logical antibiotic/bacteria combination to use. *S. aureus* was already shown to adapt

poorly to meropenem *in vitro*. Use of a different species or antibiotic could demonstrate more steady *in vivo* adaptation, which would lend a lot of value to this model as a valid concept. Unfortunately, none of the other *in vitro* results provide ideal alternatives. *K. pneumoniae* rate of adaptation was also slow, and the unsuitability of *P. aeruginosa* due to its intense virulence in *G. mellonella* has already been discussed (Jander *et al*., 2000; Hill *et al*., 2014). Further investigation here is sensible.

The results from *in vivo* adaptation cover a duration of 5 passages. This differs from the 15 passages *in vitro*. Beyond passage 5 there was an increase in incidence of death across different groups of larvae. This was not seen in either the inoculation negative or PBS control groups. When draining haemolymph to passage into the next group of larvae, individuals were selected based on visible health. This was to avoid reinjection with haemolymph from dead larvae or those with high levels of melanisation, which implies systemic infection and breakdown of tissues (Kay *et al*., 2019). It was speculated that injection with overly large infectious doses of *S. aureus* found in these individuals, or necrotic larval tissues, could inversely affect survival. The cause of increased death of larvae beyond passage 5 is not clear. It is possible that over the duration of experiment, the concentrations of TNFα or meropenem injected into each group of larvae at each passage could progressively accumulate in haemolymph. To our knowledge, there have been no studies on the elimination of meropenem of TNFα by *G. mellonella*. To improve this model for future use, this should be investigated. Accumulation of meropenem or TNFα in haemolymph could not just affect larvae survival but could also affect the results of experiment and disguise the true adaptation rate. As the component of interest in extracted hemolymph is the *S. aureus*, purifying this from

the haemolymph could in theory provide a simple solution. The method included a centrifugation step to separate the bacterial component from larval tissues and supernatant. However, it cannot be guaranteed this separated meropenem or TNFα adequately, as these are not immediately visible.

To track changes in *S. aureus* sensitivity, haemolymph extracted from *G. mellonella* larvae was spread onto MH agar with a 10μg MEM disc. This adds an additional passage to all presented results, in an *in vitro* system. There is evidence of resistance being lost in bacteria after removal from selective pressure (Andersson and Hughes, 2011; Simner *et al*., 2018; Dunai *et al*., 2019). The change from the *in vivo* to *in vitro* system for this additional passage may lead to results not entirely reflective of the sensitivity of *S. aureus* in *G. mellonella*.

In Chapter 3, *in vitro* experiments showed that *S. aureus* growth was enhanced by 3000pg/mL TNFα in sublethal concentrations of MEM. *S. aureus* in this adaptation experiment were injected into *G. mellonella* along with TNFα and MEM. If *in vitro* changes in growth translate to *in vivo*, it is possible that results of adaptation may have been impacted by unforeseen enhancement of *S. aureus* growth.

The larvae used throughout the projected were sourced from a reliable commercial supplier. However, commercial suppliers do not always provide larvae in optimal conditions, or control for the absence of infection (Trevijano-Contador and Zaragoza, 2019). Larvae can be purchased from suppliers specifically for research use, which are controlled for infection, feeding, and size, but these are far more expensive. Throughout the project, we tried to control for absence of infection by picking visibly healthy larvae with minimal melanisation or darkening of the cuticle. A sample of larvae were also weighed, with an average mass of

230mg. For experimental purposes, only larvae with a mass ±10% of 230mg were selected for use, as large differences in body weight could skew results.

To conclude, the *G. mellonella* infection model was utilised to develop a method for monitoring adaptation of bacteria to antibiotics *in vivo*. This model was utilised to investigate the effects of TNFα on the adaptation of *S. aureus* to meropenem. Larvae were found to tolerate well the concentrations of TNFα and meropenem used over the 24-hour period utilised for each passage. Results of *in vivo* adaptation possibly demonstrate a relationship between higher TNFα concentrations, particularly 300pg/g, and slower rate of adaptation. However, incidental death of larvae beyond passage 5 could not be controlled for, and the increased incidental death in experiments of longer duration is unexplained. Additionally, *S. aureus* had displayed poor adaptation to meropenem *in vitro*, and alternate combinations of antibiotic and bacteria may provide clearer results in future experiments.

The use of *G. mellonella* as a model for *in vivo* antibiotic adaptations is not without limitations, but initial results demonstrate its promise. The *G. mellonella* infection model has good record of successful use for modelling HMI and pathogenesis in infectious disease (Mukherjee *et al*., 2010; Cook and McArthur, 2013; Champion *et al*., 2016; Six *et al*., 2019; Torres *et al*., 2020; Dinh *et al*., 2021). Improvement of this model for tracking the role of HMI in development of AMR is worthy of consideration, it is possible that we could learn to predict changes in sensitivity to antibiotics prescribed for VAP, based on inflammatory response. This could inform clinicians when making prescribing decisions, for example, the application of combination therapy for an infection expected to develop resistance rapidly. Given the wider implications of VAP for the development of AMR (O'Neill, 2016; Nora

and Póvoa, 2017), being able to predict where and how quickly resistance might arise in response to treatment would be valuable for preserving the efficacy of antibiotics in this setting.

Chapter 6 | General discussion

This research has investigated the role of proinflammatory cytokines as mediators of phenotypic change in VAP bacteria, as part of a wider effort to better understand HMI in VAP. It has demonstrated a relationship between elevated proinflammatory cytokines, specifically TNFα, and enhanced growth; enhanced biofilm formation; increased resistance to meropenem; and changes in *in vivo* adaptation rate to meropenem, in bacteria implicated in VAP.

As VAP develops in critically ill patients undergoing MV, there are significant early increases in cytokines TNFα, sTNFαRI, IL-6, IL-1α, and IL-1β. This increase is compartmentalised to the site of infection in the lungs (Millo *et al*., 2004). Thus, the causative bacteria are exposed to these immune elements early in infection. Based on this the findings of this thesis, this may result in TNFα-mediated enhancement of growth, biofilm formation, and increased resistance very early in the infection process. This initial response may be important for the early establishment of infection in these patients. Likewise, the implementation frequent measurements of cytokines in MV patients, particularly TNFα may predict imminent or ongoing development of VAP. This could in turn allow for earlier decisions to be made regarding management and treatment of each patient.

There is already an understanding of the cytokine networks and patterns in several the comorbidities requiring MV. As these patients are at elevated risk of VAP, applying this knowledge as a predictor for VAP and its management has likely benefit. Two examples have been discussed previously; in ARDS patients TNFα is highly elevated, and in severe trauma patients there are significant increases in circulating IL-6 and IL-1ra (Bauer *et al*., 2000; Binkowska *et al*.,

2018). There is a wide panel of proinflammatory cytokines upregulated in COPD. IL-6 is upregulated in those with moderate COPD, representing airway inflammation (Bucchioni *et al*., 2003). In COPD patients with 3 or greater exacerbations per year, levels of IL-6 and IL-8 in sputum exceed those with fewer exacerbations (Bhowmik *et al*., 2000). TNFα is also significantly elevated during exacerbations in these patients (Aaron *et al*., 2001). Together, this cytokine response to COPD appears rather nonspecific, which may hamper its diagnostic use. However, significantly increased expression of the cytokine IL-32 has been demonstrated in the lungs of COPD patients, where it is colocalised with TNFα, and may provide a useful distinctive marker (Calabrese *et al*., 2008). If IL-32 could be related to enhancement of bacteria implicated in VAP, as others have been previously, it may provide a useful example marker for increased VAP risk. Additionally, despite being nonspecific between different conditions necessitating MV, the presence of elevated TNFα represents an increased risk of VAP in these patients based on this study's results.

Once a better understanding of cytokine effects on relevant bacterial phenotypic changes has been established across the principal species in VAP, it could be applied to personalised medicine. There would be value in rapid and simultaneous detection of proinflammatory cytokines and bacteria in the lungs. This would need to cover both pathogens that arrive from the ICU environment as well as the microbiota that provide a potential reservoir for VAP (Zakharkina *et al*., 2017; de Carvalho Baptista *et al*., 2018). Selection of appropriate empirical therapy for treating VAP is challenging and presently necessitates broad-spectrum antibiotics. If cytokine fingerprints can be rapidly correlated to a likely enhancement of AMR or biofilm formation, it could increase effective treatment. Being better informed by

where and which treatments may fail, due to enhanced resistance or additional recalcitrance of biofilms, could change prescribing behaviours. This would need combining with local epidemiological data, to understand the AMR profiles of bacteria in the specific ICU environment.

The benefit of a personalised approach to the patient would be in reduced morbidity and mortality, and more ventilator free days. The benefit to the healthcare institution is in reduced costs and development of AMR if targeted antibiotics become feasible as the initial treatment. A major challenge to the implementation of this would be in rapid identification of the implicated bacterial species. Firstly in that there is no guarantee that a potential pathogen is actually contributing to disease even if present (Gastmeier *et al*., 2009). Secondly in that it takes time for microbiological data to become available via conventional culture, so more rapid diagnostics would be necessary. The use of biochemical tests, immune assay, and molecular diagnostic tests could provide the solution, providing much more rapid detection and identification of bacterial species. The use of such techniques for rapid diagnosis of ESBLs in Enterobacteriaceae was subject to recent review (Decousser *et al*., 2017).

The mechanism by which proinflammatory cytokines mediate change in bacterial phenotype remains an important question. This is not just true for TNFα, but for a range of cytokines where similar phenomena have been described in literature. Elevated TNFα concentrations have been implicated in driving cytotoxicity in numerous lung pathologies. This includes asthma, chronic bronchitis, COPD, and ARDS. In these contexts its role is the worsening of oxidative stress through stimulation of reactive oxygen species and depletion of the antioxidant glutathione (Mukhopadhyay *et al*., 2006). In the results of this thesis, as well as in the *in vitro*

results of previous literature, the potential for TNFα and other cytokines to modify bacterial phenotype away from the influence of immune effectors or other elements of the host has been observed. This represents an alternate avenue by which TNFα may contribute to pathogenesis of lung disease.

How the potential mechanism of DNA binding of TNFα would result in phenotypical changes is unclear, but may be through changes in promoter architecture, which can up or down regulate RNA polymerase binding, or modulate the binding of transcription factors (Mahdavi *et al*., 2013). It is possible that the observed changes in *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* are a consequence of similar DNA binding activity and subsequent changes in gene expression. Meropenem, to which a significant change in *P. aeruginosa* sensitivity was observed, targets penicillin-binding proteins (PBPs) within the bacterial cell wall (Kitzis *et al*., 1989; Sumita *et al*., 1990). Assuming this effect is a result of changes in gene regulation, it is possible the target gene for TNFα binding in *P. aeruginosa* is for PBP or other cell wall components, for example OprD. However, there was no change in *P. aeruginosa* sensitivity to imipenem, which shares mode of action with meropenem by binding PBPs. The use of RNA-Seq to investigate cytokine-induced changes in gene expression could be applied here to investigate potential up or down-regulation of cell wall components in *P. aeruginosa* (Wang *et al*., 2009). This would address the overall project of aim of describing how IL-1β, IL-6 and TNFα might influence phenotypic change in bacteria, by investigating at a genetic level.

Throughout this thesis, only TNFα produced significant change in bacterial phenotype, with a single exception of IL-6 and *K. pneumoniae* growth in Chapter 3. Whether IL-6 could produce a genuine interaction is under question. Gram-

negative bacteria have been shown to have receptors for IL-1β and TNFα, but not IL-6 (Luo *et al*., 1993; Zav'yalov *et al*., 1995). Though Meduri *et al.* (1999) did report IL-6 increasing proliferation of *P. aeruginosa* despite this. Taking this result at face value would indicate that moderate elevation of IL-6 may enhance growth of *K. pneumoniae* in patients. However, any consequence that may arise from this is likely offset by the demonstrable benefit conferred by IL-6 in *K. pneumoniae* infection (Sutherland *et al*., 2008). IL-6 knockout mice are shown to have poorer survival in *K. pneumoniae* pneumonia and sepsis (Sutherland *et al*., 2008). The potential relationship between moderate elevations of IL-6 and *K. pneumoniae* growth may warrant further confirmational study.

In Chapter 3 TNFα changes in *P. aeruginosa* growth were measured with sublethal concentrations of antibiotic. As previously discussed, carbapenem antibiotics are frequently prescribed for the treatment of VAP. It was also theorised that troughs in antibiotic therapy during treatment could enhance bacterial growth (Begg *et al*., 1999). At the higher sublethal doses of meropenem and imipenem tested, there may adjustment to a senescent phenotype, with formation of bacterial persisters to tolerate the higher selective pressure (De Leenheer *et al*., 2010). This effect may counterbalance the signal from elevated concentrations of TNFα which promote more active proliferation. In VAP, the major reservoir for ongoing infection are biofilms.

The above suggests that carbapenems may still afford the patient significant benefit, even at a point where active concentrations have fallen far below the administered dose. Even in cases where the antibiotic concentration does not fall to such low levels, a significant number of bacteria present in VAP exist within bacterial biofilms. The potential promotion of a senescent phenotype by

antibiotics, for example due to prior treatment of an existing infection necessitating MV, could be important in initiating VAP. The recalcitrant nature of biofilms to antibiotic treatment is well documented, and antibiotic penetration deep into biofilms is low, with the concentrations dropping further from the surface (Gordon *et al*., 1988; Stewart, 1996; Shigeta *et al*., 1997). In this environment the concentration of antibiotic may be far lower than the systemic concentration. Human leukocytes have been demonstrated to penetrate *S. aureus* biofilms (Leid *et al*., 2002). Thus, in some VAP biofilms there may be exposure to TNFα and therefore enhanced growth, even with high levels of circulating antibiotic.

In vivo results presented in this thesis were collected using the *G. mellonella* infection model. This was part of a deliberate approach to progress through more complex models. Initial experiments were performed *in vitro* to check for simple effects, the results of which could then be applied to *in vivo* models. With the intention of eventual progression from insect into mammalian models, and then into patients, as part of a larger study beyond this thesis. *G. mellonella* was not the only model investigated for use during this research, however. Harrison *et al.* have developed a flexible and ethical *ex vivo* pig lung (EVPL) model for the study of chronic lung infections (Harrison *et al*., 2014). This model is of more similar anatomical size, structure, and immune component to human lungs than existing rodent or insect models (Dawson *et al*., 2013). EVPL has already been used for study of biofilm formation on bronchioles (Harrison and Diggle, 2016), and for the investigation of antimicrobials in the cystic fibrosis lung (Roberts *et al*., 2019). The usefulness of this model for investigating HMI *ex vivo* is clear. In the current work, significant time and effort were spent working with this model, and the methods used are detailed in Appendix 1. The aim of using this model was to follow up *in*

vitro investigations in a more clinically relevant setting, with the additional complexity that lung tissue provides. As previously outlined, the formation of biofilms is a key pathogenic event in the development of VAP (Gunasekera and Gratrix, 2016). In Chapter 3 it was demonstrated that TNFα significantly increases the biofilm formation of *K. pneumoniae in vitro*. This finding could be highly important, but the result was taken from a system of extremely low complexity. The effects of TNFα on biofilm growth of *K. pneumoniae* as well as *S. aureus* and *P. aeruginosa* could be explored in a system with greater homology to the VAP patient's lungs. This could be done by adapting the established EVPL model for investigation of biofilm growth on bronchioles.

Using EVPL would allow for TNFα mediated enhancement of *K. pneumoniae* biofilm growth to be confirmed or rejected. Similarly, whilst there was no significant change in biofilm growth of *S. aureus* or *P. aeruginosa* with the addition of TNFα *in vitro*, this may change in a model with greater homology to human lungs in terms of spatial structure and cellular component. Confirmation of any such effect *ex vivo* would be extremely valuable. With this information it would be far more justifiable to investigate this effect in VAP patients in intensive care. Once again it could lend credibility to regular monitoring of patient cytokine levels to predict development of biofilms in patient lungs or ETT, and the subsequent acceleration of VAP development. Unfortunately, a lack of presentable data was available for inclusion in this thesis. This was due to common laboratory complications, mainly related to contamination. Additionally, due to time constraints stemming from long-term closing of the laboratory, a priority was placed on acquisition of rapid *in vitro* data.

Resuming these investigations in EVPL and adapting *in vitro* and *in vivo* insect results to this model is recommended. This would be a priority in the case of future experiments continuing from this thesis. EVPL could then be used to study other changes in bacterial phenotype in response to TNFα and beyond, including changes in sensitivity, virulence, and changes in growth. The aim of these experiments would be to translate *in vitro* findings to a realistic model of respiratory infection, and confirm these observations using it.

Other logical next steps would be to apply all existing methods over other available VAP strains. Even within the species used as part of this thesis, the only species used for *in vivo* adaptation was *S. aureus*. The concerns with using *P. aeruginosa* because of its virulence in *G. mellonella* has already been discussed, but *K. pneumoniae* was shown to be tolerated well over a 24-hour period. This thesis has also only considered single strains of three bacterial species, and in each case as a monospecies infection. Incorporation of some of the other key causative agents of VAP is logical, and would likely include *A. baumannii*, *E. coli,* and *Enterobacter* spp., as together with *S. aureus*, *K. pneumoniae*, and *P. aeruginosa,* these cause the majority of VAP cases (Jones, 2010).

Adapting existing methods to polymicrobial infections would also be in important next step. VAP is frequently polymicrobial, and microbe-microbe interactions are assuredly important in the development of these infections. Bacteria in polymicrobial infections frequently benefit from synergistic interactions. For example, *S. aureus* has been shown to have significantly increased antibiotic tolerance in planktonic and biofilm models when co-cultured with *P. aeruginosa* (DeLeon *et al*., 2014). *S. aureus* is protected from gentamicin by extracellular polymeric alginate produced by *P. aeruginosa* (DeLeon *et al*., 2014). *P.*

aeruginosa has also been demonstrated to increase expression of numerous *S. aureus* transporters, including *tet38*, which increases resistance to tetracycline (Briaud *et al*., 2019). *S. aureus* has been demonstrated to protect *P. aeruginosa* as well, with staphylococcal protein A increasing tobramycin resistance of *P. aeruginosa* (Beaudoin *et al*., 2017). If co-culture of *S. aureus* and *P. aeruginosa* leads to increased antibiotic tolerance, it would be interesting to see how this might affect results seen in Chapter 4, where elevated TNFα was shown to increase the MIC of meropenem for *P. aeruginosa*. Adapting the methods used in this thesis for this purpose would be simple in theory, as they could all support the use of bacterial co-cultures. In practice it may be more suitable to use a continuous culture model, for example a chemostat, as this can prevent complete domination of one species in a mixed culture (Xu, 2021).

To conclude, the results of this thesis demonstrate the capability for proinflammatory cytokines, particularly TNFα, to modify clinically important phenotypic traits of bacteria commonly implicated in VAP. These results form the basis for further development of models to investigate HMI in these infections. This would entail measuring phenotypic change in these bacteria mediated by cytokines in models of increasing complexity, including *G. mellonella* and EVPL. Demonstration of these phenomena in mammalian models will add more legitimacy to these findings, and if applicable in the patient, could begin to improve predictive and diagnostic methods for managing VAP in intensive care. This would reduce the development of AMR driven by broad-spectrum empirical therapy, as more targeted treatments could be deployed sooner. Improved optimisation of antibiotic prescription for VAP, and improved diagnostic and predictive methods in its management, are both in line with the objectives outlined in the UK's 20-year

vision for antimicrobial resistance. The final and most obvious benefit would be to those afflicted with VAP, who would be at reduced risk of mortality due to these infections.

Chapter 7 | References

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Appendices

Appendix 1 | Investigation of TNFα effects on biofilm formation using an *ex vivo* lung model

Appendix 1.1 | Preparation of artificial sputum media

Lung tissues and bacteria used in EVPL are bathed in artificial sputum medium (ASM) for all incubation steps. ASM was prepared by slowly dissolving 4g low molecular weight DNA from salmon sperm (Sigma, USA), and 5g mucin from porcine stomach (Sigma, USA) in distilled water. To this 250mg of each L amino acid was added (Sigma, USA) except tryptophan. 5.9mg diethylenetriaminepentaacetic acid (DTPA) (Sigma, USA), 5g NaCl (VWR international, USA), and 2.2g KCl (VWR international, USA) were dissolved into 100mL distilled water. Dissolved DNA, mucin, L amino acids, DTPA, NaCl and KCl were combined in a 1L Duran bottle. Following which the pH was measured and adjusted to 7.0 with 1M tris (Sigma, USA).

ASM was sterilised by autoclave at 121°C, following which 250mg of filter sterilised tryptophan (Sigma, USA) and 5mL egg yolk emulsion (Sigma, USA) was added. ASM was then stored at 4°C until use or for one month.

Appendix 1.2 | TNFα effects on biofilm growth on *ex vivo* bronchiole sections

Fresh lungs were sourced from Large White pigs from a local abattoir (C.S. Morphet & Sons Ltd, Cronton, UK). Lungs were refrigerated during transport to the University of Salford. Dissection tools (scissors, tweezers, and single edged razor blades) and a palette knife were presterilised by autoclave at 121°C for 15

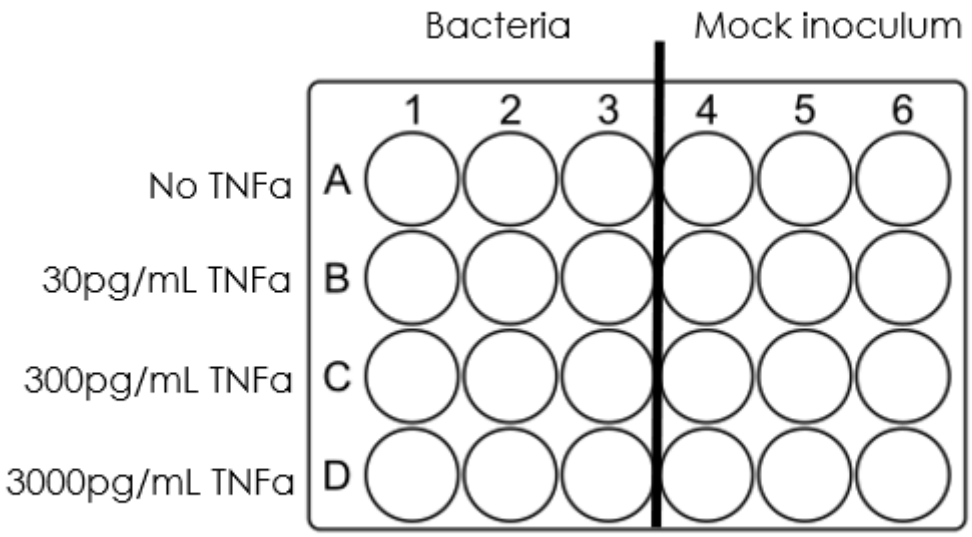
minutes. Foil and chopping boards were UV sterilised for 30 minutes prior to dissection.

Lungs were placed face-up onto UV sterilised foil on top of chopping boards, to provide a firm and sterile surface for dissection. Working in proximity to a flame, a heated palette knife was used to sterilise the pleura at the location of dissection, by touching to the pleural surface briefly. Using a razor blade, a cut approximately 5cm long was made into the tissue along the length of the bronchus, to expose the cartilage. The bronchus was then separated from the trachea by transverse cut. The razor blade was then used to gently separate a section of bronchi around 5cm long from the surrounding alveolar tissue. The size of bronchiole sections varied between dissected lungs, due to variation in frequency of branching (Harrison and Diggle, 2016).

Sections of bronchiole were stripped of attached alveolar tissue using a razor blade. Sections were then washed in petri dishes containing a 50:50 mix of Dulbecco's modified Eagle medium (DMEM) (Biosera, France) and Roswell Park Memorial Institute medium (RPMI) (Biosera, France). Dissection scissors were then used to remove any remaining alveolar tissue. Bronchioles were then cut into 5mm by 5mm squares and washed again in 50:50 DMEM:RPMI and once in ASM. Bronchiole sections were then transferred to 24-well plates (VWR international, USA) containing 400µL solid ASM supplemented with 0.8% agarose (Sigma, USA).

Bronchiole sections were inoculated with VAP isolates of *S. aureus*, *K. pneumoniae*, and *P. aeruginosa* (Supplemental figure 1). To inoculate, a sterile hypodermic needle (27G) was lightly touched to the surface of a colony grown on

MH agar. Needles were then used to prick the bronchiolar tissue to inoculate. Mock infection controls were prepared by pricking bronchiole sections with a sterile needle. ASM (500µL) was added to each well to bathe bronchiole sections. TNFα was then added to the ASM according to the assay template, to concentrations of 30pg/mL, 300pg/mL, and 3000pg/mL. Tissue sections were then incubated at 37°C aerobically on a Stuart shaking incubator SI500 (SLS, UK) at 100rpm, for 96 hours.



Supplemental figure 1: 24-well plate template for the investigating changes in biofilm growth of VAP bacteria with different concentrations of TNFα. Bronchioles in columns 1-3 were inoculated with either *S. aureus, K. pneumoniae*, or *P. aeruginosa*. Columns4-6 were mock inoculum controls and were pricked with a sterile hypodermic needle.

Following incubations, bronchioles were washed in 1mL PBS to remove loosely adherent cells. Tissues were transferred to 2mL screw cap microcentrifuge tubes (VWR international, USA) containing 500µL sterile PBS and a metal bad. Tissues were then homogenised using a TissueLyser II (Qiagen) at 24Hz for 30 minutes. Homogenates were serially diluted tenfold, and dilutions (100µ) were spread onto

MH agar. Plates were incubated for 18 hours at 37°C to obtain single colonies, which were then counted to provide a measure of biofilm growth.