Assessment of an *ex vivo* porcine lung model to investigate host-microbe interactions in cases of ventilator associated pneumonia.

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ABBREVIATIONS

16S ribosomal RNA (or 16S rRNA)

A

Abbreviated Injury Scale (AIS)

Acinetobacter baumanii (A. baumannii)

Acquired immune deficiency syndrome (AIDS)

Acute respiratory distress syndrome (ARDS)

Airway surface liquid (ASL)

Antigen-presenting cells (APC's)

Antimicrobial resistance (AMR)

Arginine vasopressin (AVP)

C

C-reactive protein (CRP)

Centres for Disease Control (CDC)

Centres of Disease Control and Prevention (CDCP)

Central line associated bloodstream infections (CLABSI)

Chronic Obstructive Pulmonary Disease (COPD)

Clinical Pulmonary Infection Score (CPIS)

Clostridium difficile (C. difficile)

Clumping factor A (clfA)

Clumping factor B (ClfB)

Community acquired infection (CAI)

Computed tomography (CT)

Control library (PhiX)

Cranioventral pulmonary consolidation (CVPC)

Cystic fibrosis (CF)

D

Disability-Adjusted Life Years (DALY's)

E

Escherichia coli (*E. coli*)

Endotracheal tube (ETT)

Extracellular polymeric substance (EPS)

G

Gut associated lymphoid tissue (GALT)

H

Haematoxylin and Eosin (H&E)

Haemophilus influenzae (H. influenza)

Healthcare-associated infections (HCAIs)

Human immunodeficiency virus (HIV)

Hospital-acquired pneumonia (HAP)

Hybridization buffer (HT1)

I

Intensive care units (ICUs)

Interferon gamma (IFN-γ)

Interleukin (IL)

Injury Severity Score (ISS)

K

Klebsiella pneumoniae (K. pneumoniae)

L

Lipopolysaccharide (LPS)

Lower respiratory tract (LRT)

M

Major heart surgery (MHS)

Methicillin-resistant *Staphylococcus aureus* (MRSA)

Microbial surface components recognizing adhesive matrix molecules (MSCRAMM)

Multidrug resistance (MRD)

Mycoplasma hyopneumoniae (*M. hyopneumoniae*)

N
National Health Service (NHS)

Next-generation sequencing (NGS)

National Institute for Health and Care Excellence (NICE)

Neonatal intensive care unit (NICU)

Noninvasive ventilation (NV)

O
Operational taxonomic units (OTUs)

P
Pattern Recognition receptors (PRR)

Pathogenicity islands (PAIs)

Pediatric intensive care unit (PICU)

Phosphate-Buffered Saline (PBS)

Polymerase chain reaction (PCR)

Pseudomonas aeruginosa (P. aeruginosa)

Q
Quantitative polymerase chain reaction (qPCR)

Quorum sensing (QS)

R
Raoultella ornithinolytica (*R. ornithinolytica*)

S
Serratia marcescens (*S. marcescens*)

Surfactant proteins (SP)

Staphylococcus aureus (S. aureus)

Stenotrophomonas maltophilia (S. maltophilia)

Streptococcus pneumoniae (*S. pneumoniae*)

T
Regulatory T-cells (T-regs)

Toll like receptors (TLR)

Tumour necrosis factor-alpha (TNFα)

U
Ultraviolet Light (UV)

Urinary tract infections (UTIs)

V
Ventilator-associated pneumonia (VAP)

Ventilator-associated tracheobronchitis (VAT)

W
World Health Organisation (WHO)

ABSTRACT

VAP is described as a pneumonic lung infection afflicting those that have been mechanically ventilated typically with an endotracheal tube. Unfortunately, there is no singular diagnostic test for VAP (Grossman & Fein, 2000) or 'gold standard' for the identification and diagnosis of the disease (American Thoracic Society; Infectious diseases Society of America, 2005). Since working with patients within hospitals is unpractical for research for several reasons, a developmental *ex vivo* porcine lung model has been theorised. The benefits of this model include immune similarities to humans and the availability of the tissue since it is normally food waste from an abattoir (Harrison, Muruli, Higgins, & Diggle, 2014). Using this model, the primary aims of this study were; to optimise the killing methodology of excised porcine lung tissue which acts as a control comparison to viable tissue highlighting the hots-microbe interactions, to establish a routine method of taking a BAL sample from the porcine lungs to therefore identify the normal microbial constituents within the porcine lungs, to establish the cell structures communally present within the lung tissue in health and to develop a time scale of the growth of a VAP clinical isolate on inoculated *ex vivo* porcine lung tissue. The results indicated that cold killing was the optimal method to kill porcine lung tissue whilst also maintaining cellular structural integrity to later investigate histological structures. A basic BAL sampling method was developed which allows for future developments, however, several different bacterial species within the lungs were identified. The inoculated *ex vivo* porcine lung tissue CFU growth curve demonstrated that the live inoculated tissue had a larger CFU than the dead inoculated tissue, suggesting that the method of killing was successful in 'knocking out' the host biome. In conclusion the *ex vivo* porcine lung model was further developed from previous studies and results indicated that it is a practical method of exploring the hoist microbe interactions in cases of VAP.

1. INTRODUCTION

1.1 - Epidemiology of ventilator-associated pneumonia

Conflict between both healthcare workers and hospital infections occurs on a frequent basis. Yet, despite many interventions in infection prevention, between 20-50% of patients in intensive care units (ICUs) acquire a nosocomial infection (Van Der Kooi *et al*., 2007). These statistics can be between two to five times higher than that of the general hospital population (Dasgupta, Das, Neeraj, & Hazara, 2015). Variability between incidence rates is believed to be heavily influenced by the prior condition of the patient (American Thoracic Society, Infectious Diseases Society of America 2005). Recently, this has been attributed to the impaired functionality of the patients' immune system prior to the development of healthcare-associated infections (HCAIs) (Hunter, 2012).

Commonly diagnosed HCAIs that ICU patients are at risk of contracting can come via various routes of infection. These include Central-Line Associated Bloodstream Infections (CLABSI), Ventilator-associated Pneumonia (VAP); Catheter-associated Urinary Tract Infections (CAUTI), and surgical site infections (SSI) (CDCP, 2014a). The damage posed by HCAIs is not only to the patient by causing significant morbidity, but also staff and visitors who circulate the busy wards which can result in community-based infections (National Institute for Healthcare and Excellence [NICE], 2011; NHS, 2017). An example of how this disperses is through healthcare staff who, as part of their duties, do home visits and are regularly in contact with those with impaired immune systems or with prolonged comorbidities. The risk of HCAIs is also influenced by environmental factors such as the layout of the ward, for example: bed spacing; contaminated air conditioning systems, which circulate potential pathogens; and poor cleaning practise (Al-Tawfiq & Tambyah, 2014). The size of hospital can also a factor, whereby in one investigation, the incidence of VAP was inversely correlated to the size of hospital (Lee, Walker, Chen, Sexton, & Anderson, 2013).

Over 80% of all HCAIs can be attributed to only six types of infection, with pneumonia/respiratory infections accounting for the largest percentile of 22% (NICE, 2016). This is then followed by urinary tract infections (UTIs) with 17.2%, then surgical site infections with 15.7%, clinical sepsis with 10.5%, gastrointestinal infections with 8.8%, and finally bloodstream infections with 7.3% (NICE, 2016). Amongst the 22% of patients suffering from

pneumonia, VAP is defined as a pneumonic lung infection that occurs in patients of whom have been on mechanical ventilation (MV) (Michetti *et al*., 2012) for 48 hours or more and which was not present before hospital admission (Wagh, & Achayra, 2009). It is a common infection resulting in a prolonged hospital stay (Centres of Disease Control and Prevention ((CDCP)), 2003). Average length of MV is around 6.1 days (Klompas, Kleinman, & Karcz, 2012), and has between a 15-45% contributory mortality rate (Bouza, Granda, Hortal, Barrio, Cercenado, & Muñoz, 2013). This rate may rise to be greater than 70% in cases where the infection is caused by multi-drug resistant (MDR) and/or invasive pathogens (American Thoracic Society, Infectious Diseases Society of America, 2005). As defined by the centre of disease control (CDC), an MDR pathogen is an isolate that is resistant to at least one antibiotic in three or more drug classes (Siegel, Rhinehart, Jackson, & Chiarello, 2006). Of greater concern, there are increasing numbers of MDR-pathogens that have developed resistance against carbapenem antibiotics; broad-spectrum antibiotics which are widely deemed as the 'last line of defence' against Gram-negative bacteria (Bryan, 2014). A patient's calculation of risk of VAP includes both modifiable and non-modifiable factors. Non-modifiable factors include sex, age, and pre-existing conditions. Examples include pulmonary diseases such as Chronic Obstructive Pulmonary Disease (COPD) and cystic fibrosis (CF), and those that directly compromise the immune system such as HIV/AIDS. In contrast, modifiable factors include the treatment and care the patient receives (Weinstein *et al*., 2004). Despite the multitude of factors that influence the patient's risk of VAP, the main links to patient mortality are pre-existing conditions/comorbidities, the pathogen inducing the infection, and the efficacy of the initial antimicrobial treatment given (Iregui, Ward, Sherman, Fraser, & Kollef, 2002).

There are a wide range of patients susceptible to VAP infection. Those receiving transplants are within the group of patients at risk of VAP. The most frequent complication following lung transplant surgery is VAP, often contributing to the overall high mortality rates (Fishman, 2007). Cases of infection can be heightened by improper lymphatic drainage, airway clearance negatively impacted by nerve damage during transplantation and the graft contact with the environment (Riera, Caralt, López, Roman, Gavalda, & Rello, 2015). A similar situation is found within burn patients, whereby 42-65% of mortalities are attributed to infection (Lachiewicz, Hauck, Weber, Cairns, & van Duin, 2017), with VAP causing the most substantial complications (Younan, Griffin, Zaky, Pittet, & Camins, 2018). Following a

comparative study of VAP in burn patients and those ventilated due to a brain injury, it has been reported that the cases of resistance were far lower in brain injured patients than in burn victims (Roquilly *et al*., 2016). These results could be explained by a depletion in local epidermal microbial ecology and the severity of the injuries (Rello, Sa-Borges, Correa, Leal, & Baraibar, 1999).

Another susceptible group are those who have recently undergone major heart surgery (MHS). Data suggests that respiratory infections contribute to 57% of all infections following MHS (Bouza, Hortal, Muñoz, Pascau, Pérez, Hiesmayr, 2006). An extensive meta-analysis highlighted the risk of respiratory infections in a far larger cohort and concluded that those who needed mechanical ventilation post MHS had an average VAP incidence of 35% (He *et al*., 2014). Of those who develop VAP, attributed mortality rates are between 15-45% (Bercault & Boulain, 2001). The type of surgery MHS subgroups receive also influences the incidence of VAP, with those receiving multiple surgeries most at risk (Hortal *et al*., 2009). Within this group unfortunately, many of the risk factors are not acquiescent for improvement, leaving this population at an extremely high risk of VAP. This further highlights the need for effective pre-emptive procedures to be in place as denoted by the American Journal of Respiratory and Critical Care Medicine (2005).

Patients also at a particularly high risk of developing VAP are trauma patients who have sustained thoracic injuries. The trauma sustained can lead to reduced levels of consciousness, and therefore patient care recommends immediate mechanical ventilation (Cavalcanti *et al*., 2006). Issues can arise, as patients who have undergone pre-hospitalisation intubation, with an initial failure, has been recorded to have a greater than three times chance of developing VAP than those who had a successful initial intubation (Mangram *et al*., 2006). In addition, the placement of the endotracheal tube (ETT) can cause varying levels of tracheal epithelial damage (Divatia, & Bhowmick, 2005). This will be elaborated on later in the publication.

When comparing the rates of VAP in critical-care units of several hospitals, one study suggested that the incidence of VAP was around four-fold higher in trauma patients than in ventilated non-trauma patients (Cook, Norwood, & Berne, 2010). Trauma patients are those who have suffered grave and life-threatening injuries which can be evaluated using the Injury Severity Score (ISS) being the oldest and best-known, consequent from Abbreviated Injury

Scale (AIS) data (Palmer, Gabbe, & Cameron, 2016). There are also increased levels of risk to developing VAP even within the type of trauma the patient has received, for example, higher levels of VAP are associated with severe head and neck trauma (Cavalcanti, Ferrer, Ferrer, Morforte, Garnacho, & Torres, 2006).

Within paediatric ICUs (PICUs), treatment of VAP amounts to over half of the total antibiotics used on the ward (Foglia, Meier, & Elward, 2007). Overall, VAP is the second most common infection acquired in PICUs and neonatal intensive care units (NICUs), with it affecting 3-10% of ventilated children (Almuneef, Memish, Balkhy, Alalem, & Abutaleb, 2004). Of the infected, the mortality rate attributed to VAP is approximately just over 10% (Ismail, Darbyshire, & Thorburn, 2012). Whilst this research does not focus on paediatric patients nor does the majority include data sets from minors (under 18), it is a group worth mentioning even if only briefly.

In summary of those who are at great risk of acquiring VAP as discussed in this chapter can be seen in Table 1 below.

Table 1.1: Denoted risk factors for development of VAP and risk specific justifications.

Risk groups:	Explanation:
ICU patients	Weakened immune status due to previous injury creating an increased risk of infection
Trauma patients	
Mechanical ventilation	Thoracic injuries/brain injuries
Those receiving at home care	A vulnerable group of often immune compromised/weakened individuals who are exposed by health care workers to HAIs
Post-surgery patients	Open wounds are ideal for opportunistic pathogens. Often on immune suppression drugs. Translocation of microbes which become pathogenic in new location post-surgery. The body is often in a weakened state after undergoing surgery creating a higher risk of infection.
Post-Transplant patients	
Mechanical ventilation	

Burn victims	Loss of normal protective endothelium barriers and lower immune function due to trauma. Often must be ventilated if tracheal injuries are sustained. Insertion of tube can also damage epithelium and lead to excessive fluid build-up around the cuff.
Non-modifiable factors	Age, sex, pre-existing medical conditions, and genetics.

Whilst it is important to discuss the groups which are commonly affected by VAP, it is also of note that that there are many exclusion categories for when researching VAP. This includes high severity patients at elevated risks of morbidity, or due to a lack of sufficient clinical or microbiological data able to be collected (Zakharkina *et al*., 2017; Rello, & Diaz, 2003). Alongside this, children under the age of 18, inpatients to a care practise other than acute care hospitals, and patients on high frequency ventilation are also frequently excluded (Magill, 2012).

Overall, there are multiple groups of patients at risk of VAP. Much of the data suggests that those within ICU wards have the greatest risk of developing VAP, and within that category, trauma patients have the highest mortalities. This suggests that prior comorbidities are a significant influence on the development and severity of VAP.

Frequent causative pathogens of VAP comprise of gram-negative bacteria such as *Pseudomonas aeruginosa* (*P. aeruginosa*)*, Escherichia coli* (*E. coli*)*, Klebsiella pneumoniae* (*K. pneumoniae*)*,* and *Acinetobacter* species, and gram-positive bacteria such as *Staphylococcus aureus ((S. aureus)* (Kollef, Shorr, Tabak, Gupta, Liu, & Johannes, 2005). Since the causative agent and MDR status varies between hospitals, regions, and countries, it is important for mass microbial surveillance data to be reported (Chi et al., 2012). This will be expanded further within this report.

1.2 - Diagnosis of VAP

Unfortunately, there are no clinical symptoms that can be singularly used in the diagnosis of VAP (Rea-Neto *et al*., 2008). The Clinical Pulmonary Infection Score (CPIS) uses a combination

of diagnostic tools including: temperature; white blood cell count; tracheal secretions; oxygenation PaO_2/FiO_2 mm hg to calculate respiratory efficiency with low levels often indicating pathophysiological states; pulmonary radiography and culture of tracheal aspirate specimen (Singh, Rogers, Atwood, Wagener, & Yu, 2000). Each category has a score from 0-2, and patients are deemed to have VAP when the CPIS exceeds 7 points or more (Singh, Rogers, Atwood, Wagener, & Yu, 2000). Diagnosis, however, with this criterion is still problematic due to the parameters measured often already being raised due to prior injury and/or other sources of non-VAP related inflammation (Kalil *et al*., 2016).

In recent years, there have been many alterations in the diagnosis of VAP, as previous methods were deemed to be subjective and inconsistent. Therefore, there is a great deal of variance in classification within the literature on the topic. The previous guidelines outlined by the CDC indicate that a patient that has been ventilated for 48 hrs or more can be diagnosed with VAP when there is evidence of new/progressive pulmonary infiltrates via the chest radiograph, alongside two or more of the subsequent criteria: fever >38.5 °C or hypothermia <36 °C, minimum leukocytosis of 12×10^9/L, purulent tracheobronchial secretions or a reduced PaO_2/FiO_2 of 15% or more, confirmed commonly by the isolation of one or more species of pathogenic bacteria (Tablan, Anderson, Besser, Bridges, & Hajjeh, 2004). However, with reference to the new CDC guidelines, VAP can only be diagnosed three days post-mechanical ventilation, and two days before/after the decrease in oxygenation alongside positively screen respiratory cultures (CDCP, 2014b). There are also further VAP definitions; early-onset VAP, considered to develop within 48hrs, and late-onset, developing at any time beyond that (Wagh, & Achayra, 2009).

Whilst diagnosing VAP quickly is crucial to avoid delay in the necessary treatment measures (Iregui, Ward, Sherman, Fraser, & Kollef, 2002), a rushed or incorrect diagnosis also can lead to further complications, such as inappropriate and unnecessary treatments (Deeks, 2001; Dellinger *et al*., 2008). When diagnosing VAP it is also important to be aware of ventilator-associated tracheobronchitis (VAT), deemed to be the intermediate stage between the colonization of the lower respiratory tract (LRT) and VAP (Piazza, & Wang, 2014). This is often associated with an increased production of infected sputum (Piazza, & Wang, 2014). Through histological data, it has been identified that VAT is a continuum to VAP (Nseir & Martin-Loeches, 2014). Key symptoms of VAP are also common in numerous other conditions, thus

resulting in more problematic diagnosis (Wiener-Kronish, 2009). Diseases that have similar clinical symptoms include acute respiratory distress syndrome (ARDS), pulmonary oedema, pulmonary contusion, tracheobronchitis, and thromboembolic disease (Gunasekera, & Gratrix, 2016).

Diagnosis of VAP tends to be based on multiple criteria, one of which is a culture-based method (Ullberg, Luthie, Molling, Stralin, & Ozenci, 2017). Quantitative cultures of bronchoalveolar lavage (BAL) or tracheal aspirates with thresholds of more than 10^3 or 10^4 colony-forming units (CFUs) can microbiologically meet the criteria of a VAP diagnosis (Willson, Conaway, Kelly, & Hendley, 2014). This is a time-consuming process, in some cases taking several days to culture certain species of bacteria (Bahrani-Mougeot *et al*., 2007). By the time colonies are present, the flora within the patient may have changed. It may also not identify any anaerobic organisms if the culture is exposed to oxygen (Bahrani-Mougeot *et al*., 2007). Under conditions where there are limited resources; bacteria will alter motility and surface factors expressed in a trade off with growth rates to survive in niche environments often in competition with other bacterium, potentially causing bacterial populations to diminish (Gude *et al*., 2020).

A BAL can be defined as a process designed to isolate the cells that are non-adhesive, in addition to the fluid lining the lungs' mucus for sampling. BAL samples within healthcare are commonly used for diagnostic and therapeutic reasons, whilst within research it is often to yield cells for *ex vivo* culture (Collins *et al*., 2014). In research, it allows for the identification of macrophages, B- and T- lymphocytes, and immunoglobulins which are active within the lungs (Fullerton *et al*., 2009). To reap the greatest benefit from samples, a minimum of 200mL of collection fluid is suggested alongside manual suction methods to reduce cellular damage (Collins *et al*., 2014). Whilst BALs are an important tool to collect data from the lungs, there are also disadvantages to this technique. As aforementioned, it can damage the lung epithelium leading to latter complications. The sensitivity and specificity of this method is also reported to have varying degrees of success. This will be expanded upon later within this report.

An alternative method of diagnosis is the chest radiograph, but this itself comes with its own difficulties acquiring both sensitivity and specificity (Koenig & Truwit, 2006). Regarding

specificity, as previously noted, there are several similar pulmonary diseases to VAP, thus, the radiographic specificity is on average under 35% (Lefcoe, Fox, Leasa, Sparrow, & McCormack, 1994). Another difficulty of note is that there are differences between the results from X-rays and computed tomography (CT) scans, as just under 30% of opacities are detected by CT scans but not via chest X-ray (Butler *et al*., 1999). Since there are no specific radiological measures for VAP, chest radiographs are left to subjectivity, leading to a lack of sensitivity and reliability (Klompas, 2007). There are, however, integral advantages of non-bronchoscopic techniques; as they are less invasive, less likely to compromise the patients access to oxygen, unlikely to increase intracranial pressure or induce arrhythmias, and do not pose a risk of contamination of the bronchoscopic channel (Koenig, & Truwit, 2006).

1.3 - Treatment and Prevention strategies

1.3.1 - Cost

In the UK, the treatment of VAP costs around £10,000-£20,000 per patient (NHS, 2016). This is similar to hospital costs in both the United States and Europe, thus indicative of a potentially global issue (Safdar, Dezfulian, Collard, & Saint, 2005; Wyncoll, & Camorota, 2012). In a survey comparing the cost of treatment of patients with VAP recovering from MHS to those without VAP, the VAP positive cases cost an extra £8829 (Luckraz *et al*., 2017). In addition, the World Health Organisation (WHO) (2016) approximated LRT infections to have been the cause of 3 million deaths worldwide and, by their estimations, in 2020, the figure will have risen to be closer to 4 million. This highlights the need for more effective prevention strategies and treatment plans to be implemented.

1.3.2 - Antibiotics

There is limited data surveying the impact of anticipatory treatments with broad spectrum antibiotics as a preventative measure to VAP (Bouza, *et al*., 2013). There are arguments for both the delay and immediate use of antimicrobial therapy in the combat of VAP, a delay can be associated with treatment failure and mortality, conversely, excessive, or incorrect use of treatment often promotes multidrug resistance leading (MDR) to global complications in future treatments and resistance to treatments within the patient (Riera *et al*., 2015).

Evidence supporting broad spectrum treatment can be observed in the comparison between patients receiving inadequate antibiotics, found to have a longer hospital stay by 15 days, than those receiving adequate antibiotic treatments (Piskin *et al*., 2012).

The most common pre-emptive antibiotic therapies for VAP include initially vancomycin for methicillin-resistant *S. aureus* (MRSA) alongside either ciprofloxacin, imipenem, cefepime or pipercillin-tazobactam for *P. aeruginosa*. The choice of combination subject to the prescribing physician and will be based on numerous factors such as patient antibiotic history and antimicrobial resistance (AMR) within the hospital (Iregui *et al*., 2002). There is also worrying evidence of further resistance in MRSA isolates as in a study involving 550 patients, over 50% were resistant to clindamycin, erythromycin, ciprofloxacin, trimethoprim-sulfamethoxazole, tetracycline, and gentamicin, whilst only being susceptible to rifampicin, chloramphenicol, and vancomycin (Rocha, Ribas, Darini, & Filho, 2013). Prescription errors may be avoided by new rapid nucleic acid amplification protocols which identify resistance genes; however, this does not provide information on the phenotypic antimicrobial susceptibility and cannot distinguish if the pathogen sequenced is dead or alive (Timsit, Esaied, Neuville, Bouadma, & Mourvllier, 2017).

Further complications arise as patients may not respond well to certain antibiotics. Whilst it is still very much a grey area as to why, it may potentially be due to the ineffectiveness of most common therapies such as β-lactams, aminoglycosides, and vancomycin at penetrating the lung tissue to target the pathogenic bacteria (American Thoracic Society, Infectious Diseases Society of America, 2005). These antibiotics target the synthesis of the peptidoglycan layer of the cell wall surrounding in various ways (Majiduddin, Materon, Palzkill, 2002) but there are several other types of antibiotic classes which have different target mechanisms as described in Figure 1.1.

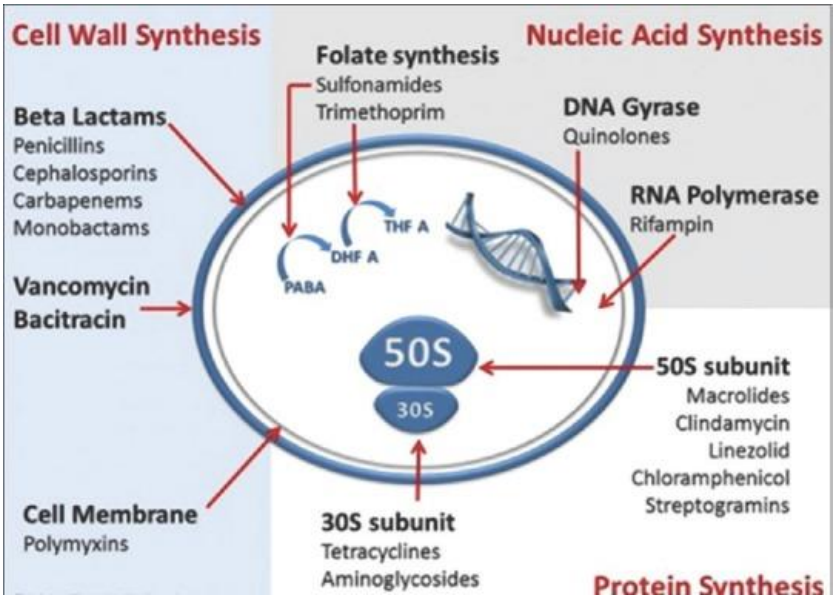

Figure 2.1: Different classes of antibiotics segregated by their mechanisms (Source: Kapoor, Saigal, & Elongavan, 2017).

β-lactams and glycopeptides inhibit cell wall synthesis by disrupting the peptidoglycan layer leading to the lysis of the bacterium (Džidić, Šušković, & Kos, 2008). Glycopeptides bind to D-alanyl D-alanine portion of peptide side chain of the precursor peptidoglycan subunit thus inhibiting cell wall synthesis (Džidić, Šušković, & Kos, 2008). There are also inhibitors of protein biosynthesis which target the 30S or 50S subunit of the bacterial ribosome (Johnston, Mukhtar, & Wright, 2002), for example Oxazolidinones, Macrolides and Chloramphenicol all target the 50s subunit. Aminoglycosides main target of action is the bacterial ribosome which requires oxygen, meaning they are most effective in aerobic conditions and have poor activity against anaerobic bacteria whilst Tetracyclines act upon the conserved sequences of the 16S r-RNA of the 30S ribosomal subunit to prevent binding (Yoneyama & Katsumata, 2006). Some inhibit DNA replication such as Quinilones whilst others inhibit the folic acid metabolism such as Sulfonamides and Trimethoprim, each inhibiting distinct steps in the folic acid metabolism pathway (Yoneyama & Katsumata, 2006).

On average it has been reported that for a third of those on intravenous antibiotics the treatment will fail (Wood, 2016). The use of combination therapies is an alternative approach, achieved by combining different classes of antibiotics, this method has been proven to be significantly effective against MDR Gram-negative bacteria (Martínez *et al*.,

2010). Combination therapies that involve the use of fluoroquinolones have been shown to reduce the rates of relapse or reinfection of VAP characterised by *P. aeruginosa* or *Enterobacteriaceae* (Planquette *et al*., 2013). However, the effectiveness of the fluoroquinolones against MDR Gram-negative bacteria appears to be inconsistent and highlights the risk of MDR bacteria in the lung microbiota (de Lastours *et al*., 2014).

According to a recent list published by WHO the most antimicrobial-resistant include carbapenem-resistant *Acinetobacter baumannii (A. baumannii)*, carbapenem-resistant *P. aeruginosa* and members of the family Enterobacteriaceae which are carbapenem-resistant and containing extended spectrum β-lactamases, all of which are Gram-negative pathogens (Willyard, 2017). Most organisms harbour multiple resistance mechanisms such as drug inactivation and drug modification to reduce efficiency to act on target (Arzanlou, Chai & Venter, 2017), there are several other antimicrobial resistance mechanisms which have been demonstrated in Figure 1.2

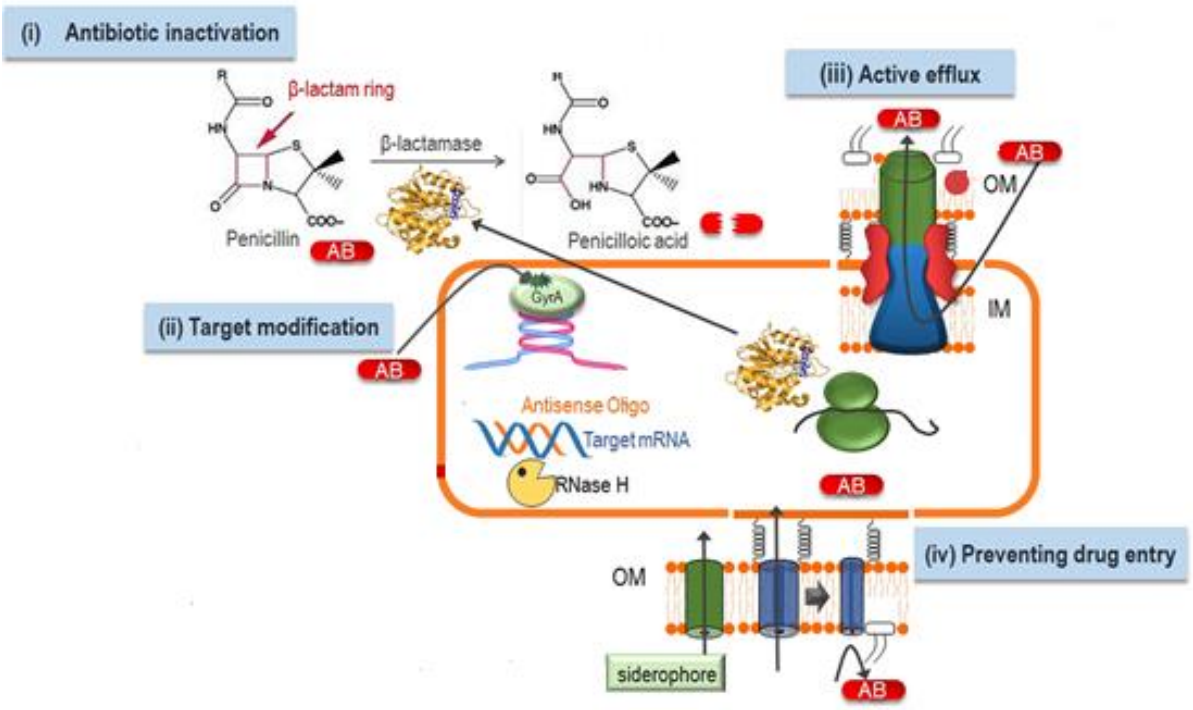

Figure 1.2: Various antimicrobial mechanisms deployed by bacteria (Source: Venter, 2019).

The blue boxes are the four main antimicrobial resistance mechanisms deployed by Gram-negative organisms, such as; (i) antibiotic inactivation, eg. via β-lactamase enzymes that hydrolyse the β-lactam ring thereby deactivating some antibiotics, (ii) target modification, resistance to fluoroquinolones is conferred via GyrA protein, (iii)active efflux, efflux pump removes antibiotic from within bacterial cell to a sub-toxic level, (iv) prevention of drug entry, often via mutation or loss or porins (Venter, 2019).

As mentioned, the highest risk of VAP acquisition is damage to the epithelium when the ETT is inserted. This is partially due to damaged tissue expressing higher quantities of the receptor asialoGM1 which bacteria can adhere to (Bucior, Mostov, & Engel, 2010). In addition to the physical trauma ETTs cause, they may also dampen the effectiveness of antibiotic ability, by impairing the physiological host defence mechanisms for clearance of pathogens whilst also providing a mechanism for the transportation of oropharynx microorganisms to the previously sterile tracheobronchial network (Barnes, Feit, Grant, & Brisbois, 2019), which may perhaps be a factor for why there is a high level of antibiotic resistant bacteria found to be the cause of VAP (Gil-Perotin *et al*., 2012). This offers another explanation as to why some classes of antibiotics impact the respiratory microbiome, while others appear to have little effect since the dampening effect will vary depending on the main target of the antibiotic. When not in a state of trauma the epithelium is normally resistant to *P. aeruginosa* infection, which explains the opportunistic capabilities of the bacterium when damage to the epithelium occurs (Galle, Carpentier, & Beyaert, 2012). Another risk due to the ETT is the capability for some bacteria to utilise this surface and form biofilms, thereby persisting in the tube, and providing a means for a pathogenic species to colonise the patient's lungs (Zur, Mandell, Gordon, Holzman, & Rothschild, 2004).

1.3.3 - Alternatives to antibiotics

More commonly non-invasive ventilation (NV) is increasingly utilised in VAP patients, with results reporting reduced mortality rates, intubation periods, further complications, and overall hospital duration (Lightowler, Wedzicha, Elliott, & Ram, 2003). Unfortunately, this form of treatment is not yet applicable to all respiratory diseases, however, there is ongoing research into its usage in patients with pneumonia and other types of hypercapnic failure (Shneerson, & Simonds, 2002). Alternatively, an effort to reduce the biofilm capabilities of the bacteria is to coat the endotracheal tube (ETT) with silver due to its broad-spectrum

antimicrobial activity and the reduce ability of bacteria *P. aeruginosa* to adhere to it (Ahearn *et al*., 2000). This technique has been demonstrated to reduce the scale of colonization from *P. aeruginosa* (Olson, Harmon, & Kollef, 2002). However, when reviewing experiments deploying this strategy whilst VAP has been significantly delayed in onset, the disease still occurs (Kollef *et al*., 2008). This finding is suggestive that the disease is not singularly caused by colonization of the ETT.

Other preventative measures including revolving the ETT and the efficient drainage of subglottic secretions produced, also reduce the incidence of VAP (Hunter, 2006). Contaminated fluids collecting above the cuff and gaining access to the LRT via a fold in the wall of the cuff is associated with incorrect positioning and pressure (Gunasekera, & Gratrix, 2016). Ensuring the cuff is functioning and not a potential risk to patients can be confirmed by repeated checks of the cuff pressure for it to be between a minimum of 25cm and a maximum of 30cm of water and to also prevent tracheal injury (Ricart, Lorente, Diaz, Kollef, & Rello, 2003). To reduce biofilm formation there have been attempts to modify the ETT via coating in silver or other nanoparticles, results have been suggested to show a decrease in incidence in VAP (Garland, 2010). Despite this, biofilms remain and pose a serious risk to patient health indicating the need for new effective preventive and therapeutic anti-biofilm developments (Hazan *et al*., 2006).

The reduction of the patient's ability to clear the accumulation of tracheobronchial secretions through coughing due to the ETT, elevates the risk of colonization and therefore pneumonia (Kollef, 2004). There is also a risk of contamination if healthcare staff try to remove the fluid (Williams, Dehnbostel, & Blackwell, 2010). Many investigations into these reservoirs of secretory fluids have identified that the cuff on the ETT may lead to microaspirations of the fluid causing tracheobronchial colonization resulting in VAP (Smulders, van den Hoeven, Weers-Pothoff, & Vandenbroucke-Grauls, 2002; Dezfulian *et al*., 2005).

The incidence of VAP has also been linked to the development of bacterial colonies within the oral cavity, especially the accumulation of pathogenic bacteria on the teeth, and subsequent plaque build-up (Rello *et al*, 2002; Schleder, 2003). Therefore, there have been many studies investigating the effect of more rigorous oral hygiene techniques, many of which indicate a reduction in VAP cases within ICU facilities (Coffin *et al*, 2008). Within a cohort of 90 patients

who were ventilated, the test group were subjected to a rigorous oral hygiene procedure including washing with an antiseptic solution of 1.5% hydrogen peroxide whilst the control group had the traditional rinse with 0.2% solution of chlorhexidine gluconate (Lev, Aied & Arshed, 2015). The results indicated that the control group had four times as many cases of VAP recorded than the study group, meanwhile the mean ventilation and days in hospital were lower in the study group than the control (Lev, Aied & Arshed, 2015). Similar studies also support these findings, as they also report further reductions in duration of ventilation, hospital stay and mortality with more rigorous oral hygiene procedures (Garcia *et al*, 2009).

Alongside the preventative measures relating to the host include that the mouth of the patient is cleaned repeatedly; that equipment such as the ventilator tubing is clean and replaced between patients and that the head of the patient's bed raised to a 30-to-45-degree angle, medical conditions permitting (Centres for Disease Control and Prevention, National Centre for Emerging and Zoonotic Infectious Diseases (NCEZID), Division of Healthcare Quality Promotion (DHQP), 2019).

There are no vaccinations specifically for VAP, partially due to the numerous causative pathogens, there are vaccination programmes which have reduced the occurrence of VAP caused by *H. influenzae* type B, *Streptococcus pneumoniae* and *Influenza virus* (Herceg, 1997). Vaccinations may prevent HCAI and may be considered before ventilation and prior to discharge for those at high risk of a subsequent respiratory infection (Wagh & Achayra, 2009).

Overall, whilst there is little evidence for treatment of late onset VAP there has been in cases of early onset. Despite the support for preventative interventions, clinical compliance can vary between 30-64% (Bouadma, Wolff & Lucet, 2012). A primary aim of healthcare professionals should be in reducing the modifiable risk factors for example, the endotracheal and nasogastric tubes, tracheotomy, reintubation, enteral nutrition, corticosteroid administration, gastric pH-modifying agents, supine positioning, prior antibiotic usage, poor infection control practice, and contaminated respiratory equipment, medications, or water (Koenig & Truwit, 2006).

1.4 - Pathogens associated with VAP

1.4.1 - Common clinical isolate pathogens

Currently there are believed to be four primary routes for bacteria to reach the LRT and cause VAP: spread due to proximity; hematogenous spread; inhalation and aspiration (Rello, & Diaz, 2003). Inhalation can refer to both microbes from contaminated air, and from the oropharynx, a principle supported by the efficacy of oral antibiotics in VAP prevention (Price, Maclennan, & Glen, 2014). The complicated interactions between the ETT, patient risk factors, virulence factors of the invasive bacteria and the integrity of the hosts immune system, also all influence the etiology of VAP (Kalanuria, Zai, & Mirski, 2014). The pathogens linked to causing VAP have the potential to vary between hospitals, patient populations, previous antibiotic exposure and change rapidly over time highlighting the need for regular isolate surveillance within ICUs (Golia, Sangeetha, & Vasudha, 2013).

The common bacterial causes of VAP are noted to be MRSA (44%) combined with *Acinetobacter baumanii (A. baumannii)* (30%), followed by *P. aeruginosa* (12%), *Stenotrophomonas maltophilia* (7%), *K. pneumoniae* (6%), and *Serratia marcescens* (2%) isolated from BAL samples of patients with VAP (Chi *et al*., 2012) as seen in Figure 1.3.

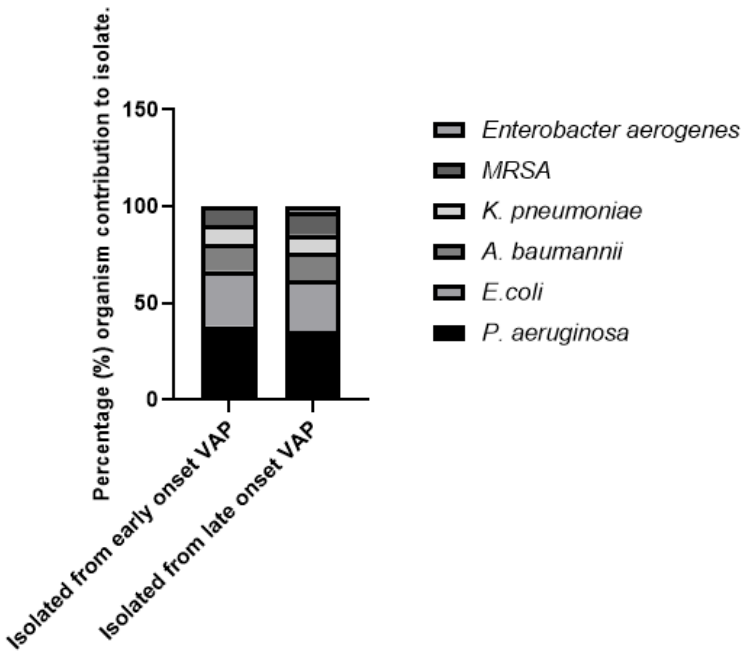

Figure 1.3: A comparison of the isolated pathogens in late and early onset VAP. The results demonstrate that in both the late and early onset cases of VAP *P. aeruginosa* was the

primary shared isolate, of 33%, followed by *E. coli,* 25.42%, then *A. baumannii*, 13.56% (Source adapted from: Golia, Sangeetha, & Vasudha, 2013).

There was no recorded significant difference between bacterial causes of late and early onset VAP demonstrated by Figure 1.1, which represents a comparison of bacterial isolates taken from patients with early and late onset VAP (Golia, Sangeetha, & Vasudha, 2013). Even between these two studies there is a difference between the highest percentile colonizing organism. This broad range of potential causative pathogens is a contributory factor as to why diagnosing the specific cause of VAP is so difficult and why appropriate antimicrobial treatment is vital.

Most Gram-negative bacteria produce lipopolysaccharide (LPS), which creates a permeability barrier on the cell surface and is a primary contributor to antimicrobial resistance whilst also stimulating the immune system (Bertani, & Ruiz, 2018). LPS structure is relatively conserved between bacteria, but there are slight variations within species and strains, and it can also undergo modifications in reaction to environmental conditions (Klein & Raina, 2015). Overall LPS frequently plays a key role in bacterial pathogenicity (Scott, Oyler, Goodlett & Ernst, 2017).

Many clinically relative gram-negative bacteria also use quorum sensing (QS) to regulate their expressed virulence factors, for example *S. aureus* uses the paradigmatic Agr system to adjust adhesion and production of virulence factors, whereas *P. aeruginosa* uses two circuits arranged in tandem to control virulence factor production and biofilm formation, both of which cause persistent disease (Rutherford & Bassler, 2012).

A risk factor which has a significant impact on the transmission of MRSA and has therefore, implications in VAP is the colonization pressure (Williams *et al*., 2007). Colonization pressure is defined as the percentage of patients colonized with a pathogen within the same environment during a specific time and can be used to quantify the level of antibiotic resistant bacteria and be used to estimate the probability of cross transmission within the area (Ajao *et al*., 2012). This understanding can also be used in a more therapeutic approach since evaluating the colonization pressure could be used to determine the most relevant course of antimicrobial therapy for each specific ward.

1.4.2 VAP Pathology

Of the patients within an ICU who contract hospital-acquired pneumonia (HAP), 90% are mechanically ventilated (Pinzone, Cacopardo, Abbo, & Nunnari, 2014). The typical barriers which help the host prevent respiratory infections include anatomical barriers, cough reflexes and mucociliary clearance (Lillehoj, & Kim, 2002). However, the presence of the ETT used in mechanical ventilation frequently dampens mucocilliary clearance and limits the cough reflex, creating an increase in tracheobronchial secretions (Gil-Perotin *et al*., 2012). The placement of the ETT can be seen in Figure 1.4.

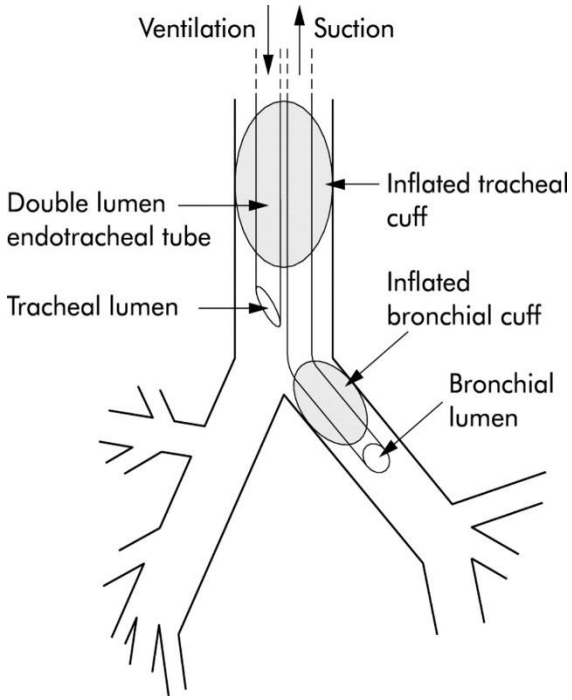

Figure 1.4: The bronchial lumen is positioned in the left main bronchus allowing ventilation of the left lung and the tracheal lumen is positioned above the carina, facilitating ventilation of the right lung (Source: Lordan, Gascoigne, & Corris, 2003).

In disease, excess mucus is also produced due to additional mucosal pathways being activated via positive feedback loops, this then coupled with the inability to remove the secretions via coughing, allows the inhaled pathogens to be deposited on the luminal surface, creating opportunity for host infection (Nadel, 2013), thus heightening the risk of contracting pneumonia. Since mechanical stimulation of the upper airway can activate the afferent and efferent limbs of the vagus nerves causing secretory cell stimulation, this may also contribute to the problem of excessive mucus secretion (Jardine, Bhutta, & Inglis, 2011). The presence

of the tube can also facilitate the movement of gastric secretions around the ETT and then into the LRT (Flanagan *et al*., 2007). The reduction of mucus removal efficiency allows greater admission of bacteria from the upper to the LRTs which enables colonization of the lower airways thought to be a potential causal factor of infection (Xu *et al*., 2015).

VAP by nature is polymicrobial with mixed bacterial-fungal biofilms colonizing the ETT surface, the variety in microbial interactions is deemed to contribute to enhanced pathogenesis leading to the high mortality/morbidity rates, increase antimicrobial resistance rates forcing clinicians to come up with novel therapeutic strategies to treat VAP successfully (Rodrigues et al., 2017).

Whole-genome sequencing has identified not only numerous antibiotic drug resistance determinants but also several pathogenicity islands (PAIs) (Fournier *et al*., 2006). PAIs are genomic islands acquired via horizontal gene transfer that contribute to the microorganisms' ability to evolve (Schmidt & Hensel, 2004). Additional relevant genes to the pathophysiology caused include those involved in the cell envelope, pilus biogenesis, alongside iron uptake and metabolism (Smith *et al*., 2007). For example, a *gacS*-like gene encoding a sensor kinase was identified which is believed to be involved in virulence determinants in other gram-negative species such as *Pseudomonas*, as well as attachment and biofilm mutants (Dorsey, Tomaras, & Actis, 2002).

Mortality rate is highly influenced by the previous impairment of the host immune system (Safdar, Crnich, & Maki, 2005) failed early treatment strategies (Lodise *et al*., 2007), and the virulence factors of the pathogen (Hauser *et al*., 2003).

1.4.3 - Biofilms

A biofilm is commonly characterised as a complex and tightly adherent microbial community, surrounded in a matrix of extracellular polymeric substance (EPS), principally formed of polysaccharides, proteins, nucleic acids, and lipids (Flemming & Wingender, 2010). Statistically biofilms are present on 90% of ETT tubes within seven days of intubation (Solomon, Wobb, Buttaro, Truant, & Soliman, 2009). This is primarily an issue as the ETT-biofilms are associated risk factors for increasing upper respiratory tract infections (Perkins, Mouzakes, Pereira, & Manning, 2004). Regarding VAP, ETT-biofilms are one of the primary causes (Tarquinio, Confreda, Shurko, & LaPlante, 2014; Gil-Perotin *et al*., 2014).

The primary causative bacterium of these biofilms is *P. aeruginosa* (Strateva & Mitov, 2011). For several years it was considered that the main transmission pathway for *P. aeruginosa* infections was via the environment, however, more recently in the last decade the emergence of a variety of different transmission methods, sometimes posing a greater risk to mortality, has caused a shift in view on how the disease is transmitted and how clinicians should treat patients accordingly (Fothergill, Walshaw, & Winstanley, 2012). Infections from *P. aeruginosa* are thought to cause higher rates of mortality than other organisms primarily due to common infectiosnn occurring in those with vast amounts of comorbidities (Parkins, Gregson, Pitout, Ross, & Laupland, 2010).

The presence of biofilms has several negative impacts on a patient's outcome as they; make infection eradication far more challenging, commonly cause chronic inflammation and their formation is associated with antimicrobial tolerance/resistance (Ciofu, Tolker-Nielsen, Jensen, Wang, & Høiby, 2015). In *P. aeruginosa* the switch to growth via biofilm formation allows the bacteria to have a greater survival rate within the host as it enables tolerance to inflammatory defence mechanisms, the lack of oxygen in the aerobic respiratory zones and to the areas which contain aerobic sputum (Bjarnsholt *et al*., 2009). In cases of biofilms caused by *P. aeruginosa*, their structure and stability are denoted to three different polysaccharides: alginate, Pel and Psl (Ryder, Byrd & Wozniak, 2007). Of note, alginate is associated with water retention and nutrient accretion within the matrix (Sutherland, 2001). From the biofilm itself, bacterial cells can detach and be transported down to the LRT via ventilator gas flow and respiration (Gil-Perotin *et al*., 2012).

It is of note that it is not only *P. aeruginosa* that forms biofilms, *A. baumannii* strains have also been found to also have environmental resistance due to its biofilm (Ioanas *et al*., 2004). *P. aeruginosa* and *S. aureus,* often have reciprocal effect on biofilm formation sometimes creating polymicrobial biofilms or sometimes causing intense levels of competitions between the two (Magalhães, Lopes, & Pereira, 2017). However, in a study by Gil-Perotin *et al*., (2012) despite over half of the patients having evidence of biofilm formation only 20% developed VAP, suggesting that whilst biofilm formation is essential for bacterial colonization, it alone is not enough to equate to infection. Whilst this study is only small scale it does illuminate the need for further investigations into the complicated dynamics between bacteria and the host regarding the onset of VAP.

1.4.4- *Staphylococcus aureus*

As mentioned, there are several bacteria deemed to be pathogenic when in association to VAP. In a healthy person *S. aureus* is a commensal and can be seen to colonize various ecological niches (Paling *et al*., 2017). The pathogenic *S. aureus* associated with VAP, are believed to have had to reach a critical load in the altered environment before causing disease (Chastre, & Fagon, 2002). *S. aureus* commonly utilises the Type I toxins to cause diseases, for example it has been noted in cases of superantigen-mediated toxic shock syndrome (Proft, Sriskandan, Yang, & Fraser, 2003). In ICUs *S. aureus* is attributed to high rates of morbidity and elevated financial costs (Bloemendaal *et al*., 2009). The primary reservoir for *S. aureus* is in fact infected patients, it is spread via contaminated health workers, patient to patient, or the environment (Williams, Callery, Vearncombe, & Simor, 2009).

The main reason *S. aureus*, including methicillin-resistant *S. aureus* (MRSA), is so dangerous is that it can survive for extended periods of time outside the host on nearby surfaces which act as a secondary reservoir and is a main component of horizontal microbial transfer. (Rocha, Ribas, Darini, & Filho, 2013). This also puts any visitors at risk who may contact the surfaces surrounding the patient and may increase the accidental spread of the disease from visitor to patient if proper hand washing is not followed. VAP occurs far more frequently in patients with acute respiratory distress syndrome (ARDS) and is often delayed due to the immediate use of antimicrobial therapy, however, this often results in colonization by strains that are resistant; such as MRSA and other MDR microorganisms (Chastre, Trouillet, Vuagnat, Joly-Guillou, Clavier, Dombret, & Gilbert, 1998). Severe infections normally cause an adaptive immune response within a week of the initial infection; however, an advantageous adaptation of *S. aureus* is the ability to cause repeated re-infections believed to be a result of the toxins released altering the hosts T cell activation pathway (Lee *et al*., 2002).

There are a variety of mechanisms of which the *S. aureus* pathogen can deploy to adhere and invade the host epithelial cells which are blanketed under the term microbial surface components recognizing adhesive matrix molecules (MSCRAMM) (Liu, 2009). Two important functional components within the MSCRAMM bracket are cell-wall protein clumping factor B (ClfB) and wall-associated teichoic acid, which both promote adhesion to epithelial cells (Wertheim *et al*., 2009). Another pathogenic mechanism that *S. aureus* deploys is the production of coagulase, an invasive enzyme, which provides an antigenic disguise by allowing

bacterial cells to be resistant to phagocytes, this enzyme is almost always associated with pathogenic S. aureus (Tam & Torres, 2019).

However, it is not essential for the bacteria to enter the host's cell to survive. A capacity which commonly enhances the ability of pathogenicity of *S. aureus* is its capability to thrive both inside and outside of the host's cells (Liu, 2009). The presence of *S. aureus* was also noted to be adhered to the ETT, this may indicate that the bacterium gained purchase during the handling of the tube, thus providing evidence possibly to support the bacterium being a secondary reservoir when it has colonized the local environment (Rosenblatt, Reitzel, Jiang, Hachem, & Raad, 2014).

1.4.5 - *Acinetobacter baumanii*

The incidence of *Acinetobacter* species has increased rapidly over the past twenty years and has been attributed to causing severe infections (Kanafani *et al*., 2018). It is becoming a global problem with multidrug-resistant *A. baumannii* (MDR-Ab) resulting in HCAIs which lead to severe morbidity and even mortality (Sunenshine *et al*., 2007), this is especially prevalent in ICUs (Siegel, Rhinehart, Jackson, & Chiarello, 2007). It is of note that *Acinetobacters* are part of the normal healthy human skin flora (Peleg, Seifert, & Paterson, 2008). However, *A. baumannii*, the relevant nosocomial species, was rarely (0.5-3%) found on human skin (Dijkshoorn *et al*., 2005).

There have even been reports of *A. baumanii* clinical isolates that are resistant to all known classifications of antibiotics (Peleg, Seifert, & Paterson, 2008). The success of this opportunistic may be due to it being able to prevail on both moist and dry surfaces (Espinal, Martí, & Vila, 2012). Almost 70% of critically ill patients are anaemic (Heming, Montravers, & Lasocki, 2011). This is most likely due to the disintegration of the erythropoietin response as a response to cytokines as well as frequent removal of blood for sampling (Yepes *et al*., 2006). *A. baumannii* has adapted to survive such normally toxic environments via secretion of low-molecular-mass ferric binding compounds, which considerably vary between clinical strains (Dorsey, Tomaras, & Actis, 2002).

From epidemiological surveys, most positive cultures of *A. abumanii* were found within respiratory secretions, followed by wound colonizations, blood samples and finally urine samples, which is represented internationally (Maslow, Glaze, Adams, & Lataillade, 2005).

There was also a higher association of resistant strains with patients in the ICU that have respiratory failure and sepsis (Kanafani *et al*., 2018). In extended ICU stays of up to 30 days (the average being 4) there was a recorded increase in resistance of *Acinetobacter* isolates believed to be due to an elevated acquisition of extended spectrum Beta lactamase (ESBL), which are enzymes that confer resistance to most beta-lactam antibiotics, such as Carbapenems (Araj *et al*, 2012). In a financial evaluation those with an *Acinetobacter* colonization accrued greater financial costs than those with different pathogens, of note *Acinetobacter* VAP infections often result in a further 14 days hospitalisation, with additional costs (Kanafani *et al*., 2018).

The key immunostimulatory factor was identified as *A. baumannii* lipopolysaccharide (LPS) which binds to the toll like receptor- 4 (TLR4) (Knapp *et al*., 2006), which when activated leads to the intracellular signalling pathway NF-κB and inflammatory cytokine production (IL-8) which in turn activates the innate immune system (Vaure & Liu, 2014). *A. baumannii* LPS, also stimulates TNFα (Erridge, Moncayo-Nieto, Morgan, Young, & Poxton, 2007) indicating that the *A. baumannii* endotoxin potentially induces a strong inflammatory response during infection (Peleg *et al*., 2008). TLR2 was also highlighted as important in the signalling pathway (Erridge, *et al*., 2007).

Since *A. baumannii* can form biofilms on surfaces and inanimate objects, it may explain its success and rapidly increasing prevalence in hospitals as the biofilm formation is linked to long term survival in both moist and dry surfaces (Tomaras, Dorsey, Edelmann, & Actis, 2003). The *csuE* gene associated with biofilm formation has also been linked to the successful adherence of the pathogen to human bronchial epithelial cells and erythrocytes (Lee *et al*., 2006). Post adherence *A. baumannii* induces apoptosis through an outer membrane protein, Omp38, which initiates both caspase-dependent and independent pathways of apoptosis, whilst an OMP38 mutant caused a reduction in the rate and extent of cell death (Choi *et al*., 2005). Alongside biofilm formation exopolysaccharide production is also believed to protect the bacterium from host defences (Joly-Guillou, 2005).

1.4.6 - *Pseudomonas aeruginosa*

P. aeruginosa is an incredibly successful opportunistic pathogen which displays intrinsic MDR capabilities but also can acquire alternative mechanisms via its fluid genome

(Fothergill, Winstanley & James, 2012). During infection, the pathogen may form biofilms to reduce efficiency of antibiotics whilst also giving it the ability to thrive in a multitude of different environments and harbours an arsenal of virulence factors often secreted by a variety of different effects making it hard to target using anti-microbials (Fothergill, Winstanley & James, 2012).

The type III secretions (T3S) are used in many gram-negative bacteria, including the commonly cited *P. aeruginosa* (Hauser, 2009). Within the T3 system the main apparatus used is the 'injectisome' which enables the bacteria to translocate effectors from the bacterial cytosol to the host cell via a needle like structure (Bridge, Novotny, Moore, & Olson, 2010). The movement of effector toxins between the bacteria and the host cell is so efficient that only 0.1% is leaked into the extracellular space (Sundin, Thelasus, Broms, & Forsberg, 2004). The transported products are associated with disease since they facilitate the circulation of the pathogen around the bloodstream which in severe cases can eventually lead to septic shock (Coburn, Sekirov, & Finlay, 2007). The main proteins transported are; ExoS; ExoT; ExoU; and ExoY (Hauser, 2009). A more detailed investigation on their effects on the host will be discussed later in this report within 'Systemic Response'.

1.4.7 - Rare pathogenesis

Due to the advances in modern detection and sequencing methods, bacterium that was previously disregarded in cases of human infection, is now being increasingly identified as the causative agent, a prime example is the bacterium *Raoultella ornithinolytica (R. ornithinolytica)* (de Jong, de Jong, & Rentenaar, 2013). Despite their being several risk factors associated with *R. ornithinolytica* infection such as solid cancers, diabetes mellitus, immunodeficiency, post-invasive procedures, and post-urethra trauma (Seng, Boushab, & Romain, 2016), until this year there had been no known cases of VAP identified to be caused by *R. ornithinolytica* within immunocompromised patients. However, two have been isolated in a recent study (Van Cleve, Boucher, Smith, & Croce, 2018).

R. ornithinolytica, is incredibly virulent due to its ability to form biofilms and the capability to adhere to human tissue (Djeribi, Bouchloukh, & Menaa, 2012). In both presented cases the bacterium was resistant to aminopenicillins (Zheng, Li, Zhang, Ji, & Fang, 2015). In further analysis of the identified cases, the patients were admitted months apart and no other cases

of the isolate were identified indicating the infection is unlikely to be caused by cross contamination or part of the unit's normal microbial flora (Van Cleve *et al*., 2018). Which poses the question, where did this pathogen appear from and what the relevant method of transmission in when causing VAP.

1.4.8 - Transfer of bacteria on insertion of the ETT

It has long been hypothesised that the oral cavity pathogens and those that cause VAP overlap, and in a recent study this idea was supported since there was an 88% overlap between the two sites (Bahrani-Mougeot *et al*., 2007). This research has also been seen in studies of CF whereby the translocation of oral bacteria such as *Prevotella*, *Veillonella spp* and oral *Streptococci* can be linked to the polymicrobial pathogens causing the chronic illness (Filkins, & O'Toole, 2015). This is a critical discovery regarding the diagnosis of VAP since most cultures are not suitable to anaerobes and yet many of the flora within the oral cavity are specialized anaerobes. It also introduces the element of risk of transferring the oral bacteria into the LRT when inserting the ETT (Perkins, Woeltje, & Angenent, 2010).

A risk factor associated with *S. aureus* colonization is the establishment of the nasal cavity, particularly in patients in ICU (Bloemendaal *et al*., 2009). On analysis of the isolates taken from paediatric VAP patients, *Prevotella* was identified to be the most common genus followed by *Streptococcus* and *Staphylococcus*, which are members of the oropharyngeal microbiota which suggests they may have been relocated into the LRT on insertion of the ETT (Leroue, *et al*., 2017). Within samples from healthy patients, there is a high degree of shared bacteria between the lungs and the mouth, but interestingly, there is little conservation between the lungs and the nose (Bassis *et al*., 2015).

1.5 - Immune response to VAP

1.5.1 - Systemic response

Patients in ICU/hospital wards frequently experience lower immunocompetency due to several reasons: the illness they are already battling; negative side effects on microbial communities due to treatments; and for some, this is worsened by genetic predispositions (Weinstein et at, 2004). Often patients in ICU experience reductions in both their adaptive and innate immune systems in the forms of reduced antigen presentation and endotoxin

tolerance which are crucial factors in the development of nosocomial infections (Boomer *et al*., 2011). Whilst it is commonly accepted that critically ill patients reside in an immunosuppressed state, more recent evidence suggests that this is also true of those with VAP (Boomer *et al*., 2011).

The impairment of the innate immune system can be expressed in decreased phagocytosis activity, associated with the negative connotations of the of the anaphylatoxin C5a, which reduces neutrophil phagocytic activity (Morris *et al*., 2011). Within the critically ill there are also predispositions which increase the likelihood of HCAIs such as, elevated levels of regulatory T-cells (T-regs), deactivated monocytes (measured by monocyte HLA-DR expression) and dysfunctional neutrophils measured by CD88 expression (Conway Morris *et al*., 2013). Since there is no link between nosocomial infection and other markers of neutrophil activation (CD11b, CD64 and L-selectin), it is indicative that the association between CD88 expression and neutrophil dysfunction is not simply induced via common activation (Conway Morris *et al*., 2009).

Largely the immunosuppression within the critically ill is believed to originate from the overstimulation of counter-regulatory mechanisms, this is demonstrated via the raised level of regulatory helper T-regs (Venet *et al*., 2008). In patient case studies, T-regs are believed to damage the effect of lymphocytes (Venet, 2009). Data collated from studies testing immune-components as biomarkers for VAP, have identified that in cases of VAP there are elevated levels of: interleukin-1β (IL-1β); IL-8; matrix metalloproteinase (MMP)-8; and MMP-9, with a combinational sensitivity of just under 95% (Hellyer *et al*., 2015). See Table 1.2: below which describes each cytokine and its primary function.

Table 1.2: Primary source and action of the elevated cytokines identified in cases of VAP. (Source: Zhang, & An, 2007).

Cytokine	Primary Source	Principle Activity
IL-β	Macrophages and other antigen presenting cells (APCs)	Co-stimulation of APCs and T cells, inflammation and fever, acute phase response, haematopoiesis
IL-8	Macrophages, somatic cells	Chemoattractant for neutrophils and T cells

MMP-8	Pre-synthesized by neutrophils and stored in granules	Tissue damage by releasing matrix degradation products upon secretion
MMP-9	Secreted by a wide number of cell types, including neutrophils, macrophages, and fibroblasts	Reduced IL-2 response; Anti-inflammatory; Enhanced collagen affinity and Bioavailability of TGFβ

There is evidence to suggest that even amongst the critically ill immunoparalysis is more severe in patients with VAP. This is indicative from the decreased levels of CD3+/CD4+ cells, the increase in monocyte apoptosis, and the reduced release of pro-inflammatory cytokines (primarily IL-6, TNFα) post stimulation with LPS in the group of patients with VAP (Christaki, 2009).

Increased mortality rates have recently been associated with disproportionately increased IL-10 in relation to TNFα production, which is believed to be of a genetic origin, however, the specifics are yet to be recognised (White *et al*., 2010). Following on from this, the overproduction of IL-10 has also been related to an increased rate of severe sepsis (O'Dwyer *et al*., 2006). This may stem from the increased IL-10 gene expression being the reason for reduced interferon gamma (IFN-γ) production in antigen-presenting cells (APC's) since IL-10 is thought to be an inhibitory factor (Fiorentino *et al*., 1991). IL-10 is an immunosuppressive cytokine which in normal quantities of a healthy patient, is used to avoid an excessive immune response and avoids self-damage by the immune system, however, in dysregulation it prevents an immune response from being mounted by the host, making them more susceptible to infections (Cadwell, 2015). Assessed cumulatively both the data surrounding TNFα and IL-10 concentrations, would suggest that as the circulating cytokine volume increases so does the severity of pneumonia (Millo *et al*., 2004).

The endotoxin tolerance is believed to be due to a decreased cytokine production in response to lipopolysaccharide (LPS) after initial stimulation (Weijer, Lauw, Branger, van den Blink, & van der Poll, 2002). LPS are also powerful stimuli for macrophages (Zhang, & Morrison, 1993). Endotoxin tolerance has been considered an example of immunoparalysis (Biswas, & ELopez-Collazo, 2009). Which can be defined as a state of prolonged and severe acquired immune deficiency (Frazier, & Hall, 2009). Present evidence suggests that there is an increased level of immunoparalysis in patients with VAP than in those with alternate bacterial infections (Christaki, 2009) and is demonstrated by the reduced count of CD3+/CD4+ cells, rise in

monocyte apoptosis, and the poorer discharge of pro-inflammatory cytokines, namely tumour necrosis factor-alpha (TNFα) and interleukin 6 (IL-6), from monocytes after stimulation with LPS (Pelekanou *et al*., 2009).

There are also genetic factors associated with the likelihood of development of VAP. There have been certain genes found to be depressed within those who have developed VAP such as those involved in the cAMP and calcium signalling pathways within the complement system, which are integral to producing the cellular immune response synapsing between antigen presenting cells and lymphocytes (Keane & Martin-Loeches, 2019). Furthermore, gene expression levels are typically inverse to a high CPIS result which is indicative of pathology, suggesting patients who develop VAP are immunosuppressed and that ddPCR quantification of genes partaking in the immunological synapse may help diagnostic efforts of differentiating between patients with and without VAP that would otherwise be hard to clinically identify (Almansa *et al*., 2018).Whilst a depression of immune factors can lead to VAP development it is also true of the inverse.

To colonize the host, the pathogenic bacteria must defend itself against antimicrobial peptides, lysozymes, lactoferrins, and antibodies such as IgA (Wertheim *et al*., 2005). There are believed to be more than fifty accessory genes which aid in parthenogenesis which code for proteins which are either excreted or presented on the bacteria's surface which enable host immune avoidance and facilitate adherence to host cells which when exposed to host tissues beyond the mucosal surface are upregulated (Novick, 2003). *S. aureus* also has the potential to utilise clumping factor A (clfA), protein A and numerous compliment inhibitors which prevent opsonophagocytosis by preventing host opsonins from adhering to the bacterium (Foster, 2005).

Since many of the common VAP pathogens are opportunistic understanding the extent of the immune failures may prove to be the understanding of novel preventative and therapeutic strategies (Williams, Dehnbostel, & Blackwell, 2010). Since *P. aeruginosa* infections are rare in those who are not immunocompromised, it is indicative that the reduction of immune functionality puts the patient at far greater risk of developing an infection (Williams, Dehnbostel, & Blackwell, 2010). Therefore, the result of the infection can be linked to the level of immune impairment within the patient (Safdar, Crnich, & Maki, 2005).

Following on from this, it is not surprising that those with HIV are at an extremely great risk of a *P. aeruginosa* infection and are also more likely to become infected than the average intubated patient (Afessa & Green, 2000). Similarly, a common occurrence in a pseudomonal infection is the elevated level of neutrophils, however, in neutropenic patients, frequent in HIV positive cases and those undergoing chemotherapy, until the development of carbenicillins, gentamicin and carbapenems, said infections were nearly always fatal (Chatzinikolaou *et al*., 2000). Whilst there are several cases of resistance in *P. aeruginosa* to commonly used antibiotics, there is evidence to suggest that whilst they may not eliminate the infection they may 'hold back' the full force of pathogenicity until the hosts immune system is at a higher functioning level often linked to the removal of the ETT (Weber *et al*., 2007).

Primarily, gram negative bacteria such as *P. aeruginosa*, initiate a response from the host immune system through their interactions with TLR4 (Kopp & Medzhitov, 2003). Throughout the progression from acute to chronic infection, there are changes in the lipid-A moiety acylation pattern to reduce its immunogenicity and therefore have a lesser stimulation of TLR4 (Ernst *et al*., 2007).

When the host receives the stimuli in response to an infection the posterior pituitary gland releases arginine vasopressin (AVP), created by hypothalamic neurons, and has vasoconstrictor and antidiuretic functions (Itoi, Jiang, Iwasakiz, & Watson, 2004). However, since AVP is a relatively unstable molecule it is far easier to study copeptin, of which concentrations are mirrored to AVP and which also increase alongside the severity of illness (Struck, Morgenthaler, & Bergmann, 2005). Based on this evidence it has been suggested therefore, that copeptin levels could be used as a biomarker to access the severity and onset of VAP (Müller *et al*., 2007). It would therefore be interesting to investigate if there are any bacterial influences which cause the rise of copeptin in patients with VAP.

However, it is important to note, there are difficulties in analysing the immune response in patients with VAP in that many diseases or injuries that induce the need for additional ventilatory support are already characterised by profound systemic inflammatory responses including immune cell activation and immune mediated organ damage (Cohen, 2002). Additionally, since the progressive relationship between immune dysfunction and the

infection is often clouded, it leads to limitations in the extrapolations about causality (Conway Morris *et al*., 2009).

Of relevance to this study, there is a high degree of similarity between lymphoid tissues associated with the oral cavity and upper respiratory in pigs and humans (Mair *et al*., 2014). The immune recognition factors such as pattern recognition receptors (PRRs) which are expressed on many immune cells are evolutionarily conserved (Kumar, Kawai, & Akira, 2011). Another beneficial element of the porcine immune system that is not dissimilar to its human counterpart is the thymus, functioning as the lymphoid organ mainly involved in T cell development (Swindle, Makin, Herron, Clubb Jr, & Frazier, 2012). This is the reasoning behind this study using the lungs of *Sus scrofa ex vivo* lungs as a model to attempt to replicate the physiology in cases of VAP.

1.5.2 - Lung specific response

It was long believed that the lung was a sterile environment, however, on appraisal, considering that upper respiratory tract microbes are found in abundance and aspiration of this content occurs daily, it is unsurprising that this idea has since been overturned and, in fact, it is now known that the lung has its own microbiome (Dickson *et al*., 2015). Comparably the microbiome of the lung has a far smaller bacterial biomass than that of the gastrointestinal tract however, there is still a large and diverse population within the area (O'Dwyer, Dickson, & Moore, 2017). Unlike the gut, the movement of microbes within the lungs is frequently bidirectional since there are no physical barriers between the larynx and the alveolus, therefore, the biome is more transient and dynamic than that of the gastrointestinal tract (Dickson, & Huffnagle, 2015). The comparison is visually represented in Figure 1.5.

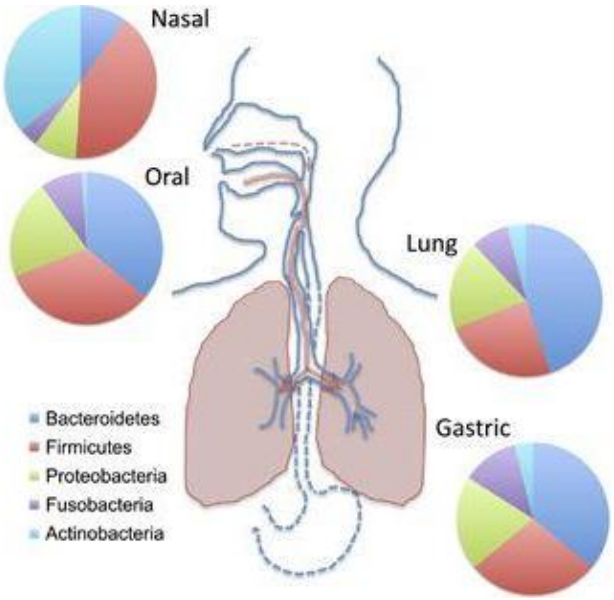

Figure 1.5: Comparative diagram of the different microbiomes of the lungs and the gut, demonstrated at a phylum level (Source: Huffnagle, Dickson, & Lukacs, 2016).

In health it is believed that it is the balance between the complex microbial constituents of the lung that help the host maintain immunocompetency in homeostasis (O'Dwyer *et al*., 2017). In fact, these endogenous bacteria are the pivotal regulators of both the host and pathogen modulation regarding infection (Global Health Sciences: Global Research Projects, 2015). Therefore, there is also an element of previous/current viral infections altering the normal flora constituents of the microbiome potentially leading to a change in bacterial populations, in turn, leading to dysbiosis which may ultimately bear an impact re infection (Cadwell, 2015).

In comparison to healthy controls, it was only seen in the diseased patients that the taxa in the LRT differed from that of the upper at a given time point (Martin *et al*., 2015). From research into the diversity of bacterial communities between healthy and diseased intubated patients it has been established that, whilst the α diversity (variance within a particular community) was lower in intubated patients than in healthy controls, it was the reverse for β diversity (variance of two communities) with there being a general similarity found amongst the healthy control microbial communities (Kelly *et al*., 2016). This loss of diversity has been linked to dysbiosis which is commonly attributed to a risk factor in disease.

Despite there being benefits in the presence of microorganisms there are also areas where, it is in fact, a disadvantage to health. Typically, in health the respiratory tract below the carina

has a low density of microorganisms, this void within the alveolar space may be linked to improved gas exchange and prevention of bacteraemia (Williams, Dehnbostel, & Blackwell, 2010). Infections of the LRT generally produce an intense inflammatory response to try and rid the host of the pathogens, since prolonged exposure is often fatal (Lau, Hassett, & Britigan, 2005). The type of infection can be differentiated into those that penetrate the host defences frequently and opportunistic pathogens, that are primarily seen in cases of impaired immunity within the host and are often commensal in states of normalcy within the host (Trautmann, Lepper, & Haller, 2005).

After the epithelial membrane has been penetrated the innate immune system is the primary defence system (Williams, Dehnbostel, & Blackwell, 2010). The innate system is made-up of microbial defences, including lung leukocytes and epithelial cells, and is generally described as any effector mechanism which aims to prevent infection (Beutler, 2004). An integral system within the innate immunity defence mechanisms is the use of pattern recognition receptors (PRR) which are encoded into the hosts germ line DNA and recognise the stimulus as pathogenic and generate an immunological reaction to it without the host having prior immunologic exposure (Williams, Dehnbostel, & Blackwell, 2010). Following its activation, the innate system often drives the adaptive system to perform functions such as, atopic pathways (receptor-mediated cell death pathways) and pathways generated by mechanical stretch (mechanical stretch accelerates cytokine production by human alveolar macrophages *in vitro*), and since each system is unique to each person this may be an explanation for the differences observed in immune responses amongst patients suffering from the same bacterial, fungal, or viral infections (Martin, & Frevert, 2005). For example, not all LRT colonizations in intubated patients result in VAP, many do not develop the disease (Durairaj *et al*., 2009), suggesting there are host factors influencing the development of the disease.

Numerous immune cells have been associated with the pulmonary defence against infections from *P. aeruginosa*, the most crucial being the neutrophil associated with bacterial clearance and therefore removal of infection (Williams, Dehnbostel, & Blackwell, 2010). T-helper cells have also been implicated in immune defence however, their role and ability to regulate neutrophilic responses remains relatively understudied (Williams, Dehnbostel, & Blackwell, 2010). There are some glycoproteins, such as IL-17 which are pivotal in the pulmonary immunoinflammatory response, for instance a lack of IL-17 is associated with an increased

vulnerability to bacterial pathogens whereas an increased quantity of IL-17 is linked to autoimmunity (Nembrini, Marsland, & Kopf, 2009). The consequences of an imbalanced quantity of IL-17 links to the wide range of cells influenced by the glycoprotein such as; neutrophils, fibroblasts, epithelial cells and endothelial cells (Flierl *et al*., 2008).

The host has several forms of pulmonary defences including structural, mechanical, chemical, and cellular strategies, alongside innate and acquired host defences (Whitsett, 2002). The epitomical lining can be defined as a 'pseudostratified columnar epithelium' formed of various morphologic cells such as: squamous; ciliated; basal; serous; goblet; neuroepithelial and non-ciliated Clara cells (Whitsett, 2002). The mechanical defence mechanisms which are so vital to respiratory health include the airway surface liquids (ASL) formed via water and mucus secretions which contain many substances that have antimicrobial properties to capture pathogens which are then repelled out of the lung via the mucociliary action. They work alongside the cellular barriers that prevent entry of organisms into the subcellular space and bloodstream. Both are often impeded during MV (Williams, Dehnbostel, & Blackwell, 2010).

In health the respiratory epithelium undergoes apostasies upon ingestion of pseudomonads to prevent greater diffusion, is a great source of microbiological agents within the ASL and has several transmembrane PPR that communicate to immune cells through via a multitude of cytokine networks (Williams, Dehnbostel, & Blackwell, 2010). Thus, highlighting why, the damage done to the epithelium by the ETT insertion has such detrimental effects.

Bacteria are translocated to the alveolar surface where they are exposed to soluble components within the ASL and alveolar macrophages (Martin, & Frevert, 2005). The soluble components include; lysozymes to disrupt the stability of the bacterial membrane, lactoferrins which interfere with iron transport, the antibodies IgA and IgG alongside the antimicrobial peptides and defensins (Lehrer, 2004). Following opsonisation from either IgG or SP-A/Sp-D, the bacteria are then ingested by alveolar macrophages whilst the toll like receptors (TLRs) aid in identifying which microbial products enter the cell (Blander, & Medzhitov, 2004).

Within the ASL four proteins, SP-A, SP-B, SP-C and SP-D, have been found to act as crucial mediators in the host defence of the lung via the regulation of surfactant lipid metabolism

and the lipid membrane organization (Kuroki, Takahashi, & Nishitani, 2007). It is noted that SP-B and SP-C are hydro-phobic and therefore are vital in the biophysical functions of surfactant (Whitsett and Weaver, 2002) whilst SP-A and SP-D are water soluble and belong to the collectin family characterised to be integral to the innate immune system (Kuroki, Takahashi, & Nishitani, 2007).

The pulmonary collectins are also known to inhibit the growth of Gram-negative bacteria via a cumulative increase in the permeability of the bacterial membrane (McCormack *et al*., 2003). Several *in vivo* studies demonstrate that the collectins also increase the uptake of pathogens (Kuroki, Takahashi, & Nishitani, 2007). SP-A and SP-D encourage phagocytosis of bacterial particulates by alveolar macrophages and granulocytes whilst also contributing to their clearance (Shrive *et al*., 2003). The complement pathway which leads to phagocytosis via opsonization is triggered by the Mannose-binding lectin 2 (encoded by MBL2) binds D-mannose and N-acetyl-D-glucosamine on the surface of the bacterium, however, MBL2 is subject to a large amount of genetic variation and low levels seem to reduce the time taken for a *P. aeruginosa* infection (Dorfman *et al*., 2008).

1.6 - Evaluation of current research approaches

Animal models provide many insights into the pathophysiology of VAP and allow for scrutiny into the efficacy and safety of current therapeutic interventions (Li Bassi *et al*., 2014). There have been various animal models developed throughout the years evaluating different pulmonary injuries, causative agents, methods for bacterial immunisation and time progression of the onset of the disease (Rouby *et al*., 2012). Primarily during the 1980s VAP was studied via the intubation of baboons (*Papio papio*) for eight days. In this study the primary causative agents were *P. aeruginosa* and *Staphylococcus aureus* (Johanson, Holcomb, & Coalson, 1982). These early models allowed for pinnacle breakthroughs in the understanding of the pathophysiology of the disease and to develop preventive strategies since there was such a close reflection in the etiology of VAP in humans (Campbell, Coalson, & Johanson, 1984). However, there are many ethical constraints to working on primates so studies that have shifted to primarily on sheep and pigs.

Of the early porcine lung models many have been proven effective into the investigation of VAP, as one study reported that of the pigs being mechanically ventilated for four days,

around 90% developed VAP (Marquette, Wermert, Wallet, Copin, & Tonnel, 1999). The causative organisms were noted to be *Pasteurella multocida* and *Streptococcus suis* which were later supported by similar colonizations of the endogenous oropharyngeal pathogens, demonstrated in models developed by the National Institutes of Health (Zanella *et al*., 2012). In said models it is of note that the animals were positioned so that the tracheal orientation was above horizontal which is reported to enhance the microaspiration of the oropharyngeal pathogens (Li Bassi *et al*., 2014). Some researchers have even induced pneumonia in pigs via bronchial inoculation of *P. aeruginosa* (Luna *et al*., 2007) and methicillin-resistant *S. aureus* (Martinez-Olondris *et al*., 2010).

The main advantages of a porcine lung model in the investigation into VAP is that it is similar in the pathogenic mechanisms of human VAP and bacteriology allowing an in-depth study to be conducted on the disease, diagnosis, and preventative strategies (Li Bassi *et al*., 2014). Another advantage is that the use of 'waste' products from abattoirs correlates with the National Centre for the Replacement, Refinement and Reduction of Animal Research (NC3R) (2021) guidelines. Using the waste tissue means that no animals were culled for the experiment itself, since they were to be slaughtered regardless, the animals were killed in a humane way and all experimentation was done after death leaving there no possibility for animal suffering due to the experiment. There has even been research into how to avoid using animals all together and to use a novel *Dictyostelium* model instead (Cocorocchio *et al*, 2018), but this needs further research and assessment before implementation into this study may occur.

1.7 - Overall aims

From the evidence the key issues within both research and treatment of VAP are that a more specific and rapid diagnosis test is needed to avoid misdiagnosis, and the development of faster ways to identify the causative organisms specific to the patient to avoid unnecessary broad-spectrum treatments which increase the risk of MDR and dysbiosis. There is a need for a more detailed analysis of the differentiation between a VAP specific response either systemically or isolated to the pulmonary system and the response to the initial cause for ventilation. These are the current problems associated with VAP that affect the clinicians, researchers, and the patients.

The aim of this study was to characterise the interactions between host and VAP pathogens in an *ex vivo* porcine lung model to help improve strategies for a more accurate and rapid diagnosis. This model has been previously developed and used to investigate virulence of respiratory bacterial pathogens, including *P. aeruginosa* and *S. aureus* (Harrison, Muruli, Higgins & Diggle, 2014), and is therefore a viable model for investigation of host-pathogen interactions in VAP.

A schematic diagram has been developed to explain visually how each element of research has been investigated within this study, see Figure 1.6 below. Each of the aims may fall into one or more of the areas within the diagram which demonstrates how the research should be viewed holistically as well as compartmentally. Since working with patients within hospitals is unpractical for research of this level a developmental *ex vivo* porcine lung model is far more practical and responsible, this issue will be further expanded on elsewhere within this study.

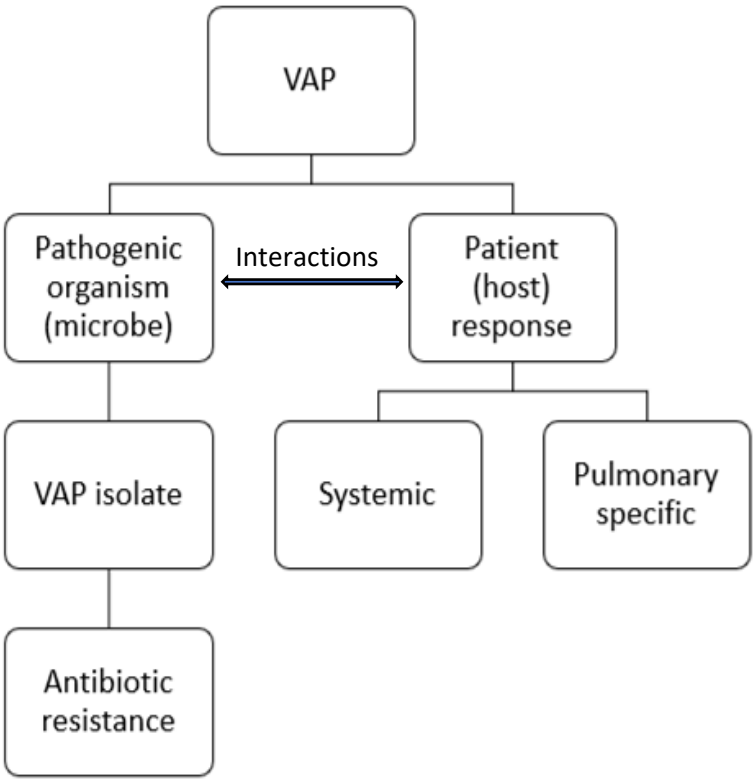

Figure 1.6: Hierarchy diagram to demonstrate the research pathways in VAP. From the top VAP is the confirmed clinical diagnosis. From this the diagram branches off to the pathogenic organism causing VAP (the microbe) which can be a variety of organisms.

Following from the causative agent is the isolation of a sample in ordered to determine the specific causative microbe. Succeeding this is the testing of the sample for antibiotic resistance to determine which antibiotics would be most suitable for the patient. On the other side below VAP is the patient (host) response which will be highly varied between patients due to several variables such as age, sex, initial cause for intubation etc. Subsequently leading to the amounted response of the patient either systemically or specific to the pulmonary organs. There is also the double headed arrow to identify that there are interactions between the host and the causative microbes.

The modifications of this study specifically are to optimise the killing methodology of excised porcine lung tissue to act as a comparison to live tissue to allow the input that the host microbes have in any interactions studied and to also assess the specific/systemic response to VAP. To establish a routine method of taking a BAL sample from the porcine lungs to therefore identify the normal microbial constituents within the porcine lungs, then by knowing the normal flora it allows later identification of foreign microbes which may lead to a species-specific treatment rather than unnecessary broad-spectrum treatments. To establish the cell structures, present within the lung tissue in health which can later be compared to diseased tissue to understand further the host-microbe interactions in cases of VAP. Lastly to generate a time scale of the growth of a VAP clinical isolate on inoculated viable and non-viable *ex vivo* porcine lung tissue to identify how much the host may impact isolate growth and in what time does colonisation of the tissue arise.

2. METHODS & MATERIALS

2.1 – Bacterial strains and the clinical Isolate

The clinical isolate was obtained from Liverpool University. Originally from a clinical patient isolate which was subculture and grown so that only the pure isolates remained. There is no patient information accessible from the sample as it is pure bacterium and contains none of the original host's DNA. The strain was stored at -80 in 50% Phosphate Buffered Saline (PBS)/50% glycerol prior to use within a 1.5mL cryo-tube. Cultures were grown on MH agar. The control strain of *S. aureus* was used as it a notably common bacteria with well documented AGE band weights whilst also having the V_1V_2, V_3V_4 gene regions (Wagner et al., 2016) and in this study was prepared via a colony boil prep method as defined by Hassanzadeh, Pourmand, Afshar, Dehbashi & Mashhadi (2016).

2.1.1 - Specifications of lungs from *Sus scrofa*

The lungs were collected from Morphets Widnes, an abattoir in Cheshire. The large white pigs (*Sus scrofa domesticus*) were culled just before picking up and then placed in a cool box till reaching Salford University, approximately 1hr later at 5-7 °C, where they were transferred to a secondary cool box with ice and stored there until use. The whole lung was excised form the larynx, including both lobes.

2.2 - Development of *ex vivo* porcine lung model

2.2.1 - Artificial Sputum Medium

All components to make ASM were purchased from Sigma-Aldric, UK. Deoxyribonucleic acid sodium salt from salmon testes (4g/L), Type II mucin from porcine stomach (5g/L), L-amino acids listed in Table 1, excluding L-Tryptophan (250mg/L), Diethylenetriamine Pentaacetic Acid (DTPA) (5.9g/L), NaCl and KCL (both 5g/L) were thoroughly mixed into 850mL of dH_2O. The solution was then made to pH 7, brought to a total of 900mL with dH_2O and autoclaved. Once cooled, filter sterilised L-Tryptophan (250g/L) was added and lastly egg yolk emulsion (5mL/L) was added to a final volume of 1L.

Table 2.1: List of all 20 naturally occurring amino acids with Tryptophan highlighted with reference to the ASM protocol (Source: Sigma-Aldric, 2019).

Alanine, Ala, A	**Isoleucine, Ile, I**	**Leucine, Leu, L**
Methionine, Met, M	Valine, Val, V	Phenyl alanine, Phe, F

Tryptophan, Trp, W	Tyrosine, Tyr, Y	Asparagine, Asn, N
Cysteine, Cys, C	Glutamine, Gln, Q	Serine, Ser, S
Threonine, Thr, T	Aspartic acid, Asp, D	Glutamic acid, Glu, E
Arginine, Arg, R	Histidine, His, H	Lysine, Lys, K
Glycine, Gly, G	Proline, Pro, P	

2.2.2 - Lung dissection

A dissection tray was prepared by sterilising with 70% ethanol and 15 mins exposed to UV light, all of which was conducted within a laminar flow cabinet. Two plastic chopping boards were placed onto two large strips of tinfoil which were placed onto the tray and UV sterilised again for 30 mins.

The first lung was removed from the cool box and placed onto one of the boards. The health of the lung was then assessed. A pictorial example of a 'healthy' and 'unhealthy' lung can be found in the Results section "3.1 Differentiation of healthy and damaged lungs".

If necessary, a BAL sample as described in the Materials and Methods Section "2.3. Bronchoalveolar lavage of porcine lung", would be conducted.

Using a hot paddle knife, the surface of lung tissue was cauterized, ensuring to be within the reach of a Bunsen on a roaring flame throughout to encourage aseptic technique.

The surface layer of the tissue was then carefully removed trying to avoid damage to deeper sections of the tissue using sterile tweezers and a single edged razor blade.

Lung tissue sections were prepared as previously described by Harrison, Muruli, Higgins, & Diggle, (2014). Using the autoclaved sterilised dissection kit and the single edged blade two horizontal lines were cut. Vertical incisions within the horizontal lines were then made. The surface alveolar tissue was removed and discarded. Tissue from the now exposed sub-dermal layers were then removed of an approximate 0.5cm^2 diameter and were immediately placed into a plate filled with ASM resting on ice. To maintain maximal similarity between the tissue sections, bronchioles and veins were avoided as much as possible. This was then repeated for the left and right lobes of the lung. The tissue was then washed in ASM twice. Research by Harrison, Muruli, Higgins, & Diggle, (2014) reports that washing causes no notable damage to the pleura or have negative connotations on the resident bacterial cells.

2.2.3 - Optimisation of killing lung tissue

Lung tissue segments were excised using the protocol defined in “2.2.2 Lung dissection”.

To six Eppendorf tubes filled with 500µl of sterile PBS (Thermo Fisher) one piece of tissue was added. After ensuring the tissue was fully submerged in the PBS. PBS is a water-based salt solution containing disodium hydrogen phosphate, sodium chloride which helps maintain a constant pH (Harrison et al., 2012). The tubes were placed into a heated block at 70 °C for 20 mins. A further six pieces of tissue were individually placed into cryovials and fully submerged into liquid nitrogen for 20 mins. A further six tissue segments were kept cool with ice, to be used as viable controls.

At the completion of the various conditions a piece of tissue was placed into each well of a pre-prepared 24 well 0.8% agarose bed plate, as seen in the layout of Figure 2.1. This was then placed into the CO_2 incubator at 37 °C.

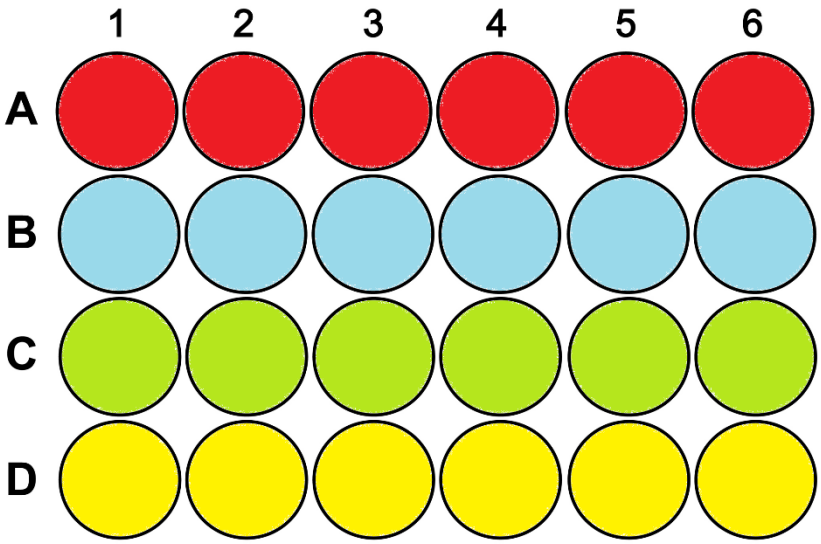

Figure 2.1: Plate layout to measure viability of ex vivo porcine lung tissue in various conditions. In row A is where the heat killed tissue pieces are placed, row B is cold killed, row C is live tissue segments placed in ASM and in row D is live tissue segments in LB.

At the time points after incubation; 1hr, 3hr, 6hr, 18hr and 24, the tissue pieces were removed from the agarose bed and added to a 24 well plate containing 1 mL ASM. A total of 10% of the total volume of Resazurin (Fisher Scientific) (from a stock solution of 0.50 g

Resazurin sodium salt dissolved into 100 ml 1x sterile PBS) was added to the well and incubated for 45 mins in the 37 °C CO_2. After the interval had elapsed 200µl of the media surrounding the tissue was removed and placed in duplicate in a 96 well plate and absorbance was read at wavelengths Ex/Em 560/590nm.

2.3 - Bronchoalveolar lavage of porcine lung

Freshly acquired lungs (>12 h post slaughter) lungs were placed into the sterile dissection zone (As referenced 2.2.2 Lung dissection). The Halyard Mini-Bal Sampling Catheter (Halyard Health) was removed from the packaging whilst maintaining sterility and inserted into the vertically suspended lungs via the larynx, down the trachea and then into the primary bronchioles where the tube was inserted into the left lobe. Using a syringe 100mL of PBS was then introduced to the lungs as a lavage via the catheter and then removed via suction excreted from the syringe. Success of the lavage could be assessed by the colour, if the fluid was red then tissue damage was likely to have occurred when inserting the tube, whereas a more yellow tinged translucent liquid indicted a successful lavage.

The extracted fluid volume was recorded and placed into a Disposable 50 x 50mL centrifuge containers (Tube Pipe Vial Lab Test- Thermo Fisher) and stored at -18 °C till further use (approximately 24hrs later). The catheter was then flushed clean with PBS and inserted into the alternate lobe of the lung as to previously. Another lavage was then completed, and the same process applied to the extracted fluid. This was then further repeated for the second lung.

2.3.1 - PCR amplification of 16SrRNA gene from BAL samples

The samples were collected by the methods referenced in “2.3. Bronchoalveolar lavage of porcine lung” and were thawed and amplified following the PCR settings in Table 2.2. The reagent MytaqRed (Bioline) was used for 16S amplicon sequencing to amplify the 16S rRNA directly from the BAL sample. Following the Highprep™ PCR 96-Well Protocol (Milipore Sigma, 2020) the samples were subjected to several rounds of bead clean up wherein 56µl of AMPure XP (Beckman Colture) beads were added to the product of PCR ensuring to mix by pipetting repeatedly for a minimum of 10 times. This was to eliminate contaminates and salts, which often act as inhibitors, therefore, leaving a purified DNA sample for later testing.

Table 2.2: PCR settings used to amplify BAL sample DNA.

Stage	Temperature	Time (seconds)	Cycles
Preliminary Denaturation	95 °C	60	1
Denaturation	95 °C	15	25
Annealing	55°C	15	
Extension	72 °C	10	

The 16S rRNA gene was amplified with primer sets of V_1V_2 and V_3V_4 modified to contain Illumina overhang sequences (16S Metagenomic Sequencing Library Preparation). These primers were used in concordance to other relevant studies of BAL samples as they are abundant in respiratory bacteria (Charlson et al., 2012 & Becker et al., 2019). See Table 2.3 for full primer sequences (Watts et al., 2017).

Table 2.3: Specific sequences of both sets of primer used including the Illumina sequencing overhang.

V_1V_2 Forward	**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGAGAGTTTGATCCTGGCTCAG**
V_1V_2 Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGCTGCCTCCCGTAGGAGT
V_3V_4 Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGAGGCAGCAG
V_3V_4. Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT

Post PCR, each of the samples was loaded (5µl) in a 1.5% Agarose gel and ran for 1hr at 70V with a 50bp hyperladder (Bioline) along with the positive control of known *S. aureus* and negative control of sterile PBS. Using the Sigma Aldrich Centricon-100 the amplicons were also purified. The Centricon-100 was set up per manufacturers manual and then 2mL deionized water was added into the column followed by the sample. The column was then spun at 1000 x g in a fixed-angle centrifuge for 10 minutes. The waste was removed, and a collection vial attached. The column was then inverted and span at 270 x g for 2 minutes. Deionized water was then added to the product to bring it back to the original volume of PCR fragments.

2.4 - Physiology of Porcine Lung tissue

2.4.1 - BAL Cytospin

A Giemsa stain was used to identify the cytoplasmic morphology of the BAL through the eosin binding to components such as the cytoplasm and granules whilst the methylene blue stains the nuclei and acts as a fixative which disallows further cellular changes and encourages adherence to the glass slide (Microbe Online, (2019). With a blank slide (Thermo Scientific™ Frosted Microscope Slides, 45°) placed into the empty clamp of the Cytospin (Thermo Scientific Shandon), 50µl of sterile PBS was pipetted around the hole of the disposable Shandon cytofunnel (Thermo Scientific). The cytofunnel was then slotted into the clamp and placed into the Cytospin and 100µl of the BAL sample was added which was spun at 700 rpm for 5 mins. Once complete the samples were left briefly to dry and Giemsa stain (Sigma Aldrich) via the Lecia Autostainer was added. Table 4 highlights the protocol the samples were subjected to.

Table 2.4: Giemsa Satin Stages of which the BAL samples post cytospin were stained in the Lecia Autostainer (Lecia Biosystems).

	Stain/Dye	**Time (seconds)**
1	Eosin 1%	00:00:07
2	Sorensens pH 6.8	00:00:03
3	Methlene Blue	00:00:05
4	Sorensens pH 6.8	00:00:07

The slides were viewed through a light microscope at 4X/0.1 objective and images taken via an iPad connected to the microscope. The images were then analysed using the software GIMP (version 2.10.14 was used, https://www.gimp.org/downloads/). A histogram of the stained portions of slide was generated using the 'Fuzzy tool select' and percentage coverage could be calculated. This was to establish if the BAL samples were working as a method to detected cellular presence within the sample, as demonstrated in the Figure 2.2

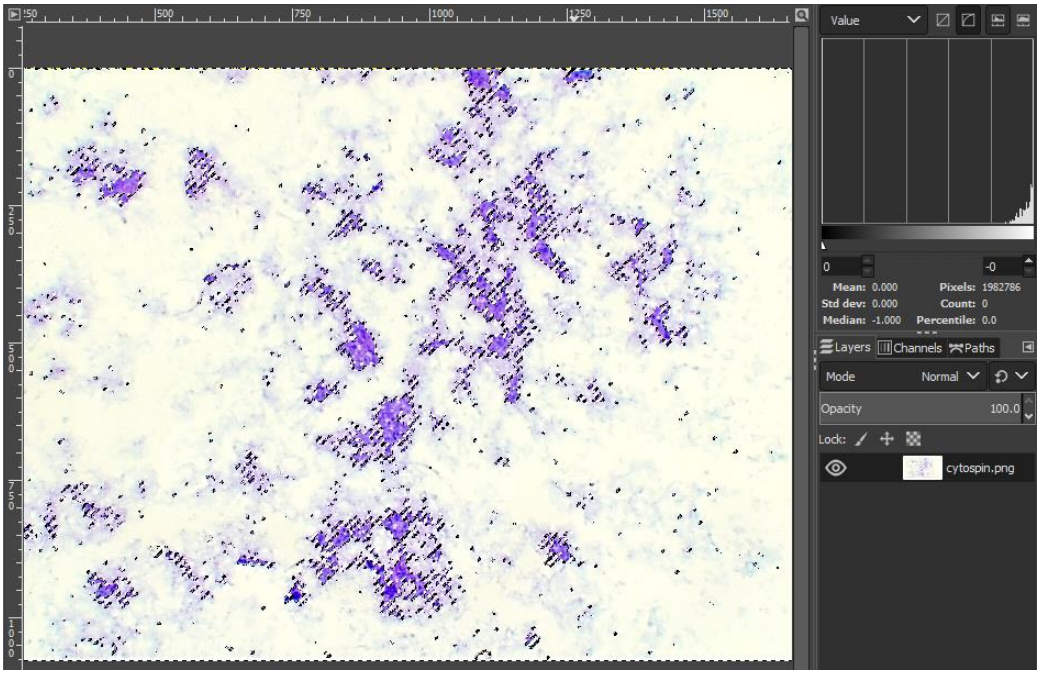

Figure 2.2: Screenshot of Fuzzy select tool within the software GIMP of the BAL cytospin Giemsa-stained slide. The right identifies the histogram and pixel count. The areas of colour are defined by the black dashed lines.

2.4.2 - Fixing of tissue in wax

Using the dissection method as stated in the "2.2.2 Lung dissection" small sections of tissue were removed from the lungs. As aforementioned in "2.2.3 Optimisation of killing lung tissue", six viable pieces of tissue were placed in Paraformaldehyde/Saline at 4 °C for 48 hours and then rinsed with PBS and placed into new Eppendorf tubes in PBS at 4 °C overnight.

The tissue samples were then removed from the Eppendorf tubes and individually placed into slotted cassettes and into the Leica TP1020 tissue processor to fix the tissue in wax.

The samples were subjected to the conditions as shown in Table 2.5.

Table 2.5: Cycle stages the tissue was subjected to in the Lecia TP1020 Tissue Processor.

	Duration (mins)	**Temperature (°C)**
Solution (Sub-X Clearing Medium, Lecia Biosytsems)	60	Room Temperature (RT)
50% Alcohol (Ethanol)	60	RT
90% Alcohol (Ethanol)	60	RT
100% Alcohol (Ethanol)	60	RT
100% Alcohol (Ethanol)	60	RT
Histoclear (Lecia Biosytsems)	60	RT
Empty	0	RT
Wax	90	60
Wax	90	60

The samples were then removed from the final wax beaker and placed in the hot wax basket.

The tissue pieces were then removed from the cassettes and added to a plastic mould case and fully embedded in paraffin and allowed to harden. A lid was placed to secure the tissue piece from moving and left on a cooler block so that the wax could solidify. The cassette was then placed into the microtome and the tissue was sectioned in thin slices. The tissue sections were floated in a water bath of 30 °C and placed onto microscope slides where they were then left to dry on the heated block of 28 °C for 2hrs. Post this interval the slides were left to further dry overnight at RT.

2.4.3 - Hematoxylin and Eosin stain

The tissue samples were subjected to the protocol outlined in “2.4.2 Fixing of tissue in wax”. The slides were then placed into the Lecia Autostainer and subjected to the protocol in table 6. The stain is preferential since it works well with a wide variety of fixatives and displays a broad range of cytoplasmic, nuclear, and extracellular matrix features with the Hematoxylin (Lecia Biosytsems) staining a deep blue, mainly targeting nucleic acids, whilst eosin stains pink and typically to the cytoplasm and various proteins (Fischer, Jacobson, Rose, & Zeller, 2008).

Table 2.6: Protocol steps used in the Lecia Autostainer on the three sections of *ex vivo* porcine lung tissue.

Step	Reagent	Duration (h:m:s)	Exact	Dip
1	Oven station	00:13:30	No	No
2	Histoclear	00:05:00	No	Yes
3	Histoclear	00:05:00	No	Yes
4	Ethanol 100%	00:01:30	No	Yes
5	Ethanol 100%	00:01:30	No	Yes
6	Water station	00:01:00	No	No
7	Carazzi Haematoxylin	00:01:00	Yes	No
8	Water station	00:00:30	No	No
9	Water station	00:00:30	No	No
10	Eosin 1%	00:00:10	Yes	No
11	Water station	00:01:00	Yes	No
12	Ethanol 95%	00:01:00	Yes	Yes
13	Ethanol 100%	00:01:00	Yes	Yes
14	Histoclear	00:05:00	No	Yes

After the slides were removed from the output tray and left to dry overnight at RT. A blank cover slip was then dabbed with a small amount of the mounting agent DPX (p-Xylene-Bis-Pyridinium Bromide) (Thermo Fisher) and the slide was then placed onto the cover slip. The slides were then left to dry further for 2hrs at 18 °C. The slides were then individually examined under a microscope and the results can be seen in "3.2.2 Identification of structures in healthy BAL sample extracted from *ex vivo* porcine lung via Hematoxylin and Eosin staining".

2.4.4 - Giemsa stain

The tissue samples were subjected to the protocol as defined in "2.3.2 Fixing of tissue in wax" with the sections being stained via the stage's outlines in table 7. The benefits of a Giemsa stain have been highlighted in "2.4.1 BAL Cytospin".

Table 2.7: Protocol steps used in the Lecia Autostainer on the sections of *ex vivo* porcine lung tissue for a Giemsa stain.

Step	Reagent	Duration (h:m:s)	Exact	Dip
1	Oven station	00:13:30	No	No
2	Histoclear	00:05:00	No	Yes
3	Histoclear	00:05:00	No	Yes
4	Ethanol 100%	00:01:30	No	Yes
5	Ethanol 100%	00:01:30	No	Yes
6	Water station	00:01:00	No	No
7	Methanol	00:00:06	No	No
8	Eosin 1%	00:00:07	No	No
9	Sorensens pH 6.8	00:00:03	No	No
10	Methylene Blue	00:00:05	No	No
11	Sorensens pH 6.8	00:00:07	No	No

The slides were then left to dry, a cover slip added and then photographed under the microscope. The results of which can be seen in section '3.2 - Physiology'.

2.4.5 - Image analysis

The lesions on the lungs may have been caused by *M. hyopneumoniae* are phenotypically purply-grey and on the upper parts of the lobes (Ceva Lung Program, 2019). To determine the extent of the infection the below methodology was employed.

Image Analysis (IA) is a subjective method which facilitates the quantification of affected lung area (%) via a photograph of the lungs (Sibila, Aragón, Fraile, & Segalés, 2014) and using the corresponding software (in this study the software GIMP version 2.10.14 was used, https://www.gimp.org/downloads/). To calculate the affected percentage, the image of lung was saved and opened in the image manipulation program GIMP. The areas of discolouration were then selected as contiguous region based on colour using the 'Fuzzy select tool', detonated in Figure 2.3. Using the colour histogram, the number of pixels selected within the discoloured regions was recorded. Using the total number of pixels for each lobe a percentage was calculated as demonstrated in the equation below.

(Right lobe infected area + Left lobe infected area) ÷ Total lung area *100.

This method is adapted from the one proposed in the study by Sibila *et al*., 2014.

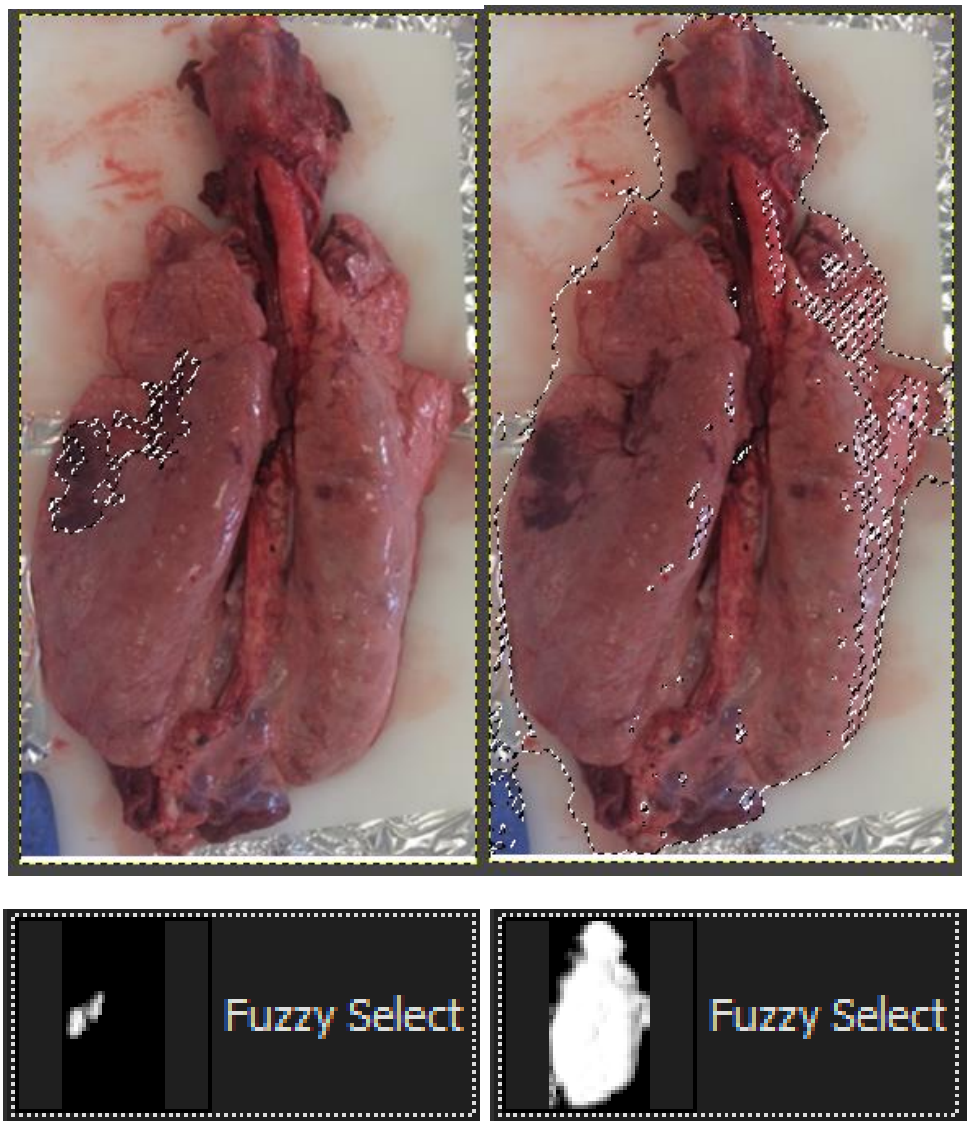

Figure 2.3: On the left-hand side demonstrates the fuzzy tool selecting the diseased area of lung by the colouration. The image below demonstrates the selected area by removing the background. On the right is the fuzzy tool selection of the whole lung whilst below it is demonstrated in black and white.

The fuzzy select tool is an objective measurement of discolouration of the lung tissue. By looking at the black and white images within Figure 2.3 it also highlights the extreme

selectiveness of the tool as it has not included any background pixels which may have disordered the percentage calculated of discoloured tissue.

To analyse the prevalence of herd infection, around 30 animals should be assessed to provide a reliable measure of severity (Sibila *et al*., 2009). Over the period from 28/08/2018-24/10/19 a total of 43 lungs were collected for this research. A total of 5 had to be discounted due to excessive lobe damage, demonstrated in Results section '3.1 Differentiation of healthy and damaged lungs'. Therefore, a total of 38 lungs were deemed healthy and therefore examined in the evaluation of potential herd infection with *M. hyopneumoniae.*

2.5 - Inoculation of porcine lung tissue with *P. aeruginosa*

2.5.1 - Understanding of optimal dilution factors of homogenised tissue.

Using the methodology described in "2.2.2 Lung dissection" sections of porcine lung tissue were excised. All tissue was then washed in ASM. Following this, 10 pieces of tissue from each lobe were placed individually into PBS (500 ul). The tissue was then placed into a heat block pre warmed to 70 °C for 10 mins. This has been determined as the optimal killing method despite not maintaining structural integrity for later histology. A further five pieces of "live" untreated tissue from each lobe were then inoculated with 5µl of a clinical VAP isolate of *P. aeruginosa* using a Hamilton's Syringe by gently pricking into the surface of the tissue, with a known diluted optical density (OD) of 0.2. The tissue was then cultured overnight in LB. Once the heat-treated tissue sections were removed from the heat block, five from each lobe were also inoculated under the same conditions. Using a premade 24 well plate with a set 500µl 0.8% Agarose/ASM nutrient agar bed filled with an additional 500µl of ASM, as described by Harrison et al (2014), five tissue pieces from each condition was added as described in Figure 2.4 and left in the CO_2 incubator at 37°C for an hour. To each well a 1/20 dilution of Ampicillin (concentration 50-100 microgram per ml) was added to suppress the host microbes since the VAP strain is known to be resistant at even the highest concentrations as documented by Harrison, Browning, Vos, & Buckling, (2006). This was evaluated by comparing the live and dead tissue colony growth counts to see if the use of the antibiotic had been successful. In column six there was no tissue added, simply 250µl more ASM to act as a negative control as displayed in Figure 2.4.

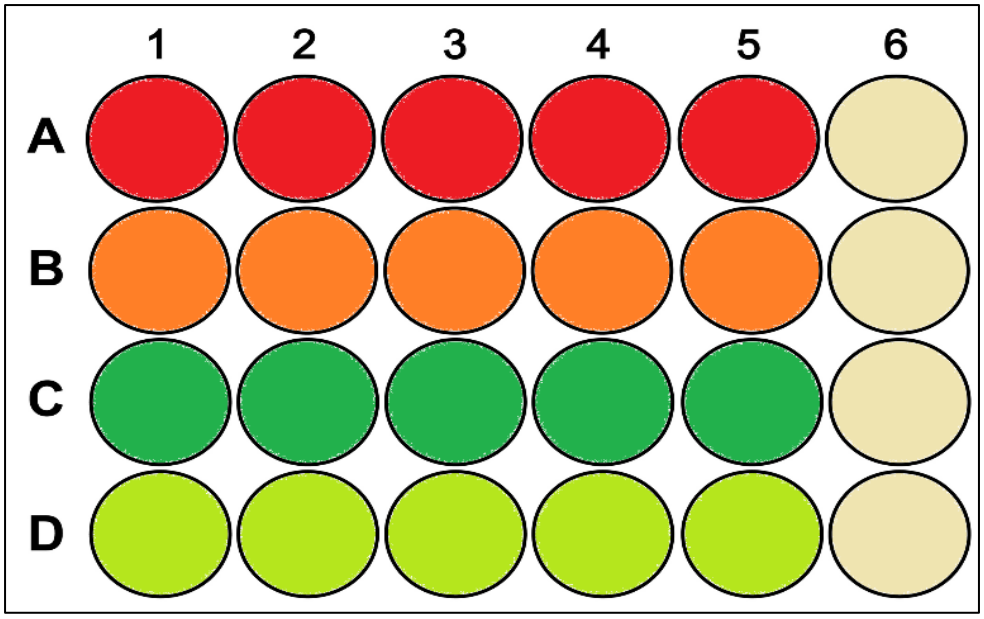

Figure 2.4: The 24 well plate plan identifying each of the four treatment conditions for the lung tissue alongside the negative control in column 6. Row 1 refers to time point 1- removal after 1hr, row 2 removal after 3 hr, row 3 removal after 6hr, row 4 removal after 18hr and row 5 removal after 24hr.

After the incubation period each segment from row 1 was removed and added to a 2mL microcentrifuge tubes with screw caps (Corning, Thermo Fisher) filled with 1mL sterile PBS and a singular sterile 6mm Diameter Grade 100 Hardened 52100 Chrome Steel Ball Bearing (Simply Bearings Ltd). The microcentrifuge tubes were then placed into the bead beater for 20 mins at a frequency of 30Hz. This time is common in collagen rich tissue samples such as lung tissue (Zelentsova, Yanshole, & Tsentalovich, 2019). From the now homogenised solution 100µl was removed and added to 9900µl of sterile PBS. This solution was then vortexed thoroughly and 20µl was removed and added to 180µl of PBS in a 96 well plate. This was repeated for all four of the conditions and both lobes. A dilution factor of 10^3 before beginning the 10-fold dilution series, ensuring to mix a minimum of five times before transferring to the next well.

From each of the dilutions 100µl was then added to an MHA plate and using an (Fisherbrand) L-Shaped Cell Spreaders a spread plate was generated. The colony growth was then analysed after 24hrs and a CFU per gram of tissue was calculated.

2.5.2 - Generation of bacterial growth curve on tissue

Using the results generated from "2.5.1 Understanding of optimal dilution factors of homogenised tissue" the methodology was refined so that the time points after incubation were: 8hrs, 18hrs, 24hrs and 30hrs. This is due to their being little to no evidence of VAP isolate growth in the first three time points of the previous experiment. The homogenate was then only diluted to 10^{-8} since there was little evidence of growth beyond this.

2.6 Genetic sequencing of BAL microbial content

2.6.1 DNA extraction

Part 1: DNA extraction

The lung was examined and identified as being in a visibly healthy state through checking for black-discolouration, excessive trauma or lacerations to the lobes, dryness or shrivelling of the tissue, swelling, and fibrinous exudate residing on the lung surface (Zuo *et al*., 2012). Using the method described in "2.3 Bronchoalveolar lavage of porcine lung" found previously in this report, a BAL sample was extracted. A total of six lungs were used to retrieve BAL samples, from each lung the left and right lung were washed making twelve BAL samples in total. The left BAL samples were pooled into one. This was repeated for the right lobes, leaving two pooled samples extracted from six lungs.

To extract the host and microbial DNA the sample was ran through a DNEasy kit (QIAGEN) and then left at -18° C overnight. The samples are lysed with the proteinase K from the kit which avoids mechanical disruption. As a negative control sterile PBS was used and for a positive, *S. aureus* ran through the DNA extraction kit alongside the BAL samples.

Part 2: Library preparation for sequencing

The samples were then left to incubate at room temperature for 5 mins and then placed onto a magnetic stand (Thermo Fisher, DynaMag™-2 Magnet) for 2 mins. The supernatant was then discarded whilst leaving the tubes on the magnetic rack. The pellet was then washed with 200µl 80% ethanol and left to stand on the magnetic rack for 30 sec. The supernatant was then discarded. This process was repeated and in the final removal of supernatant a 10µl

pipette was used to ensure all ethanol had been removed and then were left dry for 10 mins on the rack.

To the PCR tubes 27.6µl of molecular water (Milipore Sigma) was added. Proper mixing was ensured by repeated pipetting to ensure beads are fully re suspended.

The samples were then incubated for 2 mins and placed again on the magnetic rack. From this 25µl of the supernatant was removed and placed into a new PCR tube and the pellets were discarded. The samples were then stored at 4 °C overnight.

Index PCR:

A master mix of 500ul was made up of 80% My Taq Red (Sigma Aldrich) and 20% PCR grade water was formulated and then 35µl was added to each sample.

The DNA was fragmented and tagged using sequencing adapters by adding 5µl of the Nextra XT Index 1 and 2 Primers (Illumina). The index primers were added to the sample to allow sequencing of several samples at the same time, after amplification of the target region.

The samples were then run through an Index PCR with the conditions as shown in Table 2.8.

Table 2.8: PCR conditions for second PCR following the bead clean up.

Temperature (°C)	**Hold Time (seconds)**	
98	180	
89	30	Repeated 10 times
55	30	
72	30	
72	300	
4	300	

The PCR products then underwent a second bead clean up following the procedure as aforementioned which will from hereon be considered the standard protocol for a 'bead clean up'.

Library Quantification- Qubit:

Using the Qubit™ dsDNA HS Assay Kit (Thermo Fisher) the genomic library of the samples was assessed, as explained below.

Using the working solution provided in the kit the two Standard buffers were produced (190µl working buffer; and 10µl Standard). Standard 1 has an extremely low DNA concentration and Standard 2 an extremely high concentration to generate a curve for the samples tested to be compared to.

The quantity of dsDNA in each sample was then measured by making a mix of the sample and working buffer and using the standards as a scale (198µl working solution: 2µl sample). All samples were vortexed sufficiently before reading. This data allows for further calculations to equal the concentrations of DNA between each sample by diluting each sample to the lowest concentration, ideally being no lower than 2ng/µl and no higher than 2ng/µl. This created a 'working pool' of the samples.

Tape Station: Quality control and quantification of DNA libraries

After allowing the tape station reagents and the tape (Agilent) to come to room temperature (Agilent) for at least 30 mins, 1µl of each of the libraries, including the negative and positive controls, were added to individual wells of an 8-strip. To each tube 3µl of sample buffer was added, and vortexed for 60sec and briefly spun down. The tubes were then placed in the Tape Station holder and the tip rack filled. Using the TapeStation controller software on the computer, the appropriate number of wells was selected, and the samples named in the corresponding table. The run was then initiated, and the results analysed within the Electropherogram section. Traces of primer dimer and or nonspecific binding were checked for in each sample, and only if not present in the samples was the experiment continued. If there was presence of said issues, a further bead-clean up and PCR was completed, and the Tape Station repeated until the issue was resolved.

Library/PhiX denaturing and dilution- Preparation:

A Miseq reagent cartridge was defrosted in a tub of water whilst the hybridization buffer (HT1) and the control library (PhiX) were defrosted on ice.

The working pool was then diluted to 4nM with molecular H_2O. The pool was then denatured further to 1:20pM using 0.2N NaOH and 990µL HT1. This mixture was then vortexed, centrifuged and incubated at 23 °C for 5 mins.

The library was then diluted further to 12.5pM.

The PhiX library was then denatured and diluted to 4nM. Using the 10nM PhiX library stock (40%) and molecular H_2O (60%) a 5µL solution was prepared. This was then vortexed and centrifuged. This library was then diluted further to 1:20pM via the addition of 0.2N NaOH and 990µL HT1.

The PhiX library (120µL) was then combined with the denatured pool (480µL) and was inverted to mix. The sample was then kept on ice until ready to load into cassette. Making sure wash has been done prior, the machine was re started and the chip was removed and rinsed ensuring it was streak and lint free. A sequinning run was then commenced, and the outputted data was collated from the Illumina sequencer was then analysed through RStudio (RStudio Inc version 3.5.1).

2.7 - Statistical analysis

A two-way ANOVA was used to identify the degree of variation between the different groups. The standard deviation is presented by the bars and the value of *P* to indicate significance was 0.05.

3. RESULTS

3.1 - Differentiation of healthy and damaged lungs

Surplus porcine abattoir tissue was used to establish a modified *ex vivo* lung model for the investigation of host-microbe interactions relevant to VAP. The principle of this was to establish to what extent the 'host' interacts with the microbes that are associated with VAP and if this reaction could be quantifiable. Initial gross assessment of lung viability was required prior to dissection. Figures 3.1, 3.2 and 3.3 demonstrate what is defined by 'healthy lungs' within this study as they demonstrate consistent healthy colouring, have little to no damage to the lobes. Figures 3.4 what a 'damaged lung' is within this experiment, this is due to discolouration and external damage caused by removal. A general assessment was made of each lung to assess health and condition of the lung before any experiments occurred.

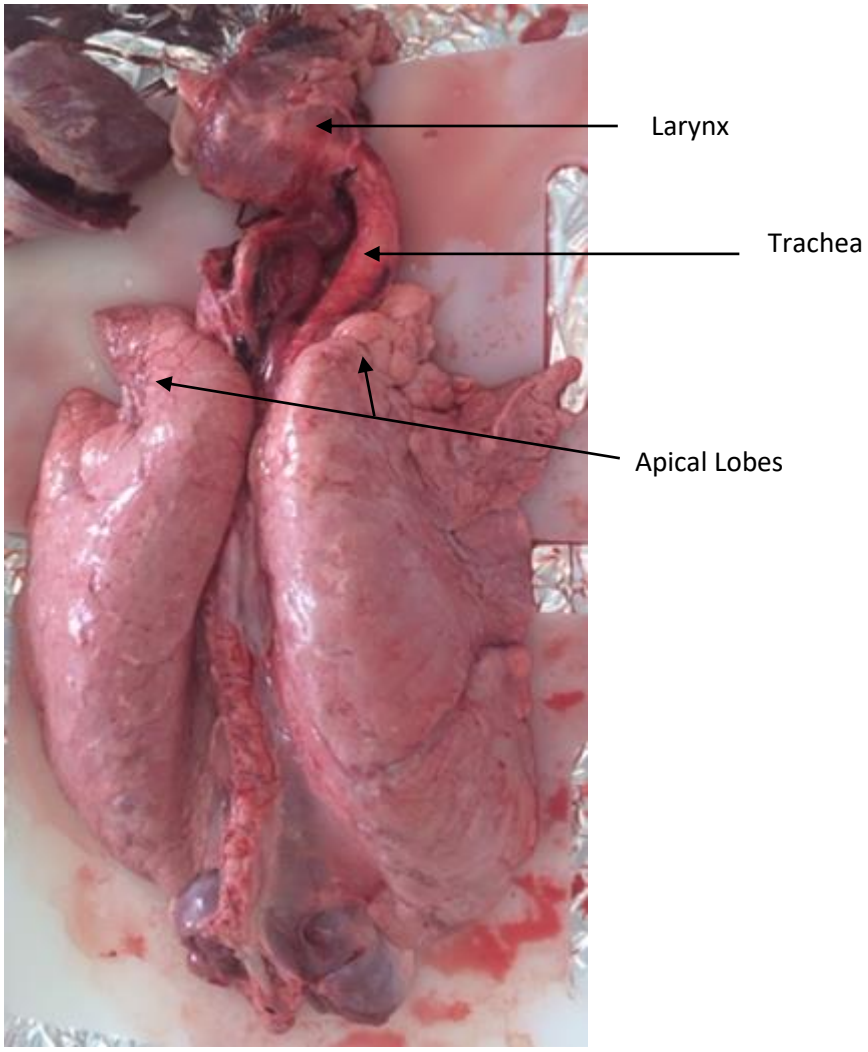

Figure 3.1: An image of the anterior face of the lungs with the labelling of the larynx, trachea, and apical lobes. The overall analysis of the lung is that this is a healthy specimen and shows little to no damage on the lobes or respiratory tract. There is no evidence of discolouration or drying out of the lobes.

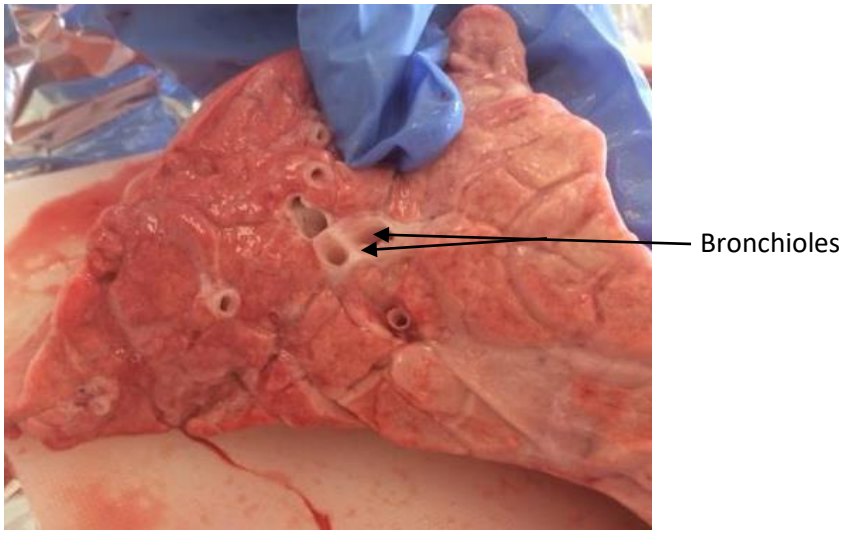

Figure 3.2: A further dissection of the right lobe of the above lung in figure 9 to demonstrate the health of the tissue continued sub-dermally. There is no sign of discolouration or tissue damage.

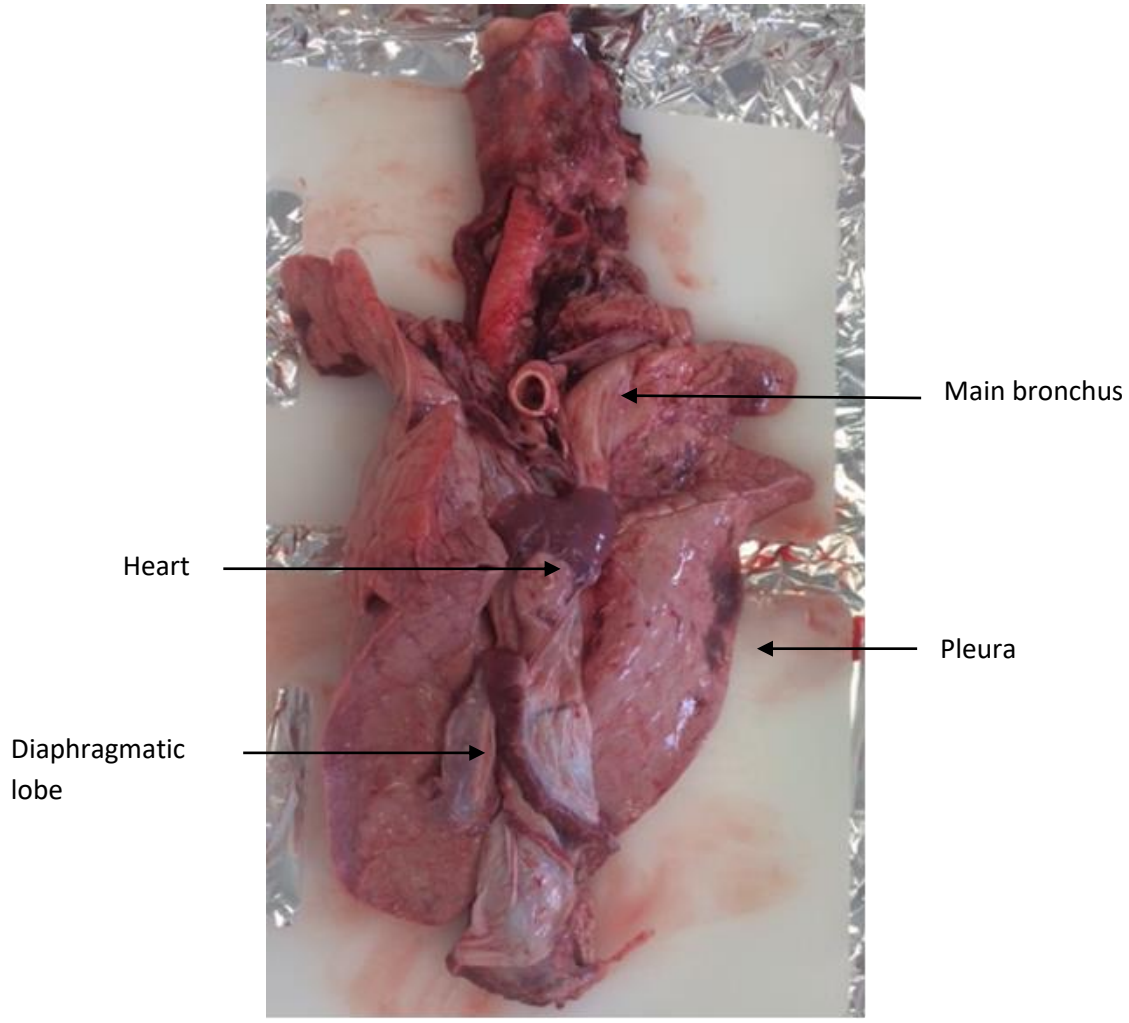

Figure 3.3: Image of the posterior face of the lungs with the main bronchus, heart, pleura and diaphragmatic lobe labelled. Again, the overall analysis of this lung is healthy and used as the standard in all experiments within this study.

However, not all lungs arrived in the conditions as described above. Figure 3.4 demonstrates the appearance of a lung subjected to severe damage. This is comprised of missing lobes and lacerations which have occurred post removal from the pig. Due to this this specific set is unfit for a BAL sample. It also excluded this set from the epidemiological analysis of the prevalence of *Mycoplasma hyopneumoniae* since there cannot be a reliable percentage of total lung infection recorded. This is especially notable as the lobes of the lungs have different weightings in terms of the infection density (Garcia-Morante *et al*., 2016). However, the tissue from the left lobe was deemed fit to be used for experimentation.

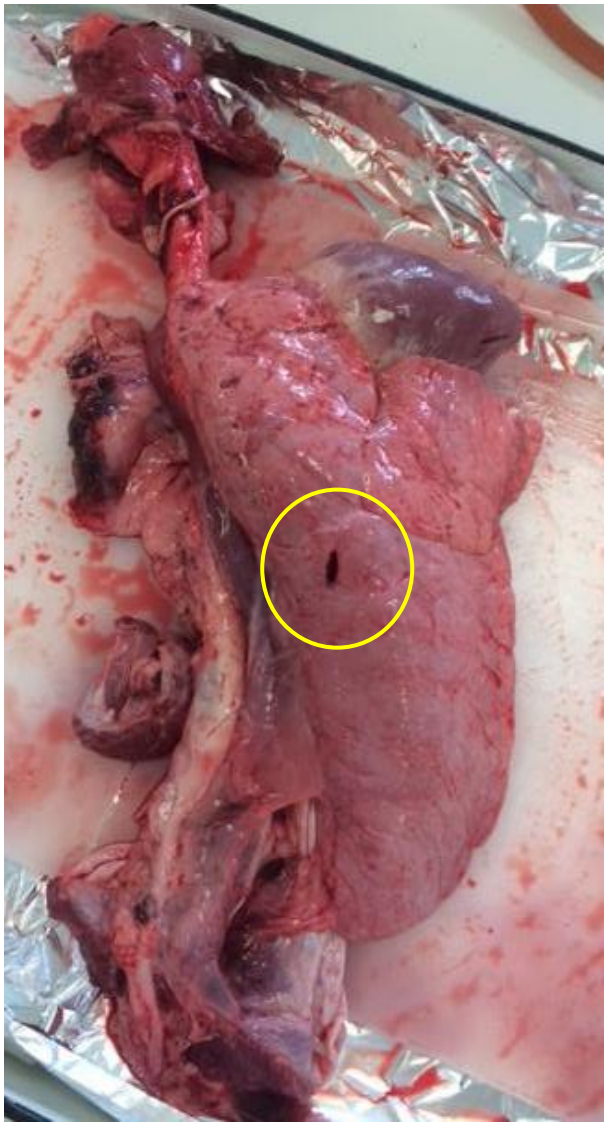

Figure 3.4: An image of what a lung which would be considered 'damaged lung' within this study. It is missing the right lobe and has a deep laceration (circled) within the left lobe. Despite the damage the remaining lobe is visually healthy due to the colouration and moisture retention.

3.1.2 - Optimisation of inducing *ex vivo* porcine lung tissue death

The impact of host viability was investigated to further the understanding of the interactions between the porcine host and the later added microbes to generate a model that can be extrapolated to human cases of VAP. The metabolism of the dissected *ex vivo* lung tissue was measured via resazurin fluorescence (in section 2.2.3 Optimisation of killing lung tissue) to establish cell viability over time and to make comparisons between the different conditions

that the tissue was subjected to: live; heat killed and cold killed. The results of which can be seen figure 3.5.

There was a significant difference between heat killed and live tissue viability for the first three hours. This is suggestive that as a method of lung tissue killing, heat is the best and most effective method. At the three-hour point there is no significant difference between cold killed and live tissue suggesting that the cold killed tissue is not dead and still viable at this point. Further reinforced by there being a significant difference between the heat killed and cold killed at the same time point.

After 18 hours there is no significant difference between live and any of the killed tissue readings, which is applicable for the latter reading at 24hrs, suggesting the tissue was no longer viable after 6hrs. There is a general trend for all conditions to have lower metabolic activity during the latter stages of the experiment than at the beginning suggesting there is a decrease in metabolic activity over time.

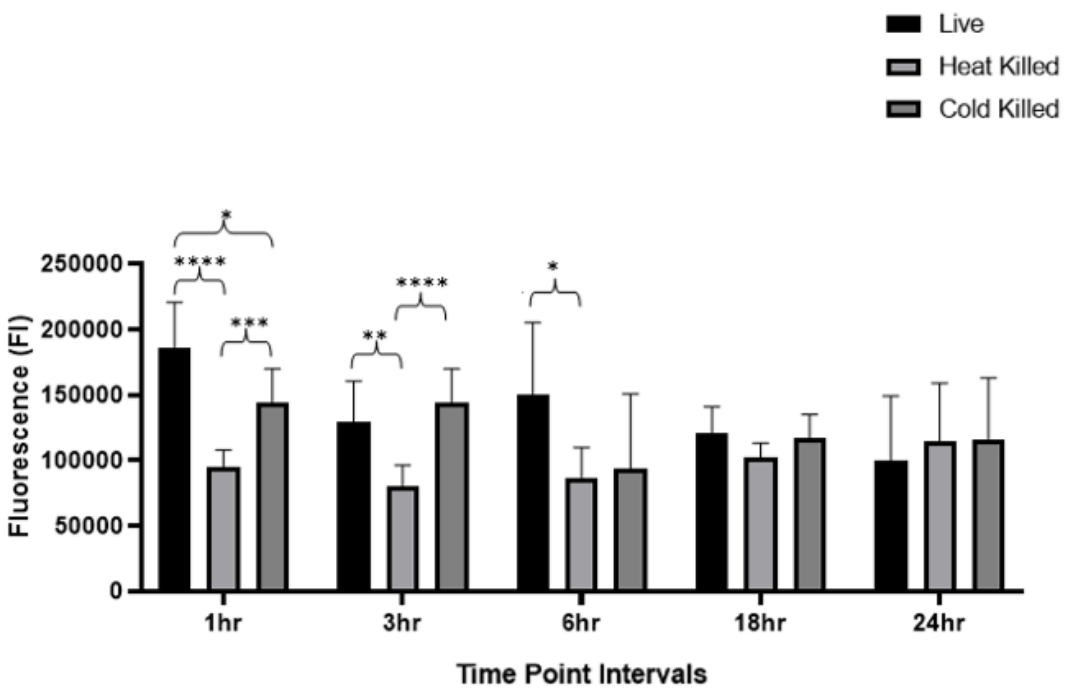

Figure 3.5: Metabolic activity of excised *ex vivo* porcine lung tissue measured by fluorescence of Resazurin read at wavelengths Ex/Em 560/590nm to evaluate methods of killing the tissue.

The statistical analysis of this data was a 2 way- ANOVA as a comparison of multiple analysis. The Tukey's test was applied.

3.1.3 - Detection of extant microbiota within BAL samples

BAL samples were prepared from the lungs of abattoir-grade pigs as described in section '2.3. Bronchoalveolar lavage of porcine lung'. Briefly, fluid was flushed into the lungs and then extracted using a mini-BAL sampling catheter, this fluid was then analysed to determine whether the isolates contained bacteria and if so what species. A PCR was performed using 16S amplicon sequencing with primer sets of V_1V_2 and V_3V_4 as described in PCR amplification of 16SrRNA gene from BAL samples. Figure 3.6 demonstrates that there are bacteria present in two different BAL samples taken from two different animals. The wells from 3-6 are 16S rRNA amplicons of the V1-V2 region from two BAL samples from two different lungs, wells 12-15 is the same BAL samples but run with the V_2V_3 primer set. The primers, V_1V_2 appear to be more complimentary to the bacterial DNA present within the BAL samples since in all four samples there are bands present whereas with the V_3V_4 only two samples have visible bands.

Wells 7,10, 16 and 17 contain the alveolar tissue and display three similar band widths in both samples. The weight is unclear since the Hyperladder did not run well, it is not the same as well 8 or 18 so it can be assumed to not be *S. aureus,* but this needs further research. Well 9 and 19 had sterile PBS however since there are bands present on the AGE it is assumed the sample was corrupted and was no longer sterile at the point of testing. This was not able to be repeated and therefore contamination of the other samples cannot be definitively ruled out.

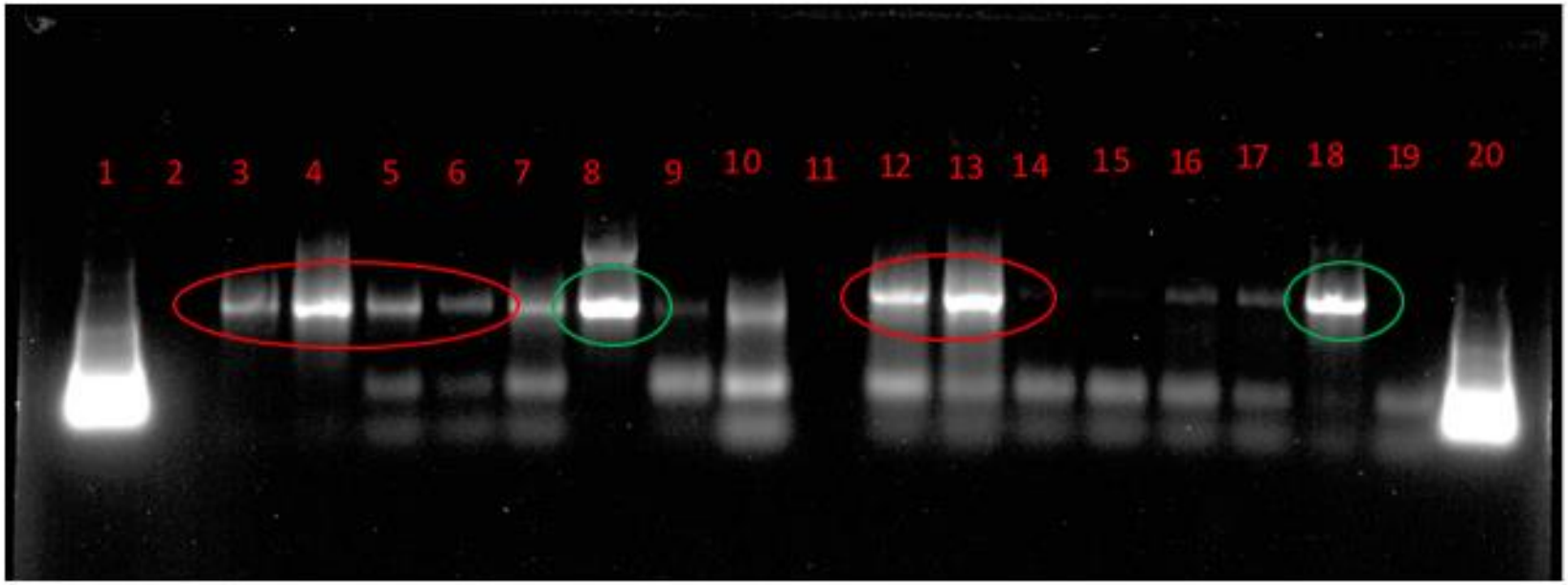

Figure 3.6: Detection of extant microbiota in pig lung. The 25bp Hyperladder in wells 1 and 20, a blank in well 2 and 11. The samples in wells 3-10 have had the V_1V_2 primers of the 16S rRNA gene and the samples in wells 12-19 have had V_2V_3. In wells 3 and 4 is the BAL sample from lung 1, wells 5 and 6 the BAL samples from Lung 2. In well 7 is the alveolar Tissue sample 2. In well 8 is the positive control of *S. aureus* bacterium and well 9 is the sterile PBS as a negative control. In well 10 is the Tissue sample 1. In wells 12 and 13 are BAL sample from lung 1, wells 14 and 15 the BAL samples from Lung 2. In wells 16 and 17 are the tissue samples from lung 1 and 2. In well 18 is the positive control of *S. aureus* bacterium and well 19 is the sterile PBS as a negative control.

3.2 - Physiology

Via tissue analysis of healthy porcine lungs, the structure and key components can be identified and then used as a control to compare to in instances of disease. These differences can highlight the changes brought about by the disease and can then be targeted by therapeutic devices.

3.2.1 Cells present in BAL sample extracted from *ex vivo* porcine lung analysed via cytospin

Samples of BAL extracted as defined in the Materials and Methods section '2.4.1 BAL Cytospin' were prepared for histological staining to investigate the cells present within a healthy porcine BAL sample. Figure 3.7 demonstrate representative images of BAL cytospins stained with Giemsa to differentiate nuclear and cytoplasmic morphology of the various blood components like platelets, red blood cells and white blood cells, such as marginated neutrophils, monocytes, and lymphocytes which were present within the sample.

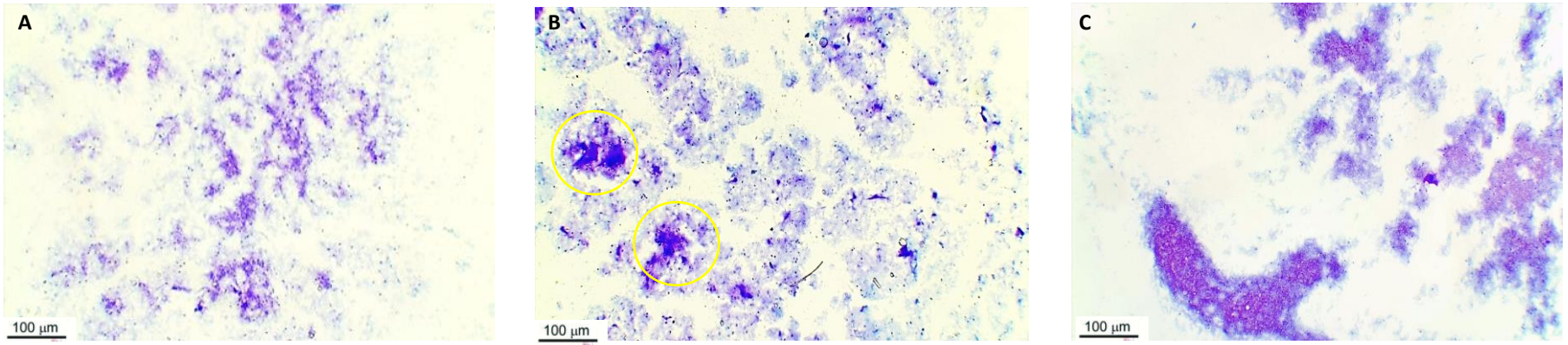

Figure 3.7: Detection of host's cells within BAL sample *of sus scrofa*. Cytospin samples of BALs were stained using Giesma stain and the percentage staining calculated using GIMP (version 2.10.14) package.

A - This representative BAL sample was taken at the objective power of 4X/0.1 and total percentage stain colouration calculated as 15%. The colouring would indicate the stained cells are most likely nuclei of host cell or white blood cells, such as marginated neutrophils, monocytes, and lymphocytes or platelets.

B - Giemsa staining of BAL cytospin with the objective power of 4X/0.1. The total stain percentage was calculated to be 47%. The yellow circles highlight areas where there may be a build-up of multiple layers of cells, so the colour is darker and individual feature are unidentifiable. The primary feature appears to be commonly found within the cytoplasm of porcine lungs.

..

C - Giemsa stain of extracted BAL sample after cytospin at the objective power of 4X/0.1. Calculated stained percentage of slide was 15%. However, the large clusters and darker colours indicate a lack of separation when spun and may be a disproportionate representation of the cells within the BAL sample. Since there is a mass of overlaying colours it is difficult to distinguish individual features.

Of the 13 total cytospin stains the average percentage covering was 30% and were most similar visually to B within figure 15. The most common colours within all the stains are dark purple, pale blue and violet. The dark purple may be nuclei of host cell or white blood cells. The pale blue potentially the cytoplasm of the porcine host, whilst the violet/purple may represent platelets. Specific protein immunochemistry and immunofluorescence assays, such as the CD-45 receptor found on most white blood cells (Inamura, 2018) could be performed to confirm the staining results in future experiments

3.2.2 - Identification of structures in healthy BAL sample extracted from *ex vivo* porcine lung via Hematoxylin and Eosin staining

Presently most tissue sections are treated with Haematoxylin and Eosin (H&E) (Titford, & Bowman, 2012; Bancroft, & Layton, 2013; Alturkistani, Tashkandi, & Mohammedsaleh, 2016). Over the years there has been little changes to the stain since it exhibits a broad array of features and works well with numerous fixatives, so much so it is often considered the 'gold standard' of medical diagnosis (St. Michael's hospital protocols, 2019). H&E stain can identify several features of tissue by the colours identified since the cytoplasm and the extracellular matrix is stained in varying shades of red whereas the nuclei are blue (Dettmeyer, 2011). The common features of lung tissue include the bronchi, bronchioles, and the pulmonary alveoli, which enable gas exchange (Zhou, & Moore, 2017) as demonstrated in Figure 3.8.

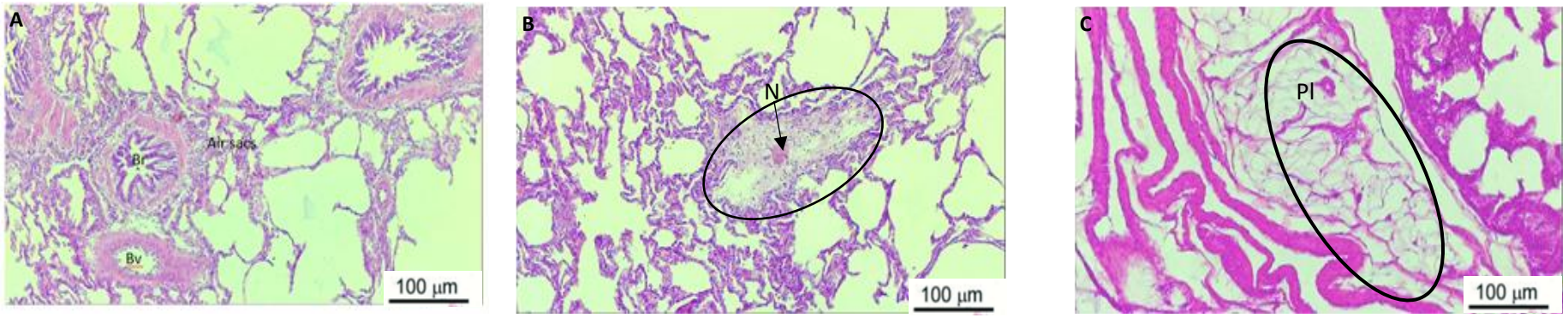

Figure 3.8: Hematoxylin and Eosin stains of excised *ex vivo* porcine lung tissue to identify the components within the tissue in a heathy state.

A - Clear section that demonstrates the healthy structures found within the porcine *ex vivo* lung tissue such as the Bronchioles (Br); Blood vessels (Bv) and Air sacs at an objective magnification power of 100X/0.1 with a light microscope.

B -Tissue section at an objective magnification power of 100X/0.1 with a light microscope. The majority is made up of extracellular matrix (circled)and nuclei (N).

C -Tissue section of the pleura(Pl) at an objective magnification power of 100X/0.1 with a light microscope.

3.2.3 Comparative method into identification of structures in healthy BAL sample extracted from ex vivo porcine lung via giemsa staining.

Giemsa staining originally developed by Dimitri Romanowsky in 1981 with its popularity rising due to the ability to stain in multiple colours and ability therefore to identify blood parasites, for which it is still used today (Alturkistani, Tashkandi, & Mohammedsaleh, 2016). Since then, the stain has been greatly modified to make it more applicable to various fixation methods (Musumeci, 2014). In this study it was primarily used to act as a comparison to H&E staining (in section 3.2.2) since the same piece of tissue was sectioned multiple times so that it was as similar as possible when comparing the two stains.

Sections of wax embedded lung tissue were stained with Giemsa as in section. This allowed for clear visualisation of the alveolar sac as demonstrated in Figure 3.9.

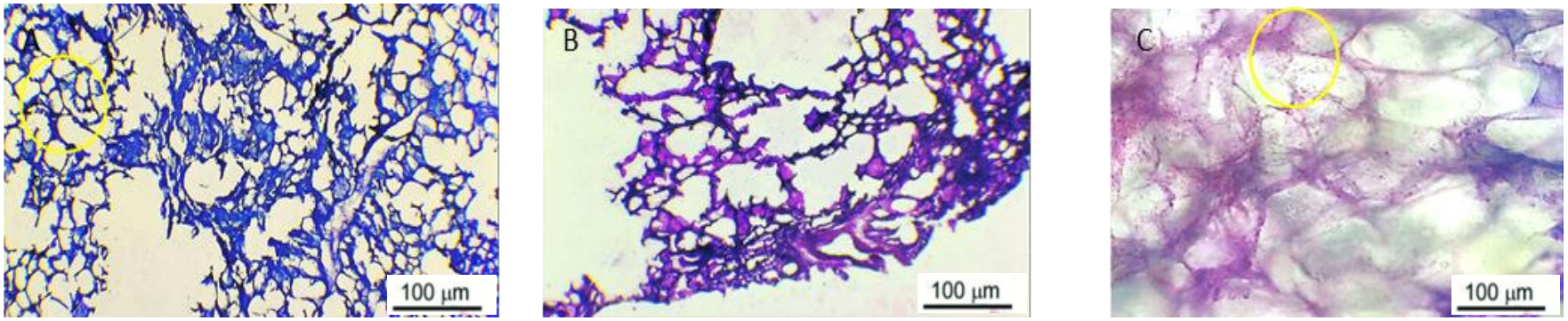

Figure 3.9: Sections of Lung tissue take from *ex vivo* porcine lung tissue and stained with Giemsa and were examined by light microscopy at various objective powers.

A - Objective power of 4X/0.1. The circled area highlights the air sacs, stained blue, which are intact and demonstrate lung health since there is no indication of damage or a desegregation of the sacs. Thus, demonstrating that the sectioning technique does not damage the structural integrity of the tissue.

B - Objective power of 4X/0.1 primarily showing the surrounding cytoplasm (pink) more so than the air sacs.

C - Objective power of 10X/0.25 mainly identifying eosinophils (circled)were present within the air sac of the alveolar. However, there is clearly wax still present on the slide making it difficult to clearly distinguish the stained features within the tissue.

3.2.4 - Prevalence of *Mycoplasma hyopneumoniae* infection within the lungs acquired

Discoloured lesions were identified on the surface of the alveolar lung tissue which could potentially be due to bacterial infection caused by a variety of pathogenic bacteria but most commonly due to *Mycoplasma hyopneumoniae* (Garcia-Morante *et al*., 2017). This discoloration was evident in some of the lungs as demonstrated in Figures 3.10 and 3.11. Pigs infected with the pathogen commonly experience damage to their mucosal clearance system, modulation of the immune system and leaves the animal more vulnerable to other respiratory tract infections since the pathogen adheres to the ciliated epithelium of the trachea, bronchi, and bronchioles stopping rapid clearance of the pathogen from the respiratory tract (Maes ET AL., 2021). But the extent of which the damage the infection does to pulmonary function is very poorly characterised and demands further research (Summerfield, 2020).

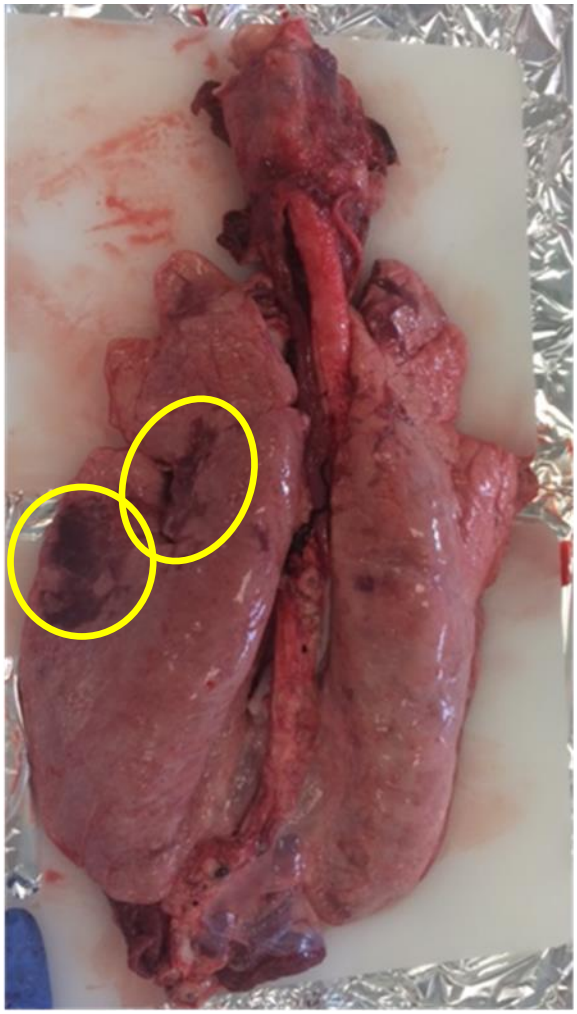

Figure 3.10: Image of the gross anatomy of *ex vivo* porcine lungs. Discolouration of lung tissue, believed to be caused by *M. hyopneumoniae*, is indicated with the circles. These lungs were discarded and not included within the analyses.

Figure 3.11 is an enlargement and focused view of the lesion in Figure 3.10.

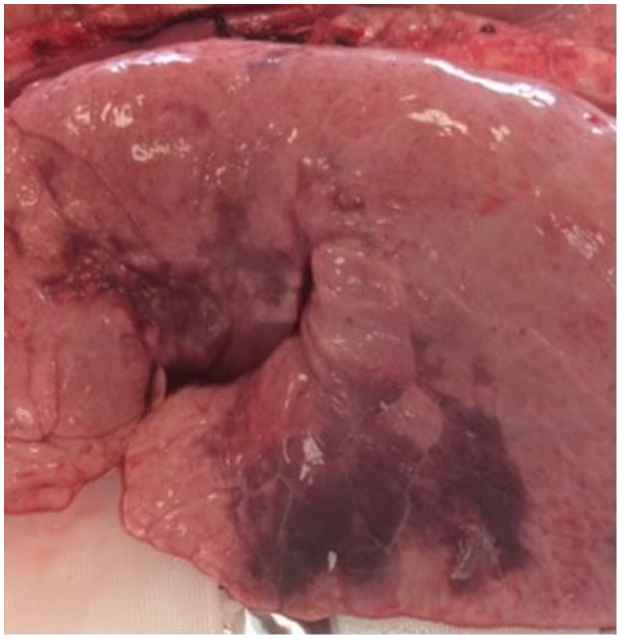

Figure 3.11: An up-close assessment of *ex vivo* porcine lungs from the abattoir. The area was located within the diaphragmatic lobe and has spread slightly into the apical lobes. The area is overall very small and takes up a marginal portion of the lung tissue.

The total prevalence of the possible herd infection can be seen in Table 3.1 where an evaluation of all lungs received was collated.

Table 3.1: Data collated to present the total potentially infected lesions from *M. hyopneumoniae,* on the *ex vivo* porcine lungs received of the course of the experiment.

Total Received	**43**
Discounted due to injury	5
Total Examined	38
Total % Infected	10.5
Average % of lesion coverage	6.71
Location of Lesion	75% Apical lobes 25% Diaphragmatic lobes

Overall, of the lungs included with the assessment just above 10% (total 4) had lesions which could be due to *M. hyopneumoniae.* Of the potentially infected the average percentage size of the lesion(s) was 6.71 % of the total pulmonary tissue area. Despite the presence of some slight discolouration as demonstrated in figures 3.10 and 3.11 the overall assessment of the lung is that it is healthy as the discolouration spans less than 10% of the total area of tissue and was avoided when dissecting sections of alveolar tissue. A histological comparison of the diseased tissue compared to the live tissue would be required in future testing to help understand further the clinical effects of the pathogen on respiratory function.

3.2.5 Proliferation of bacteria on *ex vivo* porcine lung tissue

By having established the optimum way to effectively kill *ex vivo* porcine lung tissue previously in this study, a comparable control of dead vs live tissue was able to be implemented when measuring the proliferation of the VAP clinical isolate on the excised lung tissue. The tissue was co cultured with the isolate after inoculation to establish the growth curve. To make sure it was the most accurate representation of solely VAP growth, the antibiotic Ampicillin was added since most VAP isolates are known to be resistant, however, it is of note an MIC was not done by this author on the isolate, this may also help reduce any chance of contaminating from influencing the growth of the isolate. After determining the optimal dilution range for the homogenised *ex vivo* porcine tissue, by measuring growth of the VAP isolate at various dilutions, the CFU/ml was calculated at the stated time intervals. The curves were generated for both 'dead' and 'live' tissue sections. The results of which can be seen in figure 1.12 and figure 1.13.

In figure 1.12 demonstrating the growth on heat killed tissue, the CFU/ml of the tissue demonstrates no growth for the first three time point intervals however, at the 24hr reading there is evidence of growth which increases when recorded again at the 30hr point. This is suggestive of the bacteria having a relatively long lag phase of 18rhs. The mean CFU for the heat killed tissue, no inoculum, at the 24hr point is $2x10^{10}$ which in comparison to the live tissue (not inoculated) which is $5x10^{10}$. These CFU counts present clear evidence that there is more bacterial growth on the live tissue than dead, suggesting that the killing method was successful in removing the original host biome.

The growth was at a steady rate and relatively linear. This tissue was not inoculated with VAP, so the growth is likely to be residual host microbiota which is also resistant to the added ampicillin or contamination from the extraction and homogenisation process. Further sequencing would be required to eliminate this possibility. This could be further examined in the future by using selective agars and gene specific PCRs.

Considerable difference in CFU was observed between inoculated live and dead tissue. After the 8hr point where no growth was recorded to the next time interval of 18hr there was a sharp increase in bacterial growth. Then again from the 18hr to the 24hr point there is a further steep increase in gradient suggesting the bacteria was in the exponential phase. The growth rate then falls just as sharply as it had risen between time points 24hr to 30hr suggesting the bacteria has entered the death phase. This may be due to a finite number of mineral resources supplied by the excised tissue causing bacterial starvation.

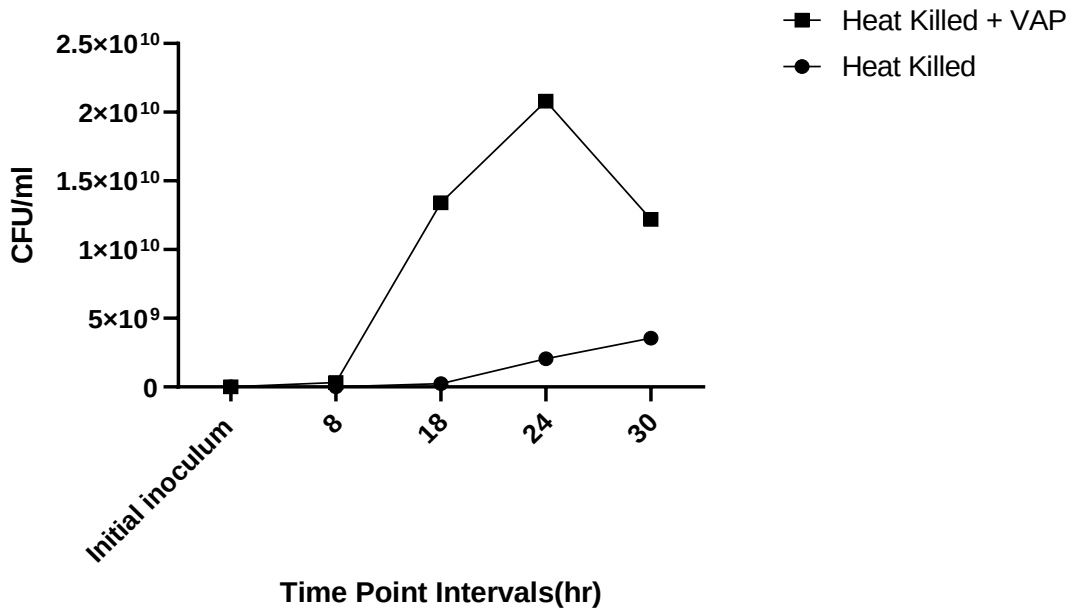

Figure 3.12: Comparative growth curve of heat killed *ex vivo* porcine lung tissue and heat killed *ex vivo* tissue inoculated with the clinical VAP isolate (Heat killed + VAP) with an inoculum 0D of 0.2 and CFU/ml of 1x106. The bacterial growth was measured at the set time points and recorded indicating there is more growth on the inoculated killed tissue than that of the killed tissue alone.

The results for the live tissue can be seen in figure 3.13 Both conditions were relatively similar after the 8hr time interval both with minimal growth recorded. However, between the 8hr to

the 18hr time point the live tissue with the VAP inoculum has a rapid and sharp increase in reported bacterial growth. This is in vast difference to the live tissue that was not inoculated which whilst does have an increase in growth of 10^9, it is minimal in comparison. Therefore, the inoculated tissue has bacterium that are rapidly proliferating in the exponential phase whereas the live tissue on its own is at a far slower rate. From the 18hr point to the 24hr time point the inoculated tissue enters a slight decline in growth.

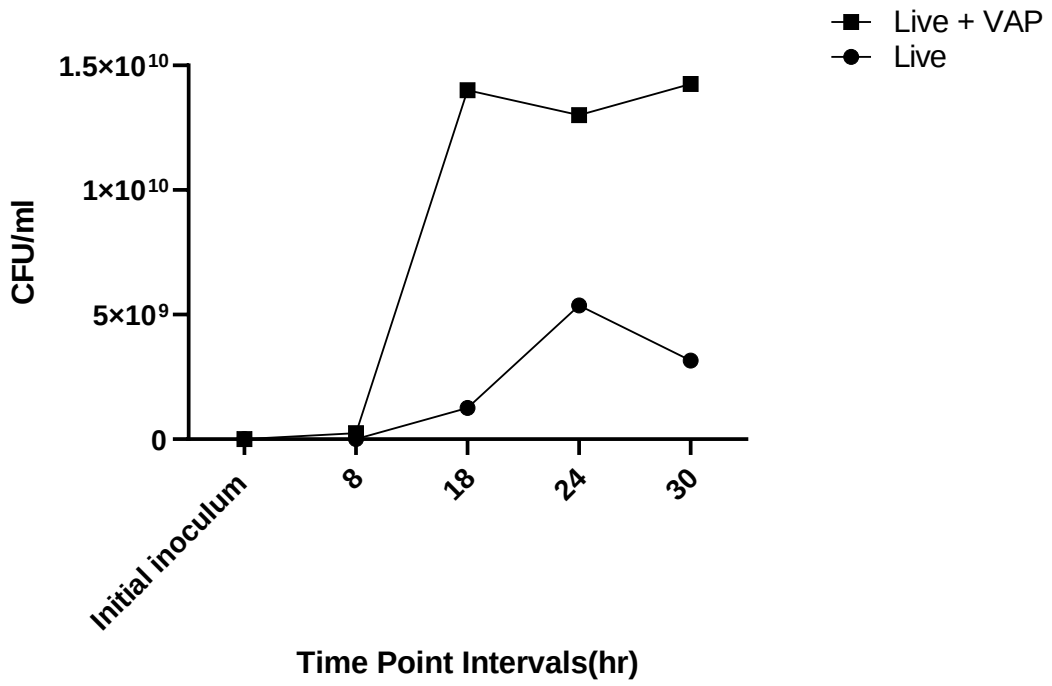

Figure 3.13: Growth curve generated via CFU/ml over time for live tissue and live tissue inoculated with the VAP clinical isolate at an 0D of 0.2 and CFU/ml of $1x10^6$ (Live + VAP). The bacterial growth was measured at the set time points and recorded indicating there is more growth on the inoculated live tissue than that of the live tissue alone.

The opposite occurs in the live tissue alone since there is a continual rise in growth of a relatively steep incline. However, this rate of growth desists, and the bacterial growth demonstrates a steep decline as it enters the death phase from the 24hr point to the 30hr. This is interestingly the opposite of what is happening with the inoculated tissue which is expressing a rise in bacterial colonies from the 24hr to the 30hr point. Whilst the results are not expressing as perfectly horizontal growth curve the bacterium may be in the stationary

phase between 18hr to 30hr on the Live +VAP condition since there is only a small change in the gradient of the curve over this time.

When extrapolating the information from figures 3.11 and 3.12 both growth curves indicate that there is a far greater amount of bacterial growth in the inoculated sections of tissue than the non-inoculated tissue in both the live and dead conditions. When comparing the non-inoculated live and dead tissue it is evident that the live tissue has a steeper initial gradient and more rapid proliferation stage at the first few recorded time intervals than the heat killed tissue, but then rapidly falls from the 24hr to 30hr time interval. The dead tissue has a far more gradual and slower proliferation stage but appears to constantly rise across all the time points. However, at the 30hr point the end growth CFU/ml recorded is not vastly different for the heat killed and live tissue.

Regarding the inoculated tissue in both the live and dead tissue conditions there are also these similarities and differences presented in the data. Within the heat killed and inoculated lung tissue, the bacterial growth appears to go straight from the steep exponential phase straight into the death phase. The live tissue that is inoculated appears to present a more gradual exponential phase but then transitions into a stationary phase towards the latter time intervals. However, again the end points after 30hrs are not too dissimilar.

3.3 - DNA extraction and sequencing of *ex vivo* porcine lung tissue

The 16S rRNA profiles of microbial communities from the pooled BAL demonstrated in figure 3.14. As aforementioned six lungs were used, from each the left and right lobes were washed. These samples (left and right) were then pooled together to make two test samples. This is to save cost and to gather a more general idea of what the normal constituents of the porcine lung microbial community. It also increases the sample size which makes the data more replicable. There are many common bacteria shared by both BAL pooled samples, but the primary shared bacterium being *Caloramator, Anoxybacillus* and *Flavobacterium.*

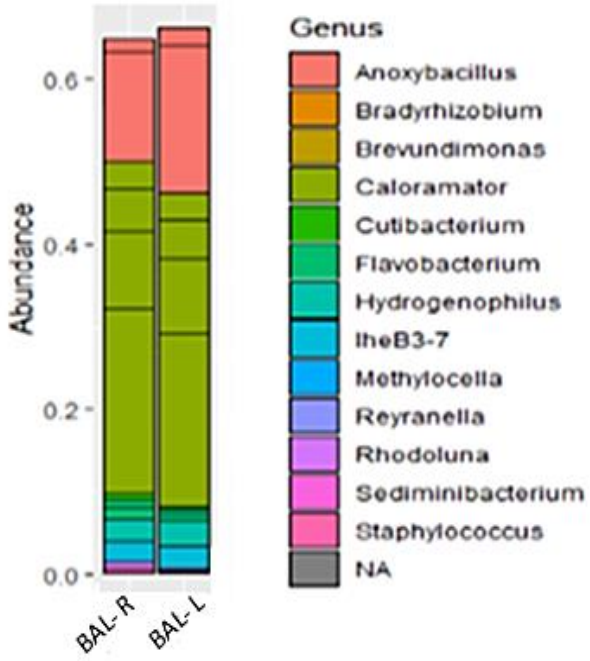

Figure 3.14: The abundance of bacterial genus's present in pooled homogenised *ex vivo* porcine lung BAL samples sequenced via Illumina technologies. BAL-R refers to the pooled samples from the right lobe. BAL-L refers to the pooled samples from the left lobes of the six lungs that underwent the BAL process.

There is very little β- diversity demonstrated in figure 3.14 however, there is a high level of α-diversity since there are thirteen different phyla identified within the pooled BAL samples. This aligns with the current beliefs in research that high levels of α-diversity are indicative of lung health whereas low levels of α-diversity and high levels of β- diversity is indicative of a pathology of some sort, be it a bacterial infection or viral (Segal et al., 2013).

4. DISCUSSION

The aim of this study was to develop the previously established *ex vivo* porcine lung model into a modified version to facilitate the investigation of host-microbe interactions relevant to VAP. The pig was used since they have vastly similar immune responses and anatomy to that of humans (Li Bassi *et al*., 2014). In developing this model, the optimisation of killing the *ex vivo* lung tissue was established. A functional method of obtaining a BAL sample from the *ex vivo* porcine lungs was developed. Via tissue histology the structures present in sections of the *ex vivo* porcine lung tissue was reported alongside an investigation into the growth of a clinical VAP isolate on the excised tissue.

Whilst collecting data for this study an internal unexpected branch of research developed as to investigate the prevalence of a potential *Mycoplasma hyopneumoniae* (*M. hyopneumoniae*) infection within the porcine lungs was identified. Since this was not necessarily an aim of this study other than reporting the initial findings, little was done in terms of exploratory research. However, this would be an interesting avenue to pursue for those looking to take this developmental model further in future research projects. The diseased lungs were not included in the primary experiment since it was unknown if the immune markers may be different to that of healthy lungs, which is also a further avenue of future exploration.

There are several elements as to why the microbiology of VAP is important, such as the ability to make a better-informed diagnosis of the patient, to provide information about the current AMR status and the microbes causing pathogenesis, whilst also being able to guide VAP prevention efforts to reduce the negative impacts of the disease (Park, 2005). Despite there being a great amount of research into the host microbiome and the pathogens that cause disease, there is little research on how these two elements are linked and how they interact in cases of disease. This is partly because there are so many variables involved which makes a holistic study difficult to singular out the cause and consequence of the applied variables.

There is also an issue that even the organisms that are normally commensal within the host are the causative agent for less than half the reported cases indicating the degree of variation in pathogenic organisms. This variety is part of the reason treatment strategies for VAP are so convoluted as each have their own individual methods of pathogenesis to the host (Akter,

S., Khatun, R., & S.M Shamsuzzaman, 2015). These mechanisms must mute the hosts microbial defences for the pathogen to establish itself and cause VAP (Bonten, Kollef, & Hall, 2004).

In initial VAP research, primates were used due to their incredible likeness to the human immune system (Pulido, Burgos, Luna, & Morato, 2017). However, there are strict restrictions when working with primates such as the substantial cost of upkeep and specialized equipment needed (Harrison *et al*., 2014). Another avenue that has been investigated is using live invertebrate models instead of mammals (Harrison, Browning, Vos, & Buckling, 2006; Brackman, Cos, Maes, Nelis, & Coenye, 2011). The benefits being that the experimental design is simple, low cost and bares far less ethical constraints however, the model itself is severely lacking in similarities with its human counterpart and their limited life spans also add extra time constraints to the research (Harrison *et al*., 2014). Overall, it was concluded as an ineffective way to study human diseases in the case of VAP due to the lack of transferability which is why most of the research has moved onto smaller mammals such as pigs and mice.

This study used *ex vivo* porcine lungs to study the host-microbe interactions to VAP. There are negatives within this avenue of research such as the loss of a more systemic and holistic view of VAP as it would be in a patient however, the specific pathophysiology caused by independent variables can be isolated and observed. This is a benefit for establishing cause and effect of specific components of VAP. Porcine organs also share great a deal of similarity in; anatomy and the organization of an amounted response to disease, as recent research suggests a similarity of up to 80% between pigs and humans which is incredibly high when in comparison to mice which share only a 10% similarity, (Dawson *et al*., 2013) thus allowing for more translational data when using pigs in research. The porcine lung model can also be used to study a variety of respiratory diseases as well as VAP such as cystic fibrosis (Harrison *et al*., 2014) and other respiratory bacterial infections (Dumigan *et al*., 2019). The ability for the porcine lung models to be so multifaceted is what makes them such excellent research tools.

The similarity between the porcine organs and immune system is imperative as it allows the data to be translational and is more likely to be applicable to clinicians dealing with patients with VAP. For research to progress to clinical trials, commonly referred to as ‘from bench to bedside’, it may take from ten to fifteen years to go through all three clinical trial phases

before the treatment can be licenced (Cancer Research UK, 2019). The ability to make frequent explorations and investigations with little cost or resource drainage via the porcine lung model means that multiple avenues can be explored quickly narrowing down what is successful and should be put forward for future trials.

With the lung tissue there are several established methods of killing extracted tissue that have been explored within popular research, most commonly by subjecting the tissue to extreme temperatures. Previous studies that investigated heat killing identified that after just one minute at 45-50 °C cell viability plummeted from around 90% to just 20% (Franco, Kothare, Ronan, Grekin, & McCalmont, 2010). In comparison, the advantages of subjecting the tissue to extreme cold via liquid nitrogen, is the ability for it to be stored and transported in a non-pressurized container allowing research to be conducted in the regular laboratory room, with no extra costs or provisions required (Nomori, Yamazaki, Kondo, & Kanno, 2017). The viability of the tissue was measured via resazurin fluorescence, which is reduced by metabolically active cells to resorufin, which is fluorescent therefore, the amount of fluorescence produced is correlated to the quantity of viable cells existing (Van Den Driessche, Rigole, Brackman, & Coenye, 2014).

Within this research, the optimisation of killing lung tissue was established using resazurin. The data identified that heat killing tissue was more successful compared with cold killing at early timepoints. However, it is of note that whilst heat may significantly reduce the viability of the tissue in comparison to live tissue, it also degrades the structural integrity of the tissue. This then makes histology impossible and is therefore useless regarding sectioning or staining in latter experiments. Whereas the cold killed tissue retains structural integrity and is metabolically significantly different to the live tissue, suggesting it has been killed. Work must now continue to identify a compromise where viability is impaired, whilst still maintaining usability for further investigations. Throughout this experiment cold freezing killing was used since it preserves the tissue for latter histology.

At the latter time points, tissue viability increases in all conditions. It is unlikely that this is due to tissue recovery, but maybe the result of growth from an opportunistic bacterium which is thriving on the decomposing tissue and/or the media itself (Shi, Mi, Wang, & Webster, 2019). However, the nutrients provided by the media and the tissue is a finite source and the

bacterium may be beginning to starve and be reducing in numbers thus explaining the varied viability results after 24hrs. This is important regarding VAP research as any bacterial components being measured after the 24hr mark may no longer solely be related to VAP and may be in fact be a by-product of the research that is unrelated to VAP in a clinical setting. This is important to be aware of to those looking to extend this research further.

Overall, the results suggest that both heat killing and cold killing are successful methods of killing lung tissue. The benefit of cold killing in comparison to heat is the ability for the tissue to remain structurally intact for future testing and therefore was used as the method for killing lung tissue throughout this experiment.

Optimising the method of killing the tissue was an important stage in the development of this model as it acts as a negative control and a comparison to the live tissue. By comparing the reactions caused in live tissue to dead it isolates the 'host' factor in the research. If the response does not happen in dead tissue but does in live, then the research can focus on specifically what is happening within the live tissue to make this difference. This is important to the patient suffering from VAP as theoretically due to the similarities between porcine and human pulmonary systems a response in one may be reflected in the other. Then once the response has been isolated it can be investigated what the host does to the bacterial component specifically. These responses are the focus of this research and the development of this model to better understand these interactions to be able to apply them to a clinical setting eventually.

Due to the relatively new discovery that the lungs are not in fact sterile as once believed the research into the lung biome is comparatively lacking to that of other areas within the body (Morris & Flores, 2017). Recent studies into the pulmonary microbiome have established that changes in the lower respiratory tract microbiota can be associated with disease (Pragman, Kim, Reilly, Wendt, & Isaacson, 2015). Bacterial diversity is commonly associated with health, and it is of considerable note that in 83% of patient's diversity decreased over the course of intubation, with dysbiosis being more significant in those who developed VAP (Zakharkina *et al*., 2017). The length of the ventilatory period was directionally proportional to the α diversity of the microbiome of the LRT (Zakharkina *et al*., 2017).

In current clinical VAP cases, through the analysis of BAL samples, it has been identified that those with microbiologically conformed VAP have a higher bacterial load than those without VAP (Huebinger *et al*., 2018). However, there is very little data on the porcine lung biome therefore, within this research it was investigated further using BAL sample techniques. This study used the Halyard Mini-Bal Sampling Catheter (Halyard Health) to obtain BAL samples. This catheter worked well within the excised lungs and is available for use within clinical settings. Thus, reducing the distance between this research and that regularly performed on humans.

By understanding the healthy pig lung biome, the effect of adding the clinical VAP isolate can be observed on tissue extracts bathed in BAL fluids. The level of microbial dysbiosis within the lungs can be observed in correlation to the bacterial load of clinical isolate inoculated into the lungs. Since they are excised, the lungs would have to be placed into a simulated *in vitro* environment to do a longitudinal study over the course of several days. This was not feasibly possible for this research study but would be interesting and more clinically relevant to be explored in the future. The use of pre-emptive therapies can also be tested, for example by knowing the main bacteria of the biome specific probiotics could be administered and their effect monitored when the lungs are later intubated (O'Dwyer, Dickson & Moore, 2017). This would be especially relevant to health care workers as if they know a patient is going to be intubated these pre-emptive therapies could be applied relatively easily and have great potential. This is an avenue of research which should be investigated in the future.

Following on from this the effect of commonly given antibiotics that patients receive could also be studied within the lungs. Whilst there are differences in the biomes there are also conserved bacteria The level of dysbiosis when VAP isolates are introduced alongside antibiotics would be clinically relevant. There may be different combinations of antibiotics which cause less dysbiosis which could be discovered by repeated testing using this model.

The conclusions of the BAL samples indicated that *S. aureus* was present within the sample. Commonly *S. aureus* strains are isolated from the nasal cavity of healthy pigs and within the trachea rather than the lungs (Moodley *et al*., 2012). The finding of *S. aureus* strains within the lung is rare as the most common bacterium within the porcine are *Methylotenera, Prevotella, Sphingobium* and *Lactobacillus* (Huang *et al*., 2019). Therefore, the *S. aureus* may

have potentially been introduced from the catheter as it was brought down the trachea rather than residing within the lung. This is of note as many cases of VAP have been associated with displaced oral bacteria that are foreign to the lung biome (Marik, 2001). When looking at other research examining the pig microbiome there are few similarities in what their research presents and this study, this may be due to such a small sample size within this but also it is of note that the quantity of literature available is very limited, alongside most studies only highlighting around six phyla for pig biomes whereas human biomes have around fifty categorised phyla (Huebinger et al., 2018 &Huang et al., 2018). This demonstrates how much more research needs to be done to establish a well published porcine lung biome for further research.

The displacement of oral bacteria is clinically relevant as it is believed to be a primary cause for VAP in intubated patients (Sands *et al*., 2016). By discovering that this is potentially happening in the *ex vivo* model not only further highlights the similarities between the model and patient cases, but once again allows for this occurrence to be studied and prevention strategies tested. For example, after changing factors of inserting the ETT the presence of *S. aureus* could then be tested, if the bacterium was not present the relocating of oral/tracheal bacterium was potentially ceased. This would be of clinical relevance as it may reduce the cases of VAP dramatically.

An agarose gel electrophoresis was used to separate the mixed population of macromolecules, such as DNA or proteins in a matrix of agarose, was used to identify the bacteria present in the BAL sample. AGE is a very simplistic approach and therefore further testing is required to identify all the species within the samples rather than just that of the control. Within this study a genetic sequencing was run on the BAL samples.

The benefits of using polymerase chain reaction (PCR) are that it is a simple procedure which generates results promptly and with a high degree of specificity (Garibyan & Avashia, 2014). However, the simplicity of PCR can potentially be its downfall since even trace amounts of DNA can result in false readings, whilst being limited to only be able to identify known genes through specific primers (Smith, & Osborn, 2009). A further limitation relating to the use of primers is the possibility for primers to anneal to sequences that are like that of the target

DNA, whilst sometimes annealing to themselves phrased ‘primer dimer’ causing a replication of non-target sequences (Garibyan & Avashia, 2014).

The samples were also subjected to the Qubit and a Nanodrop to identify if there was enough DNA for sequencing. The advantages of using a Nanodrop are the rapid turnaround time of 10 seconds for results, the small quantity (1µl) of only sample needed, the large rage of ng/µl recorded whilst also avoiding the use of cuvettes and need for diluting samples (DNA Technologies Core, 2018).

Within clinical settings the microbial composition of BAL samples is frequently characterised by 16S rRNA gene sequencing, therefore this study follows similar procedures (Huebinger *et al*., 2013; Ying *et al*., 2019). The most abundant bacteria found within this study was *Caloramator*, there is no evidence to suggest this is a normal lung microbe which suggest it is either a residual affect from pulmonary aspiration of contaminated water sources that contained this bacterium (Ogg & Patel, 2009), or it is a contaminate from the sampling technique. These methods allow for longer base sequences of DNA to be identified and are ideal for the specific bacterial isolation and identification within clinical settings (Huebinger *et al*., 2013). They also allow for the identification of individual species from mixed samples at an extremely high rate of sensitivity whilst maintaining a low cost, especially compared to that of previous methods (Hamady, Walker, Harris, Gold, & Knight, 2008).

From the results in section ‘3.3 DNA extraction and sequencing of *ex vivo* porcine lung tissue’ many of the bacterial families identified three were commonly cited in other publications. The common genus are as follows; *Flavobacterium, Staphylococcus* and *Bradyrhizobium* (Siqueira *et al*., 2017). Bacteria within the *Flavobacteriaceae* family have been discovered to be within the mucous membranes of both healthy humans and animals (Vela *et al*., 2007). However, in cases of pathogenicity this bacterium has also been linked to cases of pneumonia and other bacterial diseases in humans (Slenker, Hess, Jungkind, & DeSimone, 2012). However, it is of note that this is only a very small sample size of six lungs grouped into two pooled samples. To increase accuracy and reliability it is suggested to do this with a far larger pool of samples.

Whilst using 16S ribosomal RNA (or 16S rRNA) is still considered the go to method in biome sequencing, there are limitations in how well the method can differentiate between species

(Zhu *et al*., 2013). Due to the lower levels of bacterial biomass within the lungs there is a great risk of contamination by 'outside' bacteria either during the sample collection method or later within the experiment (Dickson, Erb-Downward, Martinez, & Huffnagle, 2016). From samples extracted from the lungs, there has been an estimated range of between 4.5 to 8.25 log copies per/ml of bacteria which equates to 1-10 bacterial cells to every 10 human cells (Charlson *et al*., 2012). This is tiny in comparison to the colon (large intestine) which has log 11 of typical concentration of bacteria (number/mL content) (Sender, Fuchs, & Milo, 2016). Due to the lack of reliability in 16S ribosomal RNA testing, full genome sequencing is often implemented either instead or alongside this as a method of bacterial detection.

Despite the recent publication of the porcine genome, there is still a lack of data surrounding the airway histology and is often limited to veterinary publications (Judge, Hughes, Egan, Maguire, Molloy, & O'Dea, 2014). The problem with a limited understanding of the porcine respiratory histology is the inability to make comparisons between shared structures with humans and the incapacity to also identify the differences that may occur which influence immune responses.

During the investigation into the pathophysiology of the extracted porcine lung tissue, most of the sections were treated with Haematoxylin and Eosin (H&E) which is a common staining method, and as such it is a well-researched and understood method (Titford, & Bowman, 2012; Bancroft, & Layton, 2013; Alturkistani, Tashkandi, & Mohammedsaleh, 2016). H&E stain can identify several features of tissue by the colours identified since the cytoplasm and the extracellular matrix is stained in varying shades of red whereas the nuclei are blue (Dettmeyer, 2011). The common features of lung tissue include the bronchi, bronchioles, and the pulmonary alveoli, which enable gas exchange (Zhou, & Moore, 2017).

The appropriate analysis is to calculate the percentage of cellular staining on the slide images. This is due to the difficulties in differentiating the cellular components after spinning. The velocity can alter morphology and create multiple layers of cells on the same section of slide which may lead to misconceiving results. However, these darker layers of colours may be simply a repeated overlay of stained cells so that they appear darker. This is a limitation of this method of cellular detection.

The wax seen on the slides is suggestive that the oven stage when staining the tissue is perhaps not long enough as the wax has not been fully melted. This is interesting as no such result was seen in the H&E staining and was not seen across all Giemsa-stained sections. Whilst using the GIMP imaging software is an objective and quantifiable method of identifying the percentage of stain coverage on the slide there are some limitations. The software struggled to identify the extremely light blue sections of stains as demonstrated within section '2.4.1 BAL Cytospin'. This may mean that the percentages calculated are perhaps lower than actual value. To overcome this limitation sourcing a more sensitive softer that can detect even minute colour changes would be optimal.

Overall, the H&E staining and analysis allows the health of the lung tissue to be analysed. They create a great base for future research to reference since there is very little in the way of research on porcine histology. Latter research could involve studying the changes in the structure of the tissue after inoculation with the VAP strain. By having a base for the tissue structure, it could also be used as an indicator of what quantity of infection must be reached before pathology occurs. Of which also the bacterial cytokines and inflammatory signals could also be discovered. Since the BAL was effective there is also the potential to study the effects of aerosol antibiotics could be investigated since there are many current cautions associated with this form of therapy.

There are cases where aerosolized colistin causes direct lung damage leading to life-threatening side effects if not administered correctly (Zarogoulidis et at., 2013a). There are also restrictions regarding the size of the droplet as it must not surpass 5µm (Zarogoulidis *et al*., 2013a), which is made increasingly difficult due to the respiratory environment of high humidity, concentration of salts and chemical compounds, the droplets tend to increase by ¼ to ½ the size of the original (Labiris & Dolovich, 2003). However, the data suggests that aerosol therapy can be successfully administered but the dose should be regularly changed, and careful monitoring of the applicable laboratory values is essential (Zarogoulidis *et al*., 2011). Therefore, further research by using this model would be relevant for clinicians to avoid causing damage to the patient.

M. hyopneumoniae is not an uncommon disease with a prevalence range between approximately 20-80% (Fablet *et al*., 2012). Delayed presentation alongside other respiratory

pathogens has been linked to high levels of morbidity and significant economic losses due to a reduced biomass (Ruiz, Utrera, & Pijoan, 2003). The main transmission pathways are vertical from sow to piglet and then is spread via proximity, especially in indoor pens (Fano, Pijoan, Dee, & Deen, 2007).

Despite the presence of some slight discoloration, the overall assessment of the lung is that it is healthy as the discoloration spans less than 10% of the total area of tissue and was avoided when dissecting sections of alveolar tissue. The areas of discoloured tissue were avoided within this study and were not used for any experimentation.

Whilst this was not an aim of the study to explore it is an interesting avenue that could be researched more extensively in another study. When relating it back to patient studies it is important to note that a large cohort of patients will have had some sort of pulmonary event, the NHS quotes that one in every five people have had a form of respiratory disease, be it a viral infection, asthma, COPD, and other instances whereby the lungs have bene influenced by an external source (NHS, 2020). More topically this could also include COVID-19 since a recorded 1.57 million cases have been reported amongst the British population (NHS, 2020). The research could therefore be focused on how these pre-existing conditions or exposure affect the development and severity of VAP. The final phase of this experiment was to generate a growth curve of inoculated porcine lung tissue with a VAP clinical isolate strain obtained from Royal Liverpool Hospital. The previous work of determining the best method for killing lung tissue was applied and therefore since the structural capacity of the tissue was not necessary heat was used. The growth curve is used to be able to simulate VAP in the lung tissue and measure the response between the host microbe interactions.

Within section ‘3.2.5 Proliferation of bacteria on ex vivo porcine lung tissue’ the heat killed tissue has a far lower bacterial viable count than that of the heat killed and inoculated tissue. This is likely due to the heating element causing tissue death and thus providing an inhospitable environment to the normal host flora, however, anaerobes which are found within the lungs are often associated with pathogenesis in pulmonary infections (Bartlett, 2012). The bacterium growing on both types of tissue is surviving anaerobically and off dead tissue/nutrients available. This ideology is supported within the research which claims that

‘anaerobes multiply well in dead tissue’ (Bowler, Duerden, & Armstrong, 2001). Since the lungs are generally considered an aerobic environment, this finding is also interesting as

When the live and live with the inoculum tissue samples were compared the non-inoculated tissue bacterial growth is far lower than that of the inoculated, however, there is still a considerable portion of growth, with a CFU of 10^9. Harrison et al (2012) published that their inoculated tissue had a mean CFU of 4 x10^9, which suggests that a CFU of 10^9 is relatively minimal and supports a successful inoculation practise within this experiment.

This measurement of growth on live but not inoculated tissue is likely caused due to the normal flora which resides on the lung tissue. Selective media was not used since it was a poly microbial isolate and therefore that would have skewed the reports. The drop of said bacterium counts after the 24hr time point may be due to the death of the tissue thus removing the normal host bacterium’s environment and leading to colony death. This is supported earlier within this report when the findings presented that the live tissue was not significantly different to that of heat killed tissue after 18hrs. This tissue death will cause a reduction in viable host flora since the majority will not be adapted to survive on the new conditions created by tissue and cell death.

A growth curve can be used as a platform to investigate the effect of antibiotics, probiotics and various other VAP prevention strategies. For example, within Salford there has been extensive testing on what types of antibiotics the VAP isolate is resistant to. Those that it is susceptible to could be added to the medium and then the bacterial growth analysed. The tissue could also be sectioned, and any differences caused could be noted. This is especially useful when testing novel or alternate treatment pathways due to the similarity of the porcine immune system to humans. Thus, allowing safe ways of testing before reaching clinical trials.

Overall, the conclusions from the curves generated is that inoculated live tissue had a far greater bacterial load than dead tissue. Live inoculated tissue had a greater bacterial load than non-inoculated tissue. To take this research further the bacterium causing the growth could be isolated and sequenced and then compared to the bacterium within the VAP isolate.

Through these findings the importance of the host is highlighted. Whilst on both types of tissue, dead and live, the inoculated sample had a greater density of bacterial growth it is of note that the heat killed tissue had a far higher colony count. Since the conditions and isolate added was the same the only difference was that one section was live, this highlights the potential 'fight' the tissue has with the clinical isolate. In fact, several of the most common microbes found within the lung microbiome of humans, such as *Proteobacteria, Bacteroidetes* and *Firmicutes* (Huffnagle, Dickson & Lukacs, 2017) are in fact thermophiles and would have survived the heat killing procedure (Selvarajan, Sibanda, & Tekere, 2017) suggesting that the tissue itself has been killed/denatured by the heat but the microbes may have survived.

The presence of thermophiles and their presence on the porcine lung tissue would need to be investigated further through sequencing and further histology to record the differences between the live and dead tissue. There could also be another stem of research into the *P. aeruginosa virulence* factors to establish if they are the same products secreted that would be inspected in the lung environment, which would be an interesting avenue to pursue. But overall, this study has provided a good benchmark for this to be investigated further. This is of clinical relevance since patients are alive when they contract VAP and therefore the live tissue response is more synonymous to what they experience clinically.

Through the research and data collected to collate this report the overall conclusions are that VAP has a variety of pathogenic causes and the need for a rapid diagnostic test is vital to reduce excessive antibiotic use and avoid MDR. When using the porcine lung model as an *ex vivo* investigation into the host microbe interactions with VAP more research needs to be done on healthy and diseased lung tissue histology to act as a comparison to any experimental designs. Despite that, the porcine model is an excellent model to study VAP.

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