An investigation of the effects of an antimicrobial peptide on the survival of *Acanthamoeba* and intracellular bacteria associated with Cystic Fibrosis

---

Bauyrzhan Tashmukhambetov

A thesis submitted for the degree of Doctorate of Philosophy

in Biological Sciences

School of Biological Sciences

University of Essex

October 2016

Abstract

Pseudomonas aeruginosa, Staphylococcus aureus and *Haemophilus influenzae* are the most notorious pathogens associated with pulmonary infections in Cystic Fibrosis (CF) lung. Treatment is problematic and re-infection is relatively common. It is vital to develop alternative therapies. Antimicrobial peptides are predominant candidates for combatting pathogenic bacteria. Free Living Amoebae is widely distributed in the environment and may also contaminate CF lung. It is well known that *Acanthamoeba* feed on and protect bacteria. The role of *Acanthamoeba* in CF lung infections has not yet been investigated. It is possible that the presence of *Acanthamoeba* in CF lung is one of the key factors for such re-infections.

In this study, antimicrobial peptide, magainin II, was tested against clinical bacterial strains isolated from CF patients and *Acanthamoeba*. Magainin II has been shown to have antimicrobial efficacy on microorganisms employed in this study.

P. aeruginosa, Escerichia coli, methicillin-sensitive *Staphylococcus aureus*, *Staphylococcus epidermidis* from CF lung showed ability to bind with, invade, survive and multiply inside *Acanthamoeba*. However, other two clinical isolates *H. influenzae* and *Streptococcus pneumoniae* were able to bind. They were found to have capsules that enables bacteria remain un-phagocytized by predators. In nature, bacteria living together with *Acanthamoeba* compete for nutrition and space. They develop strategies to combat each other. Bacterial conditioned media were tested for amoebicidal and amoebistatic abilities. *P. aeruginosa, Str. pneumoniae* and *H. influenzae* conditioned media demonstrated amoebistatic and amoebicidal effects. *Acanthamoeba* conditioned media exhibited bactericidal efficacy only against *Str. pneumoniae*. Magainin II in combination with silver nitrate or vancomycin/ciprofloxacin was successful in eradicating bacteria. It was essential to perform experiments in environment similar to CF lung. For this purpose artificial sputum medium was prepared. All bacteria and *Acanthamoeba* were able to grow in it. Results revealed sub-lethal concentrations of magainin II inhibited bacterial biofilm formation in artificial medium.

Acknowledgment

I would like to express my sincere appreciation to my Principal Supervisor, Dr. Selwa Alsam and Co-supervisor, Prof. Dr. Graham Mitchell for their constant guidance and encouragement, without which this work would not have been possible. For their unwavering support, I am truly grateful. My deep gratitude goes to the Ministry of Education of Kazakhstan Republic for awarding me a full scholarship to complete my postgraduate studies.

Many thanks go to my committee member, Dr. Brandon Reeder for his support and inspiring suggestions during the board meetings.

I would also like to express my gratitudes to Dr. Matthew Hannah, Lead Electron Microscopist in the Virus Reference Department of National Infection Service, Public Health England, London for supporting me in getting Transmission Electron Microscopy images.

I am very grateful to my previous and current lab colleagues: Dr. Mihaela Cardas, Dr. Osamah Al-Rugaie, Dr. Saleh Al-Ghamdi, Mrs. Kully Sidhu, Dr. Aiden Matthew Jayanth, Miss Eniola Olaleye, Mr. Alaa Qumsani, and Miss Amna Alotaiba; and lab technicians: Miss Lynwen James, Mrs. Julie Arvidson, and Mrs. Tania Cresswell-Maynard, for their enthusiastic support and help. I would express my best wishes to our postgraduate research administrator, Mrs. Emma Revill for her assistance, help and support. You all made my stay and studies in Colchester more enjoyable.

I would like to express my appreciation and thank my wife, Moldir Sakbayeva, and my dear daughters, Kaussar and Assem, for their constant love and continuous encouragement and patience. My deepest thanks to my mom, brothers, sister, nephews and nieces for their continues love, support, encouragements with their best wishes.

Table of Contents

List of Figures

List of Tables

Abbreviations

A. castellanii	*Acanthamoeba castellanii*
ABC	Adenosine triphosphate binding cassette
ACM	*Acanthamoeba* conditioned medium
ADP	Adenosine diphosphate
AGE	*Acanthamoeba* granulomatous encephalitis
AK	*Acanthamoeba* keratitis
AMP	Antimicrobial peptide
ASL	Airway surface liquid
ASM	Artificial sputum medium
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AZLI	Aztreonam for inhalation solution
BBB	Blood brain barrier
BCM	Bacterial conditioned medium
BHI	Brain heart infusion
CAP	Covalently attached protein
CD	Circular dichroism
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CLED	Cystine lactose electrolyte deficient
CM	Conditioned medium
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Cranial computed tomography
CV	Crystal Violet
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethyl sulphoxids
DPI	Dry powder inhaler

E. coli	*Escherichia coli*
ECM	Extracellular matrix
EDTA	Ethylenediaminetraacetic
EPS	Extracellular polymeric substance
ESBLs	Extended-spectrum β-lactamases
FBS	Fetal bovine serum
FCS	Foetal calf serum
FE	Fildes extract
FEV	Forced expiratory volume
FIC	Fractional Inhibitory Concentration
FITC	Fluorescein isothiocyanate
H. influenzae	*Haemophilus influenzae*
HBD3	Human-β defensin 3
HNP1	Human neutrophil peptide 1
HS	Hypertonic saline
HTS	High throughput screening
IFN-γ	Interferon-gamma
KGM	Keratinocyte Growth Medium
LB	Luria-Bertani
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MBP	Mannose binding protein
MCC	Muco-ciliary clearance
MIC	Minimum inhibitory concentration
MRI	Magnetic resonance imaging
MRSA	Methicillin-resistant *S. aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
NAC	N-acetyl-L-cysteine
NEAA	Non-essential amino acids
NHS	National health service

NNA	Non-nutrient agar
OD	Optical density
P. aeruginosa	*Pseudomonas aeruginosa*
PAS	Page's amoeba saline
PBP	Penicillin-binding protein
PBS	Phosphate-buffer saline
PC	Phosphatidyl-cholines
PCR	Polymerase chain reaction
PE	Pulmonary exacerbations
PhE	Phosphatidyl-ethanolamines
PFA	Paraformaldehyde
PG	Phosphatidyl-glycerol
PGS	Peptide glycine serine
PMAP	Porcine myeloid antibacterial peptide
POPC	Palmitoyl-oleoyl-phosphatidyl-choline
POPE	Palmitoyl-oleoyl-phosphatidyl-ethanolamine
POPG	Palmitoyl-oleoyl-phosphatidyl-glycerol
PTCs	Premature termination codons
PYG	Proteose peptone-yeast extract-glucose medium
QS	Quorum sensing system
RB	Rose Bengal
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
S. epidermidis	*Staphylococcus epidermidis*
SDS	Sodium dodecyl sulphate
spp	Species
Str. pneumoniae	*Streptococcus pneumoniae*
TNF-α	Tumour necrosis factor
WHO	World Health Organization

Chapter 1

Introduction

1.1 Cystic Fibrosis

CF is a disorder with an autosomal recessive pattern of inheritance with complications in multiple organs. According to the World Health Organization (WHO) there are about 70 000 people with cystic fibrosis (CF) worldwide, with incidence fluctuating by location and ethnic background. It is most common amongst people of Northern European origin and therefore its occurrence is highest in Europe, North America, and Australia (~1/3000 births). The occurrence is considerably lower in the Middle East (Bahrain 1/6000), South America (Brazil 1/7000), Africa (South Africa 1/12 000), and Asia (Japan 1/350 000) (Macneill, 2015).

Mutations in the gene, which code for the cystic fibrosis transmembrane conductance regulator (CFTR) protein result in clinical manifestations of the disorder. The CFTR is a c-AMP controlled anion channel expressed on the apical surface of epithelial cells that line the airways, pancreatic ducts, and other tissues (Bear *et al.*, 1992). In situations where its activity is reduced or is absent, bicarbonate and chloride passage are both reduced (Rich *et al.*, 1990), which leads to thick, mucoid secretions, impediment, and eventually organ death (Rowe *et al.*, 2005) (Figure 1.1).

So far, medical treatments for CF are solely aimed at organ specific complication of the disease. In the airways, inhaled dornase alfa (Fuchs *et al.*, 1994), hypertonic saline (Elkins *et al.*, 2006), and mannitol (Bilton *et al.*, 2013) are applied to improve airway mucociliary clearance; inhaled antibiotics are utilized to supress bacterial infection (McCoy *et al.*, 2008); and oral macrolide antibiotics and high doses of ibuprofen are employed to control inflammation (Saiman *et al.*, 2010). Recent novel findings in advanced technologies have facilitated high throughput screening (HTS) methods to drug discovery. And these in turn have produced orally bioavailable tiny chemical compounds that are able to target the underlying defects in CF (Saiman *et al.,* 2010).

Approximately 2000 mutations have been found in the *CFTR* gene since its discovery in 1989 (Kerem *et al.*, 1989), and nearly 242 mutations have presently been identified to cause CF. Earlier, CF patients were genotyped only to verify the diagnosis or to forecast disease difficulty (McKone *et al.*, 2003), but with the latest authorization of mutation exclusive treatments, genotypic data are considered fundamental (Ramsey *et al.*, 2011). To design novel therapies, an investigation of CFTR structure and function and how the diverse mutational classes result in ion channel malfunction is essential.

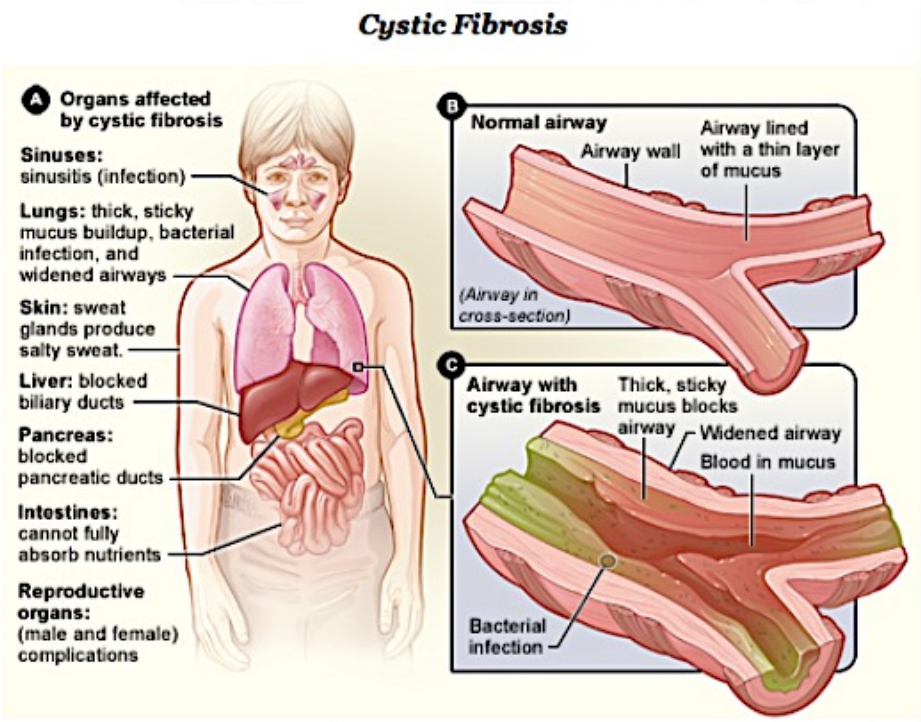


Figure 1.1 A) The organs affected by CF, with short descriptions of the difficulties related to it. B) Healthy airway showing open passages. C) A CF airway illustrating the accumulation of mucus, inflammation, and bacterial infection that will result in further difficulties (adapted from Mcdaniel *et al.*, 2015).

1.1.1 CFTR physiology in healthy people

CFTR is a representative of the adenosine triphosphate binding cassette (ABC) family of transporter proteins, categorized by two membrane spanning domains that construct the channel pore and two nucleotide binding domains that bind and hydrolyze ATP (Riordan *et al.*, 1989). In contrast to other ABC transporters, CFTR has an additional regulatory domain which regulates channel opening and locking (Riordan, 2008). Phosphorylation of the monitoring domain by protein kinase A, continued by binding of ATP and its hydrolysis by the nucleotide binding domains (Hwang & Sheppard, 2009) results in dimerization of the nucleotide binding domains and compartmental realignment of the membrane spanning domains to enable opening of the CFTR channel pore (Kanelis *et al.*, 2010).

The principal role of the CFTR protein is to transfer anions (such as chloride and bicarbonate) via the apical membrane of epithelial cells, thus creating an osmotic gradient for fluid discharge (Quinton, 1990). Moreover, the CFTR has an absorptive function in particular epithelial structures, such as the sweat gland. Deficiency or dysfunction of the CFTR lead to dehydrated, thickened secretions that block epithelium lined ducts causing tissue damage.

In the airways of CF patients, abridged airway surface liquid (ASL) volume triggers impaired mucociliary clearance and hindrance of airways with thick secretions (Cantin *et al.*, 2015). Inherent anomalies of CF mucus that intensify its gluiness and adhesion to the epithelial surface also impede the lungs and other organs. This builds a malicious cycle of mucus retention, infection, and inflammation, which additionally extends the airway damage. Furthermore, besides damage to mucociliary clearance, there is solid belief that CFTR dysfunction itself results in innate and adaptive immune deficiencies that lead to compromised bacterial clearance and uncontrollable inflammation (Birket *et al.*, 2014).

1.1.2 Classification of *CFTR* mutations

CFTR mutations can be largely classified into six categories according to the mechanisms that cause aberrant CFTR synthesis or function (Haardt *et al.*, 1999). Class I mutations include in-frame UAA, UAG, or UGA stop codons in the mRNA preceding the built-in stop codon at the 3′ end of mRNA, and are therefore suggested as premature termination codons (PTCs). The transcribed mRNA is condensed and unstable resulting in lack of protein manufacture (Mendell & Dietz, 2001). This category also comprises other

main insertions and deletions that disturb natural translation.

Class II mutations produce anomalous CFTR processing or trafficking, which lead to lower quantities of CFTR at the cell surface. For instance, the F508del-*CFTR* mutation is a three base pair deletion, which results in an amino acid deletion with consequent mis-folding of the CFTR protein. The mis-folded protein fails to transport to the cell surface because of premature degradation by the proteasome (Welch, 2004).

Class III mutations are regularly described as "gating" mutations because they result in chaotic activation of the CFTR channel. Class IV mutations display normal gating, but alterations in conductivity of the channel pore trigger irregular chloride permeability.

Class V mutations are positioned within promoter or splice sites in the gene; they lead to scarcer CFTR transcripts and abridged protein manufacture.

Class VI mutations are the latest to be discovered category and lead to lower stability of CFTR at the cell surface, resulting in increased turnover (Haardt *et al.*, 1999). Numerous mutations demonstrate features of more than one category. For instance, though the primary malfunction of F508del is aberrant cellular processing, it also expresses malfunctioning gating and an abridged surface half life (Dalemans *et al.*, 1991). R117H is generally defined as a partially active conductance alteration, but it similarly reveals partially disordered gating and is located in *cis* with mutations that influence its expression (Castellani *et al.*, 2008).

1.1.3 Disease causing alleles and CFTR activity

Individually a patient's disease phenotype is partially defined by overall CFTR activity, which in turn is defined by the net effect of both of the disease producing alleles on the amount and function of the CFTR. Usually, individuals with two loss of function alleles (classes I-III) have reduced levels of CFTR activity (<10% of normal) and more serious lung disorder and pancreatic deficiency consistent with classic CF. By contrast, those with at least one residual functional allele (classes II-VI) are likely to have residual CFTR activity (>10% of normal) and moderate lung disease and pancreatic sufficiency consistent with non-classic or moderate CF (McKone *et al.*, 2003). Furthermore, considerable phenotypic variation occurs even for patients with the identical combination of *CFTR* mutations due to environmental impacts and additional genetic variation that contributes to the ultimate phenotype (Kerem *et al.*, 1990).

1.1.4 Bacteria associated with CF

Malfunctioning muco-ciliary clearance in the lung makes CF patients vulnerable to opportunistic infections (De Soyza *et al.*, 2013b). With the introduction of upgraded culture methods and culture-independent techniques, comprising deep sequencing technology, it is obvious that the airways of patients with CF are chronically colonized with complex, poly-microbial infections. In 2003, Rogers and colleagues transformed the understanding of CF lung infections through their identification of complex bacterial communities in sputum and bronchoscopy samples exploiting a culture-independent, molecular-based procedure, terminal restriction fragment length polymorphism profiling (Rogers *et al.*, 2003). These poly-microbial communities were found to be highly individualized to each patient and promote tangled inter-microbial and host—pathogen interactions, which change the lung environment, effect response to treatment, and affect the course of disorder.

1.1.4.1 *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a Gram-positive coccus of about 1 μm diameter. Generally having a role as a commensal organism it inhabits skin, skin glands, and mucous membranes, predominantly in the nose of healthy people (Crossley & Archer, 1997). *S. aureus* is one of the leading agents causing hospital- and community-associated infections which can result in serious consequences (Diekema *et al.,* 2001). In various studies investigating pathogens in CF, *S. aureus* was identified in 4 to 60% of patients commonly in association with other bacteria, such as *P. aeruginosa*. Methicillin-susceptible strains (MSSA) cause a risk in CF sufferers, predominantly because of the production of biofilms in the infected lung in which they can escape from antibiotic therapy (Davies and Bilton, 2009).

1.1.4.2 *Haemophilus influenzae*

Haemophilus influenzae (*H. influenzae*) is a Gram-negative bacterium, playing a major role in causing community-acquired respiratory tract infections in adults and might lead to severe sinusitis and/or severe otitis media (Tristram *et al*., 2007). *H. influenzae* is isolated from the lungs of CF patients and in 2009 research stated that between 1995 and 2005 the prevalence of *H. influenzae* rose in these patients (Razvi *et al.*, 2009). In the last few decades, macrolides have been employed for the treatment of a variety of *H.*

influenzae infections (Phaff *et al.*, 2006). Antibiotics have also become significant in dealing with CF patients and attempts at employing azithromycin in CF patients chronically infected with *P. aeruginosa* have revealed enhancements in lung function (Saiman *et al*., 2003). The employment of long-term azithromycin maintenance treatment in CF patients has been related to increased macrolide resistance in CF *H. influenzae* isolates (Tazumi *et al*., 2009).

1.1.4.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative bacterium, a major opportunistic causative agent in CF patients and induces a variety of infections among other vulnerable people. Its intrinsic resistance to a range of antimicrobial agents makes it problematic to treat infections. Although the latest evidence has emphasized the diversity of this species, the majority of research on virulence and pathogenesis concentrate on a small number of strains (De Soyza *et al*., 2013a). *P. aeruginosa* infects up to 80% of adult CF patients population. Although it is still commonly presumed that the majority of CF patients acquire their infecting strains of *P. aeruginosa* from environmental sources, there is increasing evidence for the emergence of mainly effective transmissible strains (Fothergill *et al.*, 2012), some of which have been related to increased patient morbidity, mortality (Aaron *et al*., 2010) or antimicrobial resistance (Ashish *et al.*, 2012). Infections of the CF lung by *P. aeruginosa* have been related to development of a number of essential bacterial features such as induction of mucoid condition (Govan and Deretic, 1996), hypermutability (Oliver *et al.*, 2000) and alterations in cell surface virulence determinants (Cigana *et al*., 2009). It is vital to note that during chronic infections of the CF lung, *P. aeruginosa* populations are of great diversity. Therefore, there is substantial variability in virulence factor expression or antibiotic resistance between CF isolates of *P. aeruginosa*, even when they are isolated from the same sputum sample and the patient is infected with a single strain (Mowat *et al*., 2011).

1.1.4.4 *Streptococcus pneumoniae*

Streptococcus pneumoniae (*Str. pneumoniae*) is a pathogen of acute infections, many of which may possess strong epidemiological consequences, such as meningitis, otitis media, pneumonia, and sinusitis. The major area of colonization of this agent is the nasopharyngeal epithelium, where it remains as part of the commensal flora. The main

cause of pneumonia is movement of the bacterium towards the lungs through inhalation (Zeiher & Hornick, 1996). The disease-causing role of *Str. pneumoniae* in CF is disputable, and very few records were reported on its possible participation in pulmonary injury (Bauernfeind *et al.*, 1987). Isolation took place mainly in infants and young children. The isolation of *Str. pneumoniae* was related to 5% of the acute-exacerbation incidents (del Campo *et al.*, 2005). Nevertheless, the outcomes found by del Campo *et al*., suggest that CF isolates possess singular features, comprising high antibiotic resistance rates, certain clonal relationships, and predominantly increased rates of mutation frequencies in contrast to non-CF *Str. pneumoniae* isolates defined beforehand from the same institution (Morosini *et al*., 2003). Taking into account the probability that some clones of *Str. pneumoniae* may be implicated in the initial stages of lung inflammation in CF patients, a precautionary therapeutic attitude could eventually be sensible to guarantee eradication, as is recommended for *P. aeruginosa* (Rosenfeld *et al.*, 2003).

1.1.4.5 *Escherichia coli*

Escherichia coli (E. coli) is typically found in animal and human intestines. Despite the fact that *E. coli* generally behaves as an innocuous commensal, certain strains are noticeably pathogenic and have been documented as causes of food-borne enteritis. Almost every part of the human organism can be infected by *E. coli* but the urinary pathway is a commonly infected extra-intestinal location, triggering 85–95% of cases of mild cystitis and pyelonephritis in premenopausal women (Smith *et al.,* 2007). The virulence potential of *E. coli* is due to fimbriae, adhesins, toxins, siderophores, capsules, hemolysins and invasins (Croxen and Finlay, 2010, Smith *et al.,* 2007). The evolution of colonies manufacturing extended-spectrum β-lactamases (ESBLs) has grown into a severe problem for clinicians, severely decreasing the therapeutic choices. ESBLs are capable of neutralizing β-lactamic antibiotics at diverse levels (Paterson and Bonomo, 2005). According to definite antibiograms antibiotic choices have to be personalized, which may vary from one hospital to another, one city to another and one geographical region to another (Pitout & Laupland, 2008). Ultimately carbapenems, including imipenem, meropenem, and ertapenem, have become widely accepted as a primary choice drug class against infections induced by ESBL-producing Enterobacteriaceae (Paterson, 2000).

1.1.4.6 *Staphylococcus epidermidis*

Staphylococci are mutualistic inhabitants of the skin and mucous membranes of human organism (Williams &Wilkins, Baltimore, 1986). *Staphylococcus epidermidis* (*S. epidermidis*) is the major group to inhabit human epithelia, and primarily colonizes the armpits, head and nares (Kloos & Musselwhite, 1975). *S. epidermidis* species possesses genes that are expected to be responsible for defence from undesirable conditions (Gill *et al.*, 2005, Zhang *et al.,* 2003): for instance, to survive in life-threatening salty conditions and osmotic pressure (Gill *et al.*, 2005). *S. epidermidis* is representative of coagulase-negative staphylococci. (Wang *et al*., 2003, Wisplinghoff *et al*., 2003). By means of a recently upgraded scheme (Thomas *et al*., 2007), 74 sequence types of *S. epidermidis* strains were recognised (Miragaia *et al*., 2007). *S. epidermidis* is the major causative agent found on medical equipment, such as peripheral or central intravenous catheters.

1.1.5 CF lung disease treatments

Usual pulmonary treatment of CF has targeted the consequences of the disorder, specifically mucus plugging and infection. The last few years have seen the development of more advanced therapeutic agents, numbers of which are in clinical trials, or have proceeded to approved treatments. Here, pulmonary treatment opportunities are concentrated on, categorized by their type of action, giving information on those in present clinical use, and emphasizing progress in research. Treatments for other organs damaged in CF (for example, the pancreas, liver, bones and sinuses) can be found in the research of Plant and collaborators (Plant *et al.*, 2013).

1.1.5.1 Mucolytic agents

The mucolytic agent, Dornase alfa is a recombinant human deoxyribonuclease (DNase). As contributor for the inflammatory progression in CF there is a substantial buildup and breakdown of neutrophils in the lungs resulting in great quantities of extracellular DNA, which significantly intensifies the gluiness of the sputum. DNase has been shown to cleave the extracellular DNA and consequently assist in airway clearance (Konstan & Ratjen, 2012) .

Another agent, N-acetyl-L-cysteine (NAC) has formerly been considered to be mucolytic in CF and may also raise levels of the intracellular antioxidant glutathione (GSH), thus protecting from the neutrophil-driven tissue destruction in the lungs. Clinical

trials have however produced slightly conflicting results (Dauletbaev *et al.*, 2009), consequently agreement is lacking on the clinical utility of NAC for CF.

1.1.5.2 Airway clearance

One of the mainstays of treatment for CF is physiotherapy, which helps in clearance of airway secretions. Multiple diverse mimicking techniques are also applied, but the optimal approach is not equaled. A recent report found no indication in support of high-frequency chest wall oscillating devices when compared to positive expiratory pressure and essentially found a higher level of pulmonary exacerbations (PEs) demanding antibiotics related to their administration (McIlwaine *et al.*, 2013). Various interventions to endorse health and exercise have been trialed although there is no unanimous conclusion over which technique has been the most efficacious (Cox *et al.*, 2013).

1.1.5.3 Rehydration strategies for airway surface

Hypertonic saline (HS) contributes impaired muco-ciliary clearance (MCC) by raising hydration of the airway surface in the short term. As it might cause bronchoconstriction, it is regularly administered with an associated bronchodilator. When compared with a placebo, HS was nontoxic, economical and efficient in reducing the number of PEs involving intravenous (IV) antibiotics (Donaldson *et al.*, 2006).

Mannitol is a non-absorbable sugar alcohol that provides an osmotic gradient on the airway surface resulting in rehydration, and an increase in surface liquid volume, which helps in the clearance of mucus. An international phase III trial presented a sustained and clinically important advantage even with concomitant DNase utilization in patients 18 years of age and older. Nevertheless it additionally exhibited an increased quantity of adverse events such as hemoptysis and cough. Existing evidence suggests mannitol is nontoxic to use in patients who are capable of tolerating it (Bilton *et al.*, 2011).

Denufosol tetrasodium, a P2Y2 purinergic receptor agonist, induces chloride secretion and ciliary beat frequency independent of CFTR. Phase III study results were primarily promising, exhibiting a small but statistically significant increase in lung function in contrast to the placebo (Accurso *et al.*, 2011).

1.1.5.4 Antibiotics against infections

Antibiotics are administered in four different conditions in CF: prophylaxis, suppression of early infection, eradication of chronic infection and in the treatment of exacerbations. The pathogens isolated in CF lungs differ with patient's age. In infancy the most common bacteria identified is *S. aureus*, with *H. influenzae* increasing during childhood; by adolescence and young adulthood by far the commonest pathogen identified is *P. aeruginosa*. Nevertheless, the introduction of culture-independent molecular tools to identify bacterial strains has shown the poly-microbial nature of the CF lower airway. The association of the micro-biome to patients' health and disease development is currently the focus of numerous research efforts (Rogers *et al.*, 2015). Whilst clinicians will often treat an early bacterial infection with an intention of both handling symptoms and decreasing the likelihood of chronic infection, the organism for which the latter outcome is most strongly supported is *P. aeruginosa* (Langton Hewer & Smyth, 2014). If not identified and treated aggressively, this Gram-negative, opportunistic bacterium will be converted to chronicity; the resulting inflammatory response is strongly related to a decline in lung function. Eradication approaches differ between countries and even amongst sites but involve inhaled +/− systemic antibiotics. In North America, inhaled tobramycin is the first line treatment (Mogayzel *et al.*, 2014), while in Europe, a multicenter trial is presently assessing whether oral antibiotics are better, when used with nebulized colistin (Edmondson & Davies, 2016).

When bacterial infection has become chronic, the focus shifts from eradication to chronic suppression in the expectation of decreasing the inflammatory response. Conventionally-delivered antibiotics carry with them possible side effects and may result in suboptimal sputum concentrations (Waters & Ratjen, 2014). Consequently much research has concentrated on inhalation as a route of antimicrobial delivery, which by distributing the drug directly to the site of infection and in high dosages, can enhance bacterial killing at the same time as limiting side effects (Hewer, 2012). Difficulties involved, in particular cases, are an un-palatable taste, possible bronchospasm, especially with the dry powder formulations, and the time required both to use the drug and care for the equipment, which may influence adherence. The most commonly administered nebulized antibiotics against *P. aeruginosa* are tobramycin, colistin and more recently, aztreonam; in numerous circumstances, a cycling approach is applied, administering on a month-on, month-off basis or alternating drugs.

In an effort to decrease the time desirable to deliver the drug to the airway and the preparation and cleaning essential, dry powder formulations have been developed for both colistin and tobramycin. Colistin (Schuster *et al.*, 2013) and tobramycin (Konstan *et al.*, 2011) were carried using a dry powder inhaler (DPI) and short term results exhibited they were non-inferior to the nebulized forms of tobramycin. There was no indication of boosted adherence, though this is a problematic outcome to measure. It seemed there was an intensification in the reporting of cough in the DPI group (Uttley *et al.*, 2013). Advance research must be carried out to consider the long term consequences of using a DPI rather than the more conventional nebulized medicines (Tappenden *et al.*, 2013). Nevertheless, Harrison and colleagues did show improved adherence to inhaled tobramycin when compared with the nebulized form in adults with CF (Harrison *et al.*, 2014).

Numerous later agents are currently under investigation. A liposomal formulation of amikacin is being trialed as a new once-a-day substitute. An appealing feature of the liposome is its breakdown by bacterial rhamno-lipids, meaning the drug becomes efficiently delivered to the site of demand. In both phase II and III trials similar increases in the forced expiratory volume in one second (FEV_1) were seen as for tobramycin (Ehsan *et al.*, 2014). Levofloxacin inhalation solution (MP-376) has lately been revealed to be well tolerated and non-inferior to tobramycin (Stuart Elborn *et al.*, 2015). Lastly, a double-blind, placebo-controlled trial compared a combination antibiotic fosfomycin/tobramycin with a placebo after a 28-day open-label run-in course of aztreonam and showed maintenance of the substantial improvement in FEV1 in patients after the aztreonam course (Trapnell *et al.*, 2012). This proposes that constant antibiotic treatment using alternating antibiotics may be attractive in future for the treatment of *P. aeruginosa* infection, although discussion remains regarding which combination of antibiotics that should contain.

PEs are signs of symptoms and degenerating lung function; their mechanism is inadequately understood. In childhood, it is common for viruses to be identified in airway secretions at the time of PE and on occasions, a new bacterial organism might be isolated, but much more commonly, neither the type of organism isolated nor its number appear to have altered considerably. This fosters the probability that it is the bacterial behavior, for example the manufacture of virulence factors, or the host response, which has altered. PEs are treated with oral or IV antibiotics varying on severity.

Methicillin-resistant *S. aureus* (MRSA) is on the rise in patients with CF, being

found in up to 25% of patients in the USA. MRSA has been related to deteriorating lung function and higher mortality. A recent research report into the Staphylococcal Cassette Chromosome mec (SCCmec) types in children with CF showed that the SCCmec type isolated in the sputum correlated with the extent of exacerbation and the number of courses of antibiotics administered by each patient. Those chronically infected with SCCmec II MRSA isolates had higher exacerbation frequencies. The authors propose that this points out the question about whether isolated MRSA should be routinely typed to allow for direction of the eradication technique (Heltshe *et al.*, 2015). STAR-TOO is a current, randomized, controlled, intervention research comparing an early suppression protocol for MRSA with the current standard of only treating PEs. The use of AeroVanc, first inhaled antibiotic, Savara USA to reduce MRSA colony-forming units is also being assessed in a phase II randomized, double-blind, placebo-controlled trial. A recent phase III, double blind, placebo-controlled, two-part multisite investigation confronted aztreonam for inhalation solution (AZLI) with a placebo and concluded there was no noteworthy improvement in lung function. The authors hypothesized that non-study antibiotic use may have effected their results (Tullis *et al.*, 2014).

1.1.5.5 Non-antibiotic agents against bacterial infections

Chronic *P. aeruginosa* produces a biofilm in the CF airways. This comprises of the bacterium itself implanted in a complex matrix of neutrophil DNA, exopolysaccharide and airway mucins, which makes it extremely resistant to antibiotic therapy. OligoG, a dry powder formulation of seaweed-derived alginate oligosaccharide, seems to have both antibiofilm and mucolytic characteristics and is presently in phase II trials. A non-significant clinical trial has earlier proposed that IgY derived from immunized hen eggs could be capable of protecting *P. aeruginosa* infection (Kollberg *et al.*, 2003). A multi-center trial is currently investigating the benefits of this antibody directed as a gargle solution.

1.1.5.5.1 Targeting CFTR mutations

Drugs in treatments of CFTR defects could be classified in three groups: potentiators, correctors or read-through agents. Potentiators improve the activity of the CFTR channel if it is properly located. Correctors aim to correct defects such as protein mis-folding in F508del leading to easy trafficking to the cell surface. Read-through agents

stimulate the ribosome to ‘ignore’ a premature termination codon and manufacture a full-length protein. Over the last decade, drugs in all of these groups have proceeded into, and in some cases through, clinical trials (Edmondson & Davies, 2016).

1.2 *Acanthamoeba*

Acanthamoeba is a free-living protist which is widely distributed in the environment, but which is also an opportunistic pathogen. As a human pathogen it can cause skin lesions, sinus infections, keratitis and an infrequent but deadly granulomatous amoebic encephalitis (Marciano-Cabral & Cabral, 2003). Features of *Acanthamoeba* such as being an agent that causes various illnesses, an important role in ecosystems; harbouring endosymbionts; and a typical organism for investigations of unicellular movement have led to notable attention in recent decades (Figure 1.2) (Khan, 2012).

The term *Acanthamoeba* is the combination of two words, acanth (Greek "acanth" means "spikes") and amoeba. It possesses spine-like structures, which are called acanthopodia. The function of contractile vacuoles in *Acanthamoeba* is to secrete excess water to maintain osmotic pressure in the cell (Bowers & Korn, 1973). In addition there are several other types of vacuoles. These are lysosomes, digestive vacuoles and a great quantity of glycogen-containing vacuoles. The constituent parts of the cell plasma membrane are proteins (33%), phospholipids (25%), sterols (13%), and lipophosphonoglycan (29%)(Dearborn & Korn, 1974).

It was shown that colonization in intensive care unit patients' respiratory tracts with *Acanthamoeba* spp does occur. This fact may have relevance for the role of *Acanthamoeba* as a source of bacterial pathogens in CF patients' respiratory tracts (Bradbury *et al.,* 2013).

1.2.1 Classification

After its discovery, *Acanthamoeba* was categorized according to isolator, source or other criteria. Pussard and Pons (1977) divided this genus into three groups according to the characteristics of cysts. So far, according to rRNA gene sequence, 17 diverse types (T1 – T17) of *Acanthamoeba* genus have been found (Corsaro & Venditti, 2010). Last year T18-20 genotypes were identified (Corsaro *et al.*, 2015). Every type sequence displays 5% or more variance. Human infections with *Acanthamoeba* are mainly related to the T4 type. For instance, more than 90% of *Acanthamoeba* keratitis (AK) cases are associated with the same type. Equally, the T4 type has been the main type linked with *Acanthamoeba* granulomatous encephalitis (AGE) and skin infections. Currently, the reason for the T4 type prevailing in human infections is unclear (Khan, 2012).

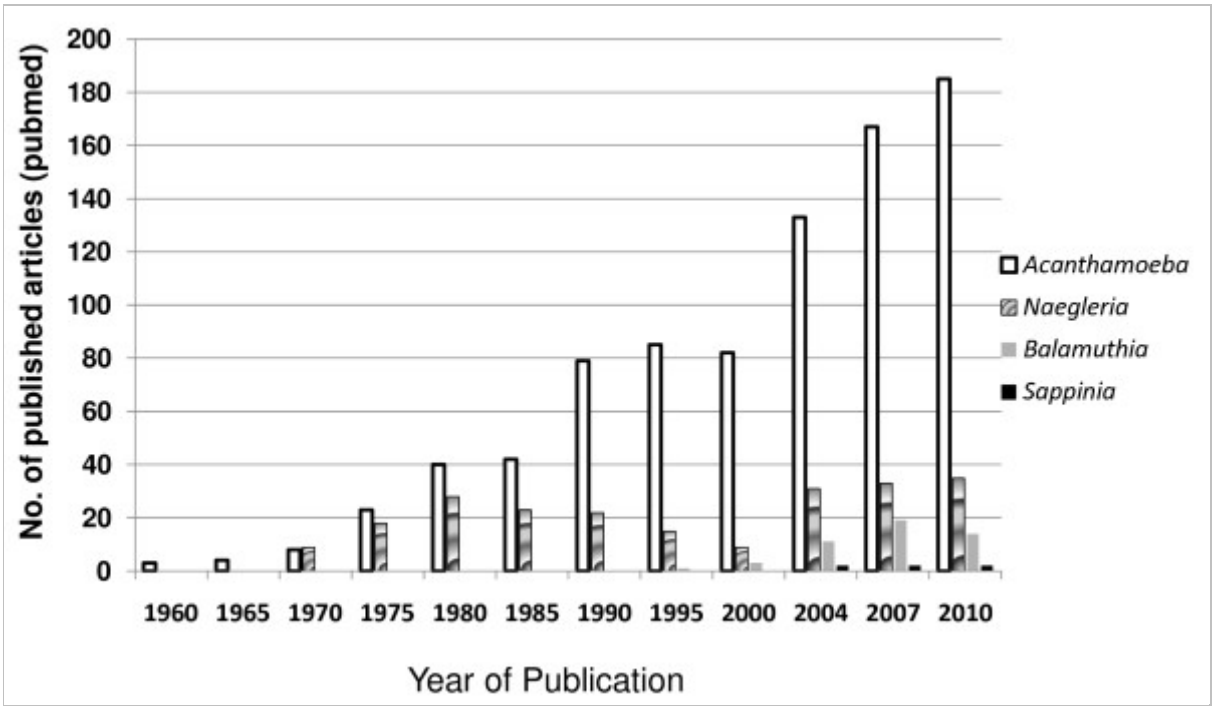


Figure 1.2 Increasing scientific interest in the field of free-living amoebae as determined by published articles over the last five decades (Khan, 2012).

1.2.2 Ecology

Acanthamoeba has been detected in various natural surroundings, products for daily use, hospitals, and as a human parasite (Castellani, 1930). In the environment, *Acanthamoeba* has two main impacts: (i) affecting the organization of the microscopic community, and (ii) intensifying nutrient recycling. Free-living amoebae are the leading bacterial eaters and reduce the overall bacterial population by up to 60% of (Sinclair *et al*., 1981). Free-living amoebae as secondary decomposers devour bacteria, which are primary decomposers and by expelling waste materials enrich the soil with minerals that were tied up in microbes. It was shown that soil containing *Acanthamoeba* and bacteria had considerably superior mineralization of carbon, nitrogen, and phosphorous to soil with bacteria but no *Acanthamoeba* (Ronn *et al*., 2002).

1.2.3 Life cycle

The diameter of *Acanthamoeba* during the trophozoite phase is 13-23 μm and during the cyst phase is 17-33 μm (Figure 1.3). In trophozoite form (Greek "tropho" means "to nourish"), *Acanthamoeba* is active, engulfs organic substances, microscopic organisms and reproduces mitotically under desirable circumstances (food supply, neutral pH, ~30°C) and 50-80mOsmol (Band & Mohrlok, 1973).

During unfavourable conditions *Acanthamoeba* differentiates into a double-walled cyst form (Weisman, 1976). The ectocyst consists of proteins and polysaccharides, and the endocyst comprises cellulose (Dudley *et al.,* 2009). Exocyst and endocyst are typically demarcated by a space, excluding some points where they make opercula in the midpoint of ostioles (exit points for excystment). It has been shown that the cyst wall of *A. castellanii* T4 type is composed of proteins (33%), lipids (4 - 6%), carbohydrates (35%) (mostly cellulose), and unidentified and ash-forming materials (28%) (Hirukawa *et al*., 1998).

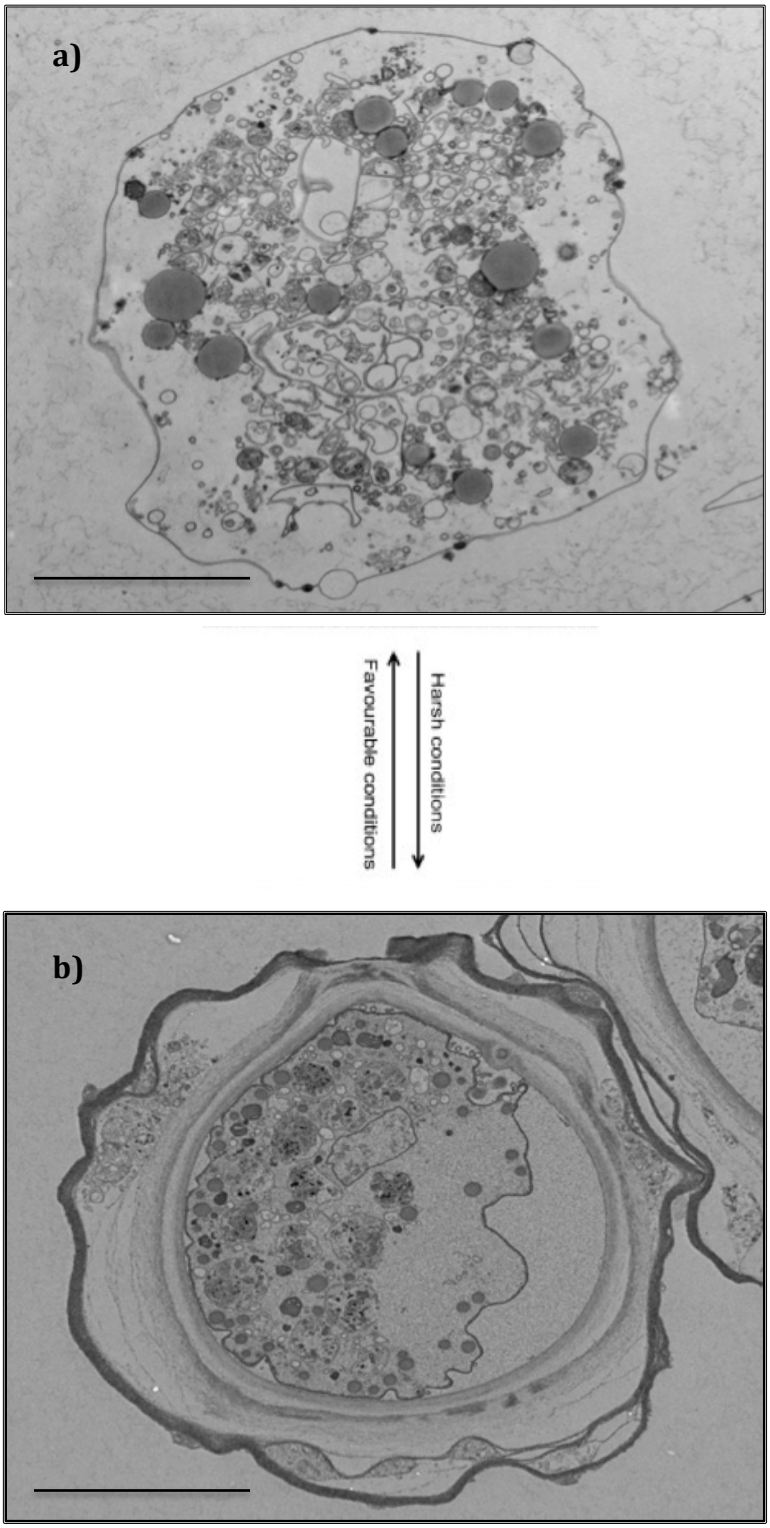


Figure 1.3. The life cycle of *Acanthamoeba* spp. Under favourable conditions, *Acanthamoeba* remains in the trophozoite form and divides mitotically (a) and is infectious, while under harsh conditions amoeba transforms into a dormant cyst form (b) that is highly resistant to harsh conditions scale bar, 2µm. Micrographs were obtained in collaboration with Virus Reference Department of National Infection Service, Public Health England.

1.2.4 Encystment

The differentiation in response to undesirable conditions from vegetative form to resting form is termed encystment (Weisman, 1976). Four morphological steps were identified by scanning electron microscopy during encystment: trophozoite, precyst, undeveloped cysts, and developed cysts (Chavez-Munguia *et al.*, 2013). Encystment can be initiated by a number of circumstances, such as starvation, osmotic stress, and as a reaction to bacteriotoxins (Cordingley *et al*., 1996). Cyst forms are specifically resistant to treatments and therefore are crucial in the survival and distribution of *Acanthamoeba*. Conversion of trophozoites into cysts initiates massive structural alterations. Cysts are spheroidal with a rigid cell wall. However, the structure of cyst walls is different among different genera (Fouque *et al*., 2012).

The inner fibrillar layer of the cyst - the endocyst - is formed after the ectocyst. Both layers comprise at least acid-insoluble proteins (Neff and Neff, 1969) and cellulose (Tomlinson & Jones, 1962) but the exact configuration has not been identified yet. One or more hole-like structures - ostioles exist in the exterior part of the cyst wall. Chavez-Munguia *et al*. (2005) suggested that excystment takes place over these ostioles after breakdown of the opercula enclosing the ostioles (Chavez-Munguia *et al*., 2005). Recently, it has been found that the ectocyst possesses an irregular surface and that there are vesicles inside its structure. The application of electron microscopy has shown that the endocyst is thinner and more fibrillar than the ectocyst and is similar to the conformation of cellulose in plant cell walls (Lemgruber *et al.*, 2010).

1.2.5 Feeding

Acanthamoeba engulfs microbes existing on surfaces, in various surroundings (Brown & Barker, 1999, Alsam *et al.,* 2006). Acanthopodia serve to seize nutrient materials, which generally are bacteria (Weekers *et al.,* 1993), and in a minority of cases algae, yeasts (Allen & Dawidowicz, 1990) and other protists. *Acanthamoeba* feeding is based on phagocytosis, which is a receptor-dependent phenomenon, and on pinocytosis, a nonspecific process, through membrane invaginations (Bowers & Olszewski, 1972, Alsam *et al.*, 2006, Cardas *et al.*, 2012). This complicated process may be associated both with endocytosis and pathogenesis of *Acanthamoeba* (Khan, 2006). Some bacteria can survive and even multiply after *Acanthamoeba* phagocytosis. This can be followed by bacterial re-

infections occurrence. Therefore treating *Acanthamoeba* is essential to stop re-infection processes.

1.2.6 Pathogenesis and Virulence factors

The factors determining the pathogenicity of *Acanthamoeba* can be categorized as contact dependent and contact-independent (Martinez, 1991). Contact dependent pathways include binding to the host cell (Alsam *et al.*, 2003), and phagocytosis performed by acanthopodia which interact with microbes via calcium pores and cytoskeletal particles (Taylor *et al*., 1995). Contact-independent pathways comprise synthesis of extracellular protease as well as phospholipases. Indirect factors facilitating interaction comprise adequate structure, thermal conditions, proper osmotic medium, no immunological resistance, and biofilm formation (Siddiqui & Khan, 2012).

1.2.7 *Acanthamoeba* Infections

There are different entry routes for *Acanthamoeba* to cause this disease including lower respiratory tract, olfactory neuroepithelium, and skin lesions (Figure 1.4) (Garajová & Mrva, 2011). *Acanthamoeba* induces two well-recognized disorders in human health: an eye associated painful keratitis that may cause loss of sight (Khan, 2006) and an infrequent *Acanthamoeba* granulomatous encephalitis concerning the central nervous system (CNS) that is associated with immune-compromised patients that practically at all times results in a fatal outcome. In contrast to AGE, keratitis can arise among healthy people (Clarke *et al*., 2012).

By restriction fragment length polymorphism analysis or total sequence analysis of 18s rDNA revealed that practically majority of clinical isolates of *Acanthamoeba* belonged to the genotype T4. A great quantity of environmental isolates from contact lens storage cases, tapped water, and ocean sediments also belonged to the genotype T4 (Kong, 2009). So, it is essential to look for T4 strain presence in the lower respiratory tract and analyse their interaction with bacteria associated with CF.

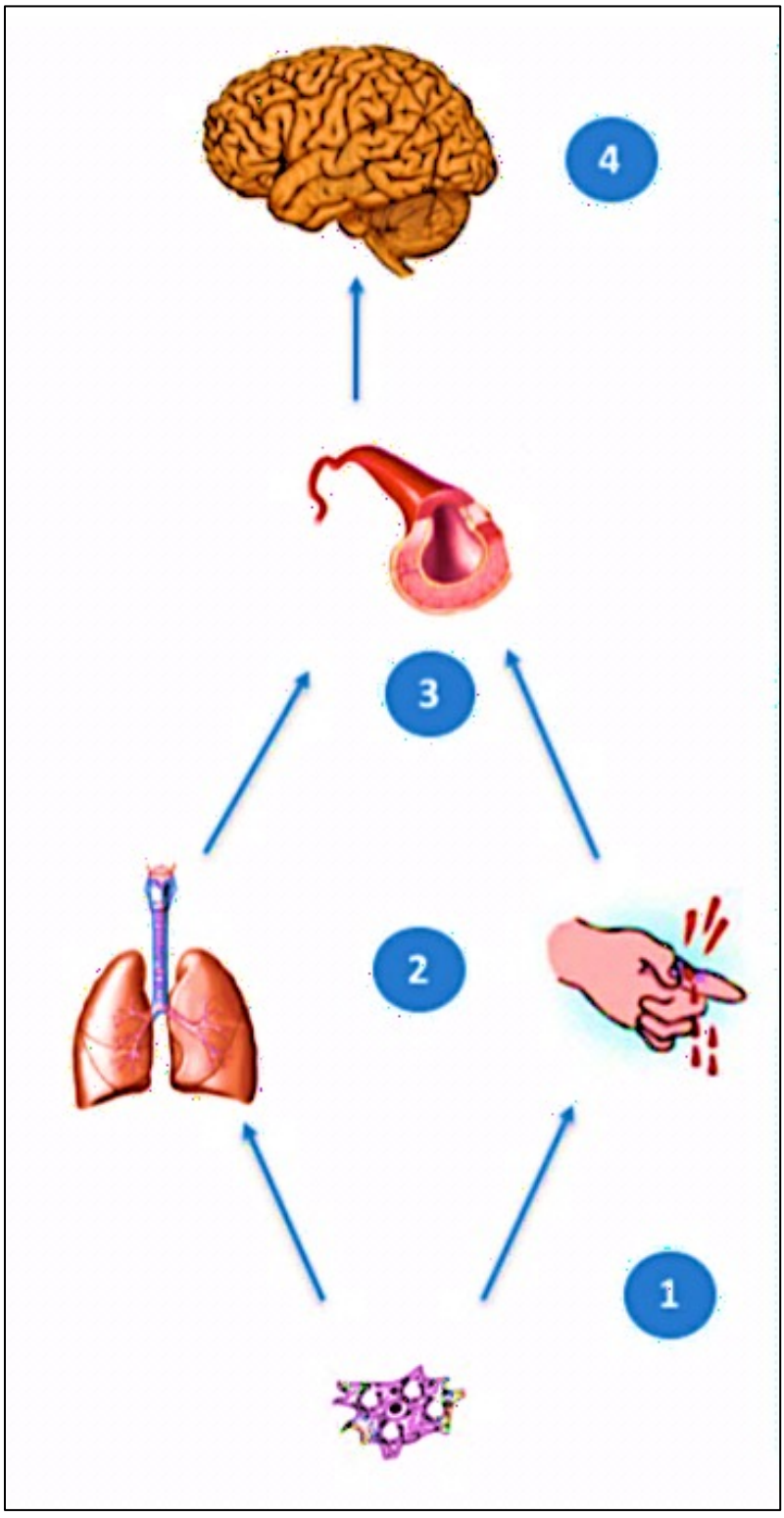


Figure 1.4 The haematogenous routes of *Acanthamoeba* (a) entry into the CNS. *Acanthamoeba* may pass through the respiratory tract or through skin injury (b) then via bloodstream (3) to blood brain barrier (4) (Khan, 2009).

1.2.7.1 *Acanthamoeba* Keratitis

Generally keratitis is identified in patients who are contact lens wearers (Figure 1.5). The reasons leading to this disorder include: usage of contact lenses for long periods, poor personal hygiene, improper cleaning of contact lenses, biofilm development on contact lenses, and exposure to infected water (Khan, 2006). Reaching the cornea *Acanthamoeba* causes failure of the epithelial barrier, invades the stroma, drains corneal keratocyte cells, stimulates an extreme inflammatory reaction, causes severe sensitivity to light and as a final point the death of stromal tissue with loss of sight (Thebpatiphat *et al.,* 2007, Panjwani, 2010).

1.2.7.1.1 Pathophysiology and treatment

Acanthamoeba keratitis requires several days to weeks to be treated, but it varies on the route of amoeba's access. The emergence of infection caused by contact lenses is very slow while, in the occasion of corneal trauma, it is fast. The foremost symptoms of AK are characterized by photophobia, pain, epithelial defects, and inflammation with redness and tearing that result in epitheliopathy, perineural infiltrates, ring infiltrates, ulceration, abscess formation, hypopyon, scleritis, glaucoma, cataract, corneal perforation and posterior segment inflammation (Alsam *et al.*, 2008; Lorenzo-Morales *et al.*, 2015). Several drugs can be employed against AK comprising propamidine isothionate, dibromopropamidine isothionate, neomycin, paromomycin, clotrimazole, ketoconazole, miconazole, and itraconazole. Debridement or penetrating keratoplasty processed at an initial period can be effectively applied in treating the damaged site (Horne *et al.*, 1994; Illingworth *et al.*, 1995; Dudley *et al.*, 2007).

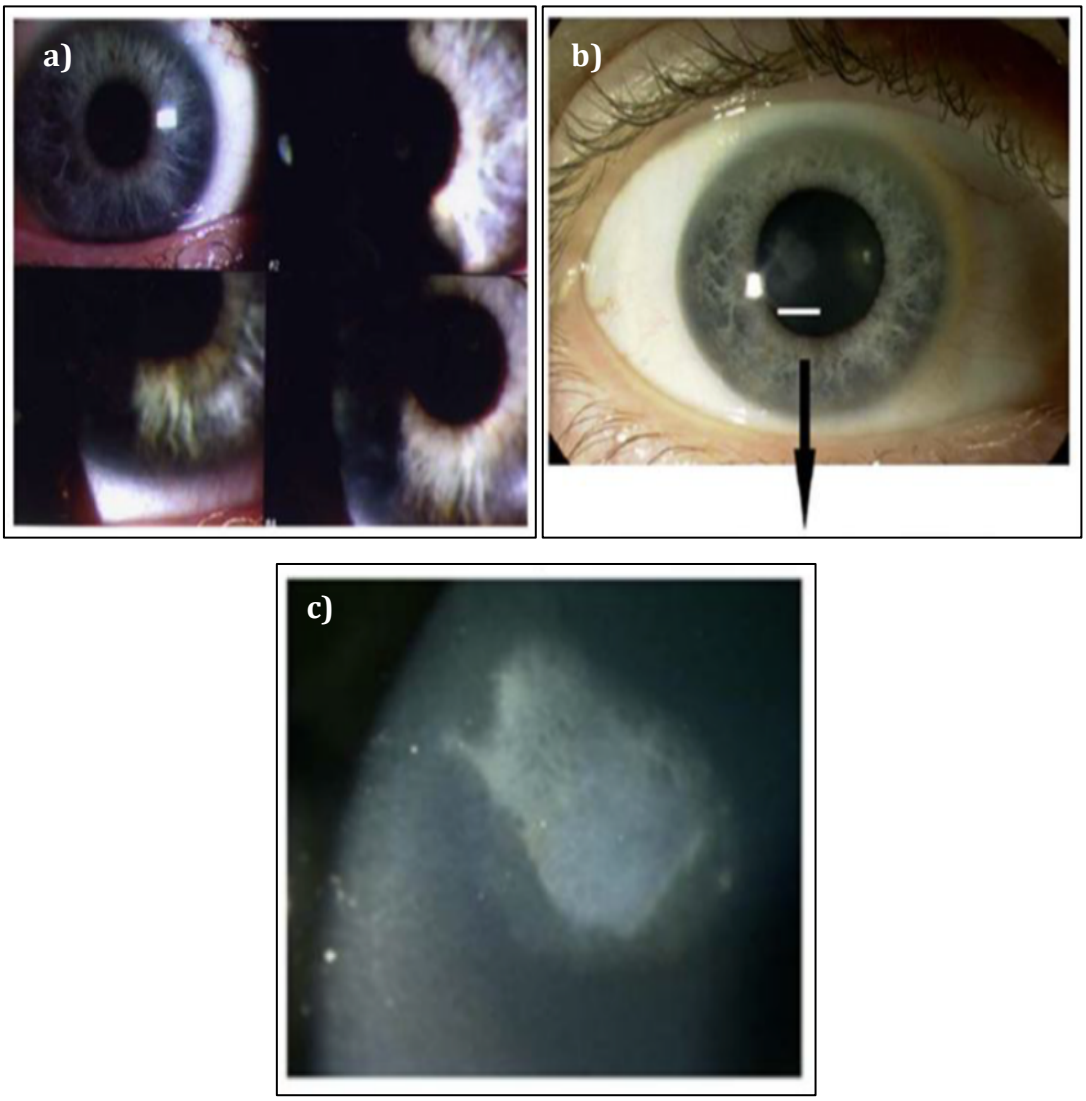


Figure 1.5 Normal eye and ulcerated epithelium and corneal opacity in *Acanthamoeba* keratitis (a). Improvement of *Acanthamoeba* keratitis after 6 months' treatment with polyhexamethylene biguanide, propamidine-isethionate and neomycin (b,c). (Alsam *et al.*, 2008).

1.2.7.2 *Acanthamoeba* granulomatous encephalitis

Normally patients receiving immunosuppressive therapy or over-usage of steroids are likely to be infected by *Acanthamoeba*. It is generally believed that the path of infection starts in the respiratory tract with cyst inspiration, leading to amoebae penetrating alveolar blood vessels, and continuing by haematogenous dissemination (Figure 1.6). It is proposed that *Acanthamoeba* passes into the CNS through the blood-brain barrier (BBB) (Martinez & Visvesvara, 1997).

1.2.7.2.1 Pathophysiology and treatment

Known manifestations of AGE comprise confusion, stiff neck, headache, lethargy, ataxia, vomiting, aphasia, nausea, seizures, cranial nerve palsies, increased intracranial pressure, coma and fatal end (Bloch & Schuster, 2005). It is problematic to diagnose AGE, as of the resemblances in its symptoms with viral encephalitis and bacterial meningitis. Though, scans by magnetic resonance imaging (MRI) or cranial computed tomography (CT) may aid in analyses and could reveal contrast enhancement sites demonstrating brain abscess or tumors (Miltner & Bermudez, 2000). Other drugs to treat AGE are trimethoprim-sulfamethoxazole, ketokonazole, amphotericin-B, rifampicin, fluconazole, sulfadiazine and albendazole (Kaushal *et al.*, 2008; Zamora *et al.*, 2014).

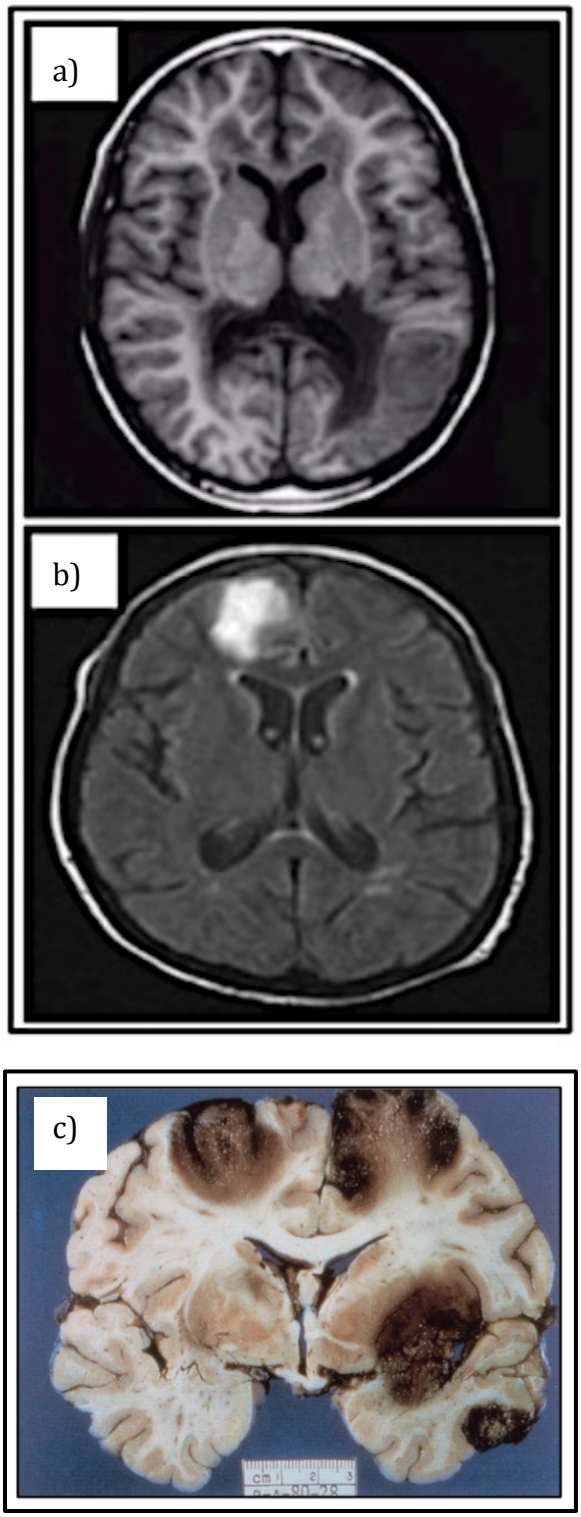


Figure 1.6 Brain cross-section images. (a) Normal brain MRI. (b) MRI of a patient's brain with AGE showing a hemorrhagic lesion in the right front lobe (Meersseman *et al.*, 2007). (c) Coronal section of the cerebral hemispheres with cortical and subcortical necrosis from a fatal human case of AGE (Marciano-Cabral and Cabral, 2003).

1.3 Antimicrobial Peptides (AMPs)

Even though humans historically have evolved to coexist in harmony with diverse microorganisms, this stable mutual relationship can occasionally shift and allow pathogenic bacteria to flourish and trigger infections. In the battle for survival, a complicated mechanism including various vital components aids in the elimination of these infectious agents. AMPs are well-conserved biomolecules among all living species, including bacteria that participate in the struggle against the invading pathogens. They are moderately short (<100 amino acid residues), positively charged, amphipathic (have both hydrophobic and hydrophilic domains) and display diversity in accordance to their structural characteristics (Jenssen *et al.*, 2006). Despite the structural variance and myriad of sequences integrating both natural and unnatural amino acids, AMPs demonstrate broad-spectrum antibacterial potencies. The potency of AMPs to eradicate or inhibit the growth of bacteria has attracted the attention of many research groups globally to investigate mechanisms of their antimicrobial action. Their amphipathic nature is considered to allow them to interact with the negatively charged compartments and hydrophobic fatty acid chains found on the target microbial cell envelopes, leading to membrane destabilization and evidently cell lysis (Wimley, 2010). Commonly AMPs are classified in four large families based on their secondary conformations in α-helices, β-sheets, mixed structures and non- α- or β-structures (Figure 1.7) (Wang, 2013).

These conformations predominantly exist upon interaction with lipid membranes. Most AMPs from both multi- and unicellular organisms originate from precursor sequences. Successively, they exhibit a number of post-translational reformations that basically modify their activity. These modifications involve: proteolytic processing, glycosylation, amidation, halogenation, phosphorylation, incorporation of unnatural-amino acids and cyclization (Andreu & Rivas, 1998). Particular AMPs are also synthesized non-ribosomely e.g., gramicidin S and lipopeptides. AMPs have also been intensively altered through synthetic chemistry in order to reach the requirements of potential therapeutic medications, therefore further increasing the structural diversity. Lately, combinations of diverse structures such as β-peptides, peptoids, β-peptoids, peptide-peptoid hybrids and others, have been synthesized and the antimicrobial activity of the resulting peptidomimetics has been matched to that of conventional AMPs. This broad research holds immense potential in analysing the detailed mechanism by which both

innate and novel antimicrobial agents can be utilized, to combat pathogenic infections. Furthermore, there is a significant interest in the development of new antimicrobials to combat infectious illnesses in parallel with the increasing evidence of their broad-spectrum potency. So far, more than 2000 AMPs have been identified from diverse cells and tissues of animals, insects, plants and bacteria (Wang *et al.*, 2009a). Broadly, known examples of AMPs belong to the families of the cathelicidins and defensins (found in many insects and plants and animals, including humans), thionins (isolated from plants), cecropins (found in the hemolymph of the cecropia silk moth) and magainins (secreted from frog skin) (Pasupuleti *et al.*, 2012).

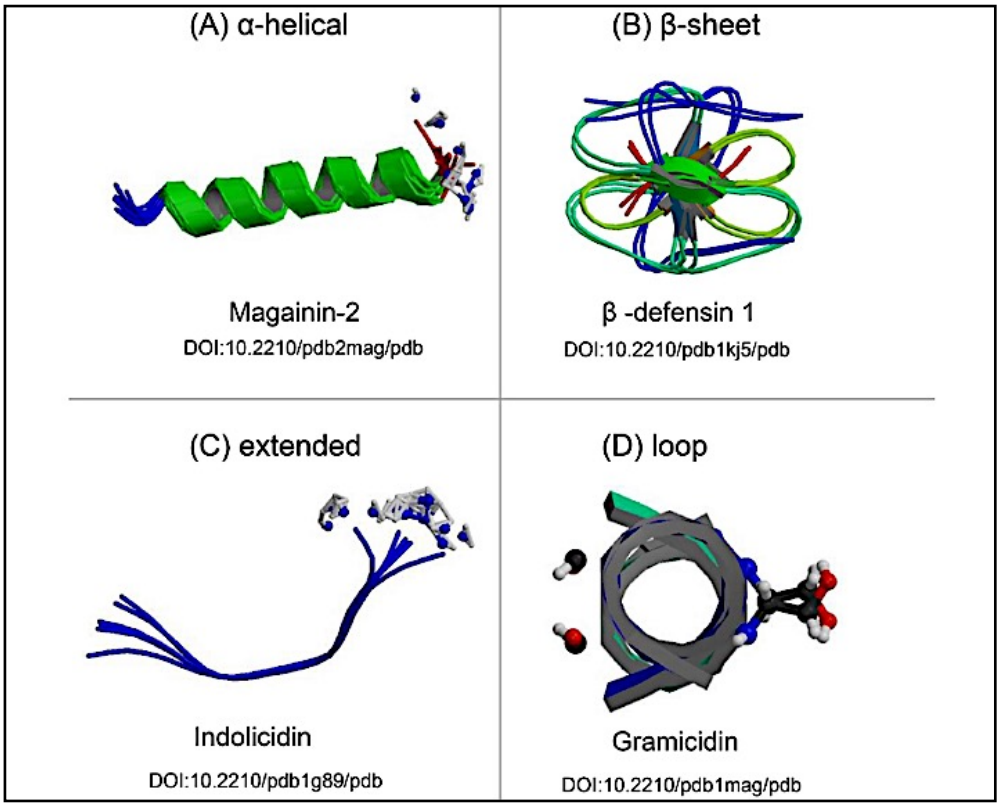


Figure 1.7 Protein models representing the structural differences of the four classes of antimicrobial peptides, (A) a-helical peptides, (B) peptides composed of a series of β-sheets, (C) peptides that adopt unconventional structures, such as extended helices, and (D) peptides that assemble into loops.(Peters *et al.*, 2010)

1.3.1 Structural properties of AMPs

The level of biological activity of any given biomolecule is related to its structural properties. Numerous studies have been performed to separate the significant elements that define peptides as antimicrobial "armaments". Extensive diversity of antimicrobial peptide sequences occurs in the environment, which contributes to the whole structural variety, however there are evolutionarily preserved characters that ensure their activity on diverse types of bacteria with diverse membrane arrangement and different targets. The secondary structure, cationicity, hydrophobicity and amphipathicity are the very substantial main components that allow characterization of AMPs.

Circular dichroism spectroscopy, X-ray crystallography and nuclear magnetic resonance spectroscopy have been exploited extensively for the structure identification of AMPs. For instance, the first X-ray crystallographic construction of native human α-defensin, the human neutrophil peptide 3, appeared in 1991, followed by a nuclear magnetic resonance construction of human neutrophil peptide 1 (Pardi *et al.*, 1992). Such structural report was found to be valuable when studying the significance of the secondary structure in deciphering the antimicrobial activity of AMPs. Investigations have established that bacterial eradication by human neutrophil peptide 1 is structure-independent (de Leeuw *et al.*, 2010). The most usual pattern noticed in numerous proteins and peptides with biological activities is the amphipathic α-helix, nonetheless, various AMPs occur as extended or unstructured conformers and only adjust to α-helical conformations upon interaction with phospholipid membranes (Yeaman & Yount, 2003). Circular dichroism investigation has indicated that in the presence of unilamellar phospholipid vesicles with diverse content of zwitterionic and negatively charged phospholipids, numerous AMPs develop well defined α-helical and/or β-sheet like structures compared to the buffer condition (Jahnsen *et al.*, 2012).

The occurrence of α-helical structures in AMPs is commonly assumed to stimulate interaction with membranes and promote membrane lysis (Epand & Vogel, 1999) and consequently specific amino acids such as alanine, leucine, arginine, lysine (Pace & Scholtz, 1998). From this perspective, Deslouches *et al.* revealed that normally, the peptides from their *de novo* library, with more than 80 % helical content, showed greatest antimicrobial potency against the examined bacterial strains (Deslouches *et al.*, 2005). In another study, Javadpour *et al.* also designed a library of highly α-helical peptides comprising lysine and leucine/alanine/glycine residues in arrangements that extended up

to 21 residues. In this library the predispositions of α-helical conformation was identified to be associated with the toxicity of the peptides on mammalian model cells. Nevertheless, no exact decision could be made for a correspondence of these peptides' conformations and their detected antibacterial potency (Javadpour *et al.*, 1996).

1.3.1.1 Cationicity

The universal characteristic of the most AMPs is the cationic property characterised by the various positively charged residues (lysine, arginine and histidine) within their configuration, which varies between +1 and +7 charges (Wang, 2013). Many studies have correlated the positively charge characteristic in AMPs with the antimicrobial potency detected. The established prominence lies largely in the interaction amongst the positive charge in the peptides and the negatively charged bacterial membrane structures through electrostatic interactions. For example, the antimicrobial activity of the majority of the defensin family seems to be associated with their cationicity. As an example, human defensin-5 interacts with the bacterial membrane through its arginine residues and consequently applies its antimicrobial potency. Substitution of arginine residues at position 9 and 28 with alanine or lysine residues decreases the antibacterial properties and the host cell interaction, the latter of which is identified as receptor mediated (de Leeuw *et al.*, 2009). The overall cationic charge might not be the foremost factor in the detected antibacterial activity as in the case of human α-defensins which normally display more activity against Gram-positive bacteria than human β-defensins, despite human β-defensins being more positively charged (Wei *et al.*, 2009). It was shown that human neutrophil peptide-1 with net charge +3 proved more efficient against *S. aureus* compared to human β-defensin-3 with net charge +11 (Schibli *et al.*, 2002). Furthermore, cationicity encounters a limit, beyond which raising the charge does not lead to boosted antibacterial activity. A study of Dathe *et al.* has established that increasing from +3 to +5 in magainin-2 derivatives, also boosted the peptides' antibacterial potency against both Gram-positive and Gram-negative bacteria, but additional increases to +6 or +7 resulted in loss of the antibacterial potency and boosted haemolytic property (Dathe *et al.*, 2001). Additionally to the net charge of AMPs, it has further been demonstrated that the location of the charged residues is likewise an essential aspect that defines the peptides overall antibacterial potency. For instance, altering the position of a few amino acid residues inside the natural structure of the linear

bactenecin, ended up in a scrambled arrangement that exhibited boosted antibacterial potency (Hilpert *et al.*, 2006). Further to the prominence of charge in mediating preliminary interaction with target membranes, in Gram-negative bacteria, the net positive charge is essential for the self-promoted uptake of AMPs. Positively charged AMPs interact with the bacterial membrane where there are divalent cations such as Mg^{2+} or Ca^{2+} cross bridging LPS molecules. Dislocation of these cations triggers disruption of the outer membrane and consequently allowing uptake of molecules (Piers & Hancock, 1994). Besides, bacteria have more negative trans-membrane potential in contrary to a normal mammalian cell membrane which enables introduction of charged AMPs into the membranes (Matsuzaki, 2009).

1.3.1.2 Hydrophobicity

Hydrophobicity is an important structural characteristic that determines the total potency of a particular antimicrobial peptide, and consequently it is constantly illustrated in the literature as a central functional characteristic. It impacts the potential of interaction amongst AMPs and diverse membrane structures and besides this directs the degree of peptide segregating into the lipid bilayer. Boosted hydrophobicity is associated with fading antibacterial specificity, leading to high toxicity towards mammalian cells. For example, Yin *et al.* revealed that replacing four alanine residues with four hydrophobic leucine residues produced higher haemolytic activity of their membrane active model peptide (Yin *et al.*, 2012). Magainin-2 derivatives with varying hydrophobicity have likewise been employed to exhibit that minimal alterations in the hydrophobicity may considerably impact and increase its membrane binding and permeabilization activity (Wieprecht *et al.*, 1997). The impact of hydrophobicity on the antibacterial and haemolytic potencies has been further showed through peptide series constructed of repeats of lysine and tryptophan residues. Introduction of a number of repeats of these two residues led to corresponding increase of both hydrophobicity and antimicrobial activity. However, when five repeated units were in the configuration, the increase produced unfavourable variation in the haemolytic profile (increased toxicity) and decrease in the antimicrobial potency because of self-aggregation (Gopal *et al.*, 2013).

1.3.1.3 Amphipathicity

The prevalence and polarization of the hydrophobic and hydrophilic domains is mirrored by amphipathicity in antimicrobial peptide configurations. A majority of the cationic AMPs exhibit a net positive charge (+1 to +7) (Wang, 2013) and comprise around 50 % hydrophobic units which contribute to the identification and interfering with the cytoplasmic membrane barrier through the cellular membranes. The cationic charge and the hydrophobic groups segregate into amphiphilic constructions. A quantitative measure of amphipathicity is the hydrophobic moment, attributed to peptides in α-helical confirmation and employed as a determinant in analysing the role of amphipathicity for peptide antimicrobial potency (Eisenberg, 1984). The prominence of amphiphilicity in the identification of the antimicrobial potencies of AMPs is contentious since diverse research groups have found positive and adverse contributions such as increasing antimicrobial and increasing haemolytic activities, respectively (Chen *et al.*, 2005). Amphipathicity has been stated as a key structural determinant for the biological potency of a small library of arginine and tryptophan rich linear and cyclic hexapeptides (Pathak *et al.*, 1995). The structural and conformational restrictions of the peptides together with the ultimate locating of the hydrophobic clusters appeared to define the antimicrobial potency and selectivity of the peptides (Chen *et al.*, 2005). In another investigation of magainin-2 and its derivatives it was revealed that the antimicrobial activity was directed extensively by the peptide amphipathicity and not by hydrophobicity or α-helicity (Pathak *et al.*, 1995). Analogously, design of cecropin A and melittin hybrid constructions has exhibited boosted amphiphilicity and helicity correlating with high antibacterial potency and lower toxicity against mammalian cells (Andreu *et al.*, 1992). In contradistinction to these outcomes are various other peptide studies, indicating that higher amphipathicity if measured as hydrophobic moment, intensifies membrane disturbance resulting in increase in both the antibacterial and haemolytic activity (Fernández-Vidal *et al.*, 2007). Amphipathicity in AMPs that occurs in β-sheet conformations is determined by the quantity of β-strands systematized by two distinct polar and non-polar domains. β-strands are typically stabilized through disulphide bonds, which deliver high conformational rigidity in aqueous solution. The polar and non-polar domains in β-strands enable AMPs to interact effectively with membranes and once linked with the membrane, the amphipathic property triggers membrane disturbance through establishment of trans-membrane channels (Yeaman & Yount, 2003). Modification in the non-polar domain of gramicidin S

analogues, which reduced hydrophobicity and the overall amphipathicity, consequently as expected diminished the peptides' haemolytic activity (Kondejewski *et al.*, 1999).

1.3.1.4 Specific amino acids in AMPs

Isolated AMPs from plants, insects, fish, frogs and mammals differ significantly with respect to their amino acid configuration. Each of the amino acid individually or in cooperation with the adjacent amino acid units contributes to the experiential antimicrobial properties kept in the peptides.

The fundamental amino acids Lysine and Arginine are highly conserved units in antimicrobial peptide structures as they allow electrostatic interactions between the peptide and the negatively charged surfaces of bacterial membranes (Shepherd *et al.*, 2001). These two amino acids vary in their side chain chemistry. Arginine has a guanidinium group which enables more distributed positive charge and suggests greater possibility of hydrogen bonding with the surrounding water molecules. The exceptional features of the arginine side chain enable formation of multiple interactions contrasted with the mono charge existing in lysine.

Another essential residue identified in the structure of AMPs is tryptophan. The specific side chain comprising an indole ring holds hydrogen-bonding potential as well as other physiochemical features such as dipole and quadrupole moments (Vogel *et al.*, 2002). Tryptophan amino acid displays strong membrane-disruptive activities by its potential to interact with the interface of a membrane anchoring the peptide to the surface of the bilayer (Khandelia & Kaznessis, 2007).

Another sulphur-containing amino acid residue is cysteine, which is strongly reactive. The thiol- group of it can be effortlessly oxidized to form a dimer, consequently generating a disulphide bond amongst two cysteines. Disulphide bridges made by cysteine residues are strongly hydrophobic (nonpolar) and play a principal role in the constructions of many AMPs. Furthermore being critical for the overall structural fold of the peptide, these bridges also raise the peptide's stability towards proteolytic degradation, like in defensins (Tanabe *et al.*, 2007).

Proline-rich AMPs are unique family of cationic peptides identified from both insects and mammals, with established antimicrobial activities precisely targeted against Gram-negative bacteria (Scocchi *et al.*, 2011).

1.3.2 AMPs' modes of actions

In pharmacology, a decent antimicrobial candidate should demonstrate highly precise biological activity followed by good pharmacokinetic profile and low immunogenicity. In order for peptides to be administered as antimicrobial agents of therapeutic relevance, it is fundamental to separate their biological activities, particularly their mode of action (Figure 1.8). It is not easy as more than 2000 biological native peptides with broad spectrum antimicrobial potencies have been identified. In spite of this fact, numerous scientific researches have provided structure-activity relationship evidence that aids the overall comprehending of how antimicrobial peptide damage infectious agents. For this purpose, investigators have employed numerous model membranes, which AMPs act on. These comprise micelles, artificial liposomes with variable lipid arrangements (Palmitoyl-oleoyl-phosphatidyl-ethanolamine (POPE), Palmitoyl-oleoyl-phosphatidyl-choline (POPC), Palmitoyl-oleoyl-phosphatidyl-glycerol (POPG), cholesterol *etc.*) and natural *E. coli* polar lipid extracts (Bobone *et al.*, 2012).

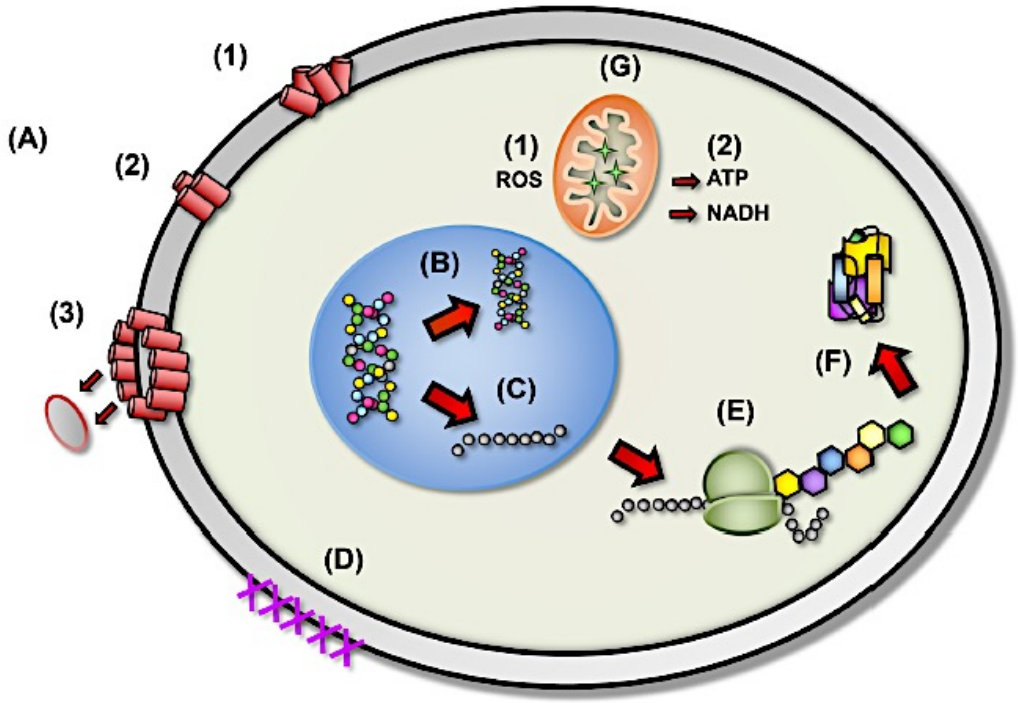


Figure 1.8 The proposed diverse mechanistic modes of actions of AMPs. (A) Disruption of cell membrane integrity: (1) random insertion into the membrane, (2) alignment of hydrophobic sequences, and (3) removal of membrane sections and formation of pores. (B) Inhibition of DNA synthesis. (C) Blocking of RNA synthesis. (D) Inhibition of enzymes necessary for linking of cell wall structural proteins. (E) Inhibition of ribosomal function and protein synthesis. (F) Blocking of chaperone proteins necessary for proper folding of proteins. (G) Targeting of mitochondria: (1) inhibition of cellular respiration and induction of ROS formation and (2) disruption of mitochondrial cell membrane integrity and efflux of ATP and NADH (Peters *et al.*, 2010).

1.3.2.1 AMP disruption activities on membrane

It is well known that after membrane binding, AMPs contribute to potential interchanges of the membrane structure such as thinning, pore formation, and altered curvature. Then loss of membrane potential eventually halts ATP manufacture and cellular metabolism and results in cell death (Bobone *et al.*, 2013).

One of the earliest models for pore formation suggested for AMPs is the barrel-stave model (Ehrenstein & Lecar, 1977) (Figure 1.9a). This model has been widely investigated and illustrates a process where following initial binding of the peptides to the membrane. They align perpendicularly to the membrane and aggregate on the surface resulting in formation of pores. It has been shown that AMPs with distinct secondary structures exploit this mode as their hydrophobic fragments interact with the lipids of the membrane and the hydrophilic part line the lumen of the pore (Brogden, 2005). Alamethicin (Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-Aib-Pro-Val-Aib-Aib-Glu-Gln-Phl) (Wang *et al.*, 2014) is an antimicrobial peptide that has been widely investigated for its capacity to disrupt membranes using this model (Laver, 1994). Lately, Bobone *et al.* identified and suggested that trichogin GA IV (n-Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol) (De Zotti *et al.*, 2012) and analogously short peptaibols act through the barrel-stave model to employ their membrane disruption powers (Bobone *et al.*, 2013).

The second hypothesis of membrane interaction by AMPs supports the toroidal pore model (Figure 1.9b) where the peptides trigger the membrane to fold inwards so that the channels made comprise peptides and hydrophilic lipid head groups (Yang *et al.*, 2001). Chaotic toroidal pore models have been detected by Sengupta *et al.* exploiting molecular dynamics simulations for melittin and DPPC membranes where peptides were found to position close to the pore centre, while other molecules were located near to the pore edge (Sengupta *et al.*, 2008).

The third model which suggests further vigorous disruption of the membrane is the carpet model (Figure 1.9c). In this model the peptides align parallel to the membrane and when they reach a particular concentration they turn the membrane into micelles, similar to a detergent–like mode of membrane permeabilisation (Bechinger, 2009). Recently, this model has been suggested for a derivative of PMAP-23 peptide (cathelicidin; RIIDLLWRVRRPQKPKFVTVWV) focussing on its potency on *E. coli* (Roversi *et al.*, 2014). Bacterial death had only occurred when a complete saturation of the membrane with

PMAP-23 molecules happened, demonstrating the suitability of the carpet model mechanism of membrane disruption as it has been defined exploiting artificial membrane systems (Roversi *et al.*, 2014).

Five other models are proposed by various research groups comprising interfacial activity (Orioni *et al.*, 2009), sinking raft (Pokorny *et al.*, 2002), leaky slit (Zhao *et al.*, 2006), lipid clustering (Epand & Epand, 2011) and sand in a gearbox (Pag *et al.*, 2008) models. Looking through these differing models, it is clear that the challenge is to deliver commonly recognisable explanations of mechanisms of action from the diverse techniques that are employed to study their complexity.

1.3.2.2 Nonspecific killing properties of AMPs

It is generally accepted that cationic AMPs interact with membranes where they disrupt the amphipathic lipid bilayer, which consequently results in disruption of fundamental bacterial metabolic activities and eventually bacterial killing. Bacterial membranes hold a large portion of negatively charged lipids and conserve high electrical potential gradients, therefore attracting positively charged compounds such as cationic AMPs. An outcome of the electrostatic interaction of positively charged peptides on the negatively charged bacterial composition, is deterioration and discharge of built-in divalent cations from the membrane. This transposition results in disturbance of the outer membrane barrier in Gram-negative bacteria.

In contrast to bacterial membranes, plant and animal cell membranes are concentrated in cholesterol and lipids, have no net charge, and keep weak trans-membrane potential (Zasloff, 2002a). Researches that mention the electrostatic interactions amongst the positively charged amino acids and the negatively charged phospholipid head groups in the membranes have shown that by rising the buffer salt concentrations the peptide potency on the negatively charged membranes is weakened (Kagan *et al.*, 1990).

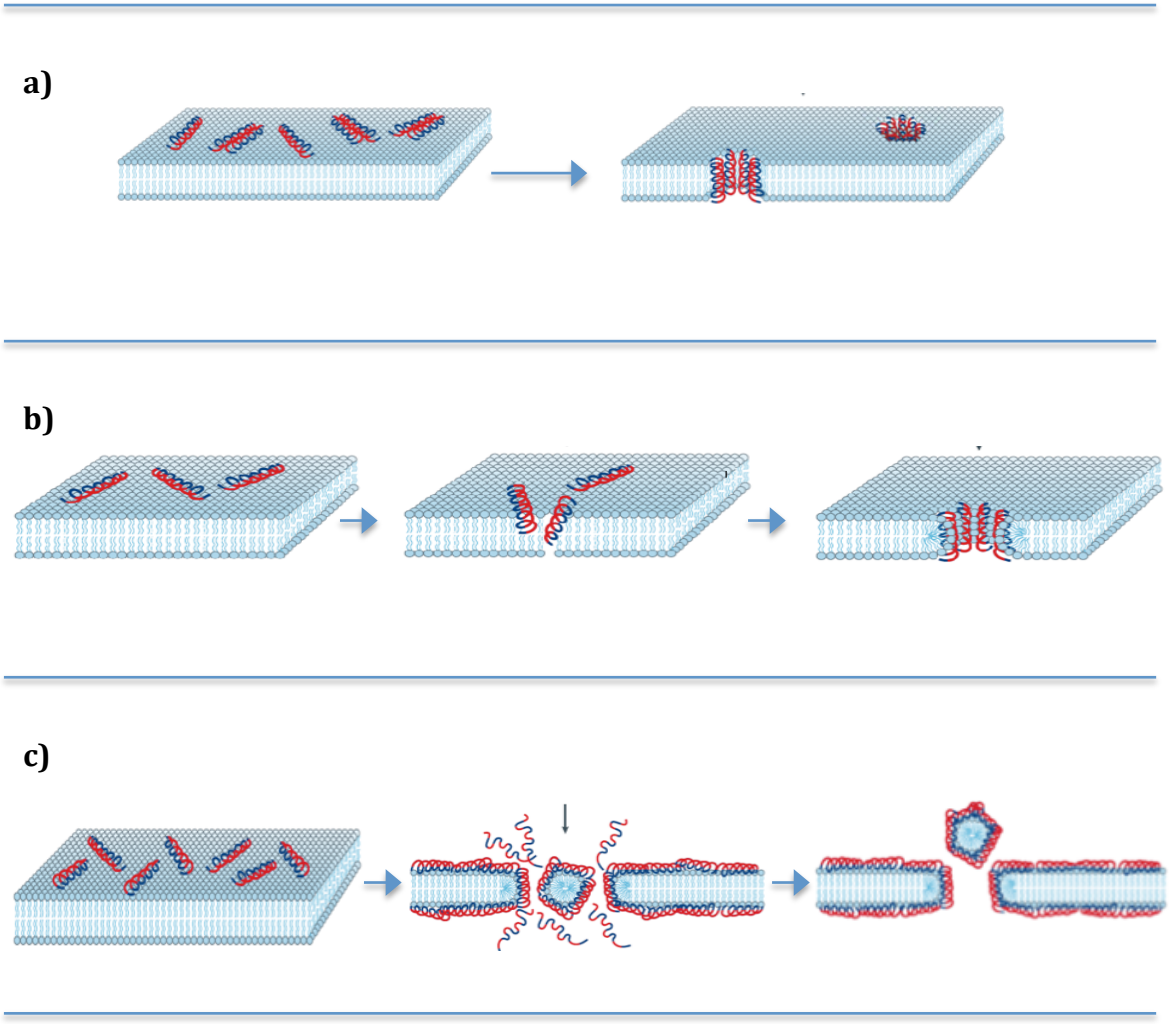


Figure 1.9 Three types of AMP insertion into cell membrane. The barrel-stave model of antimicrobial-peptide-induced killing. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue (a). The toroidal model of antimicrobial peptide induced killing. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue (b). The carpet model of antimicrobial-induced killing. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue (c) (Brogden, 2005).

1.3.2.3 AMP binding affinity to membranes

Isothermal titration calorimetry is one of the procedures exploited to obtain knowledge about the antimicrobial peptide binding affinity to model membranes. Andrushchenko *et al.* (Andrushchenko *et al.*, 2008) examined the thermodynamics of the interaction of tryptophan-rich AMPs with large uni-lamellar vesicles (LUVs). They achieved binding isotherms that might illustrate the potency of binding of the peptides with model membranes. All peptides employed in that study showed selectively stronger binding to anionic and *E. coli* membranes in contrast to zwitterionic membranes (Andrushchenko *et al.*, 2008).

Fluorescence spectroscopy is another procedure employed for investigations of peptide interaction with model membranes, which yields valuable but partial information concerning the affinity of the peptide for lipid bilayers. It could likewise be useful on peptide accumulation, localisation in the membrane and the influences on membrane integrity. Whilst using fluorescence spectroscopy on tryptophan-rich peptides, deviations of blue shifts in tryptophan fluorescence can be calculated, and related to binding to model membranes. Several tryptophan-containing β-hairpin peptides have been tested for their binding to phosphatidyl-ethanolamines (PhE)/phosphatidyl-glycerol (PG) and phosphatidyl-cholines (PC)/cholesterol-containing vesicles and their binding features specifically to PE/PG related to their antimicrobial activity (Dong *et al.*, 2012).

Lipopolysaccharide (LPS) is the endotoxin identifiable on the external surface of the outer membrane envelope of Gram-negative bacteria, which aids cell health by increasing the overall negative charge. It is associated with systemic inflammatory reaction in mammals, occasionally resulting in septic shock. The charge distribution in the majority of cationic AMPs is reported to demonstrate high affinity to LPS. Peptide interaction with LPS is enhanced by dislocation of Mg^{2+} which typically stabilizes and cross-bridges joining LPS molecules in the external membrane (Sawyer *et al.*, 1988). This interaction additionally supports the peptides in self-stimulated uptake providing them entry to the inner membrane where they can extend bacterial destruction. Nonetheless, when LPS are detected as endotoxins, the binding of a majority of cationic AMPs has the capacity of counteracting the toxin, therefore they hold potential as novel candidates for elaboration of better approaches for clinical treatment of sepsis (Andrä *et al.*, 2006).

1.3.2.4 AMP and bacterial biofilm interactions

Prolonged cultivation of bacterial organisms lead to adherence to animal tissues and inorganic materials (Costerton *et al.*, 1981). And it, in turn, enables the establishment of a biofilm, which is a multi-layered population of sessile bacterial microorganisms. Biofilms allow an advantage in persistence compared to planktonic bacteria by boosting nutrient trapping and colonization (Costerton, 1999). Presently, biofilms are an extensive medical issue in clinics and healthcare facilities. Certainly, the United States National Institutes of Health found that 80 % of chronic infections are associated with biofilms (Monroe, 2007). Furthermore, many investigations have identified that biofilms are related to dental plaque (Hojo *et al.*, 2009), endocarditis (Palmer, 2006), lung infection (Wagner & Iglewski, 2008a), and infection through medical devices (Khardori & Yassien, 1995).

Biofilm-formation by bacteria is accomplished through responses to numerous factors, such as nutritional signals, cellular recognition of binding sites on the surface, exposure to sub-lethal dosages of antibiotics, and environment stresses (Hoffman *et al.*, 2005). Biofilm-formation is commonly initiated with the attachment of planktonic bacteria to a surface via weak van der Waals forces, and the colonies are anchored firmly using pili. To enable the accumulation and attachment of other planktonic bacteria, the primary attached bacteria build numerous adhesion sites and the matrix. Bacteria are positioned within this matrix of extracellular polymeric substance (EPS), which comprises extracellular DNA, proteins, lipids, and polysaccharides with numerous conformations (Hall-Stoodley & Stoodley, 2009). These constituents are very key targets for controlling both biofilms and drug-resistant bacteria (Landini *et al.*, 2010). Throughout colonization, particular bacteria are able to communicate exploiting a quorum sensing (QS) system (Horswill *et al.*, 2007) through tiny molecules named auto-inducers and governors of overall behaviours, like bioluminescence, virulence factor manufacture, and biofilm formation (Waters & Bassler, 2005a). Auto-inducers in Gram-negative and Gram-positive bacteria are reported to be acyl-homoserine lactone molecules and oligopeptides, respectively. These are presently accepted as key target for inhibiting biofilm infection. The mature biofilm demonstrates boosted antibiotic-resistance compared to bacterial colonies, with increased cell division and recruitment. Following establishment, biofilms are distributed and the bacteria translocate to further surfaces, such as organs, tissues, and medical devices, where the biofilm formation progression appears again.

Two leading models in the inhibition of biofilms are dispersion of the biofilm EPS and disrupting of the bacteria embedded in the EPS. Naturally, lethal or inhibiting dosages of antibiotics are considerably increased by up to 1000-fold against biofilm of bacteria since they are incapable of trans-locating into EPS and consequently do not reach the bacterial populations. AMPs are considered to possess the potential for employment as anti-biofilm drugs owing to their specific mechanisms, which comprise membrane-disrupting potencies, functional inhibition of proteins, binding with DNA, and detoxification of polysaccharides. The EPSs of biofilms include significant quantities of polysaccharides, proteins, nucleic acids and lipids (Sutherland, 2001). For instance, particular AMPs can be relocated in biofilm EPS via channels or pores formed in the lipid constituent of the EPS, while others can disperse biofilms.

P. aeruginosa is the notorious pulmonary pathogen affecting patients with CF (Moreau-Marquis *et al.*, 2008a), and this bacterium develops a biofilm on medical devices and tissues. LL-37, a human origin cationic host defence peptide, displayed an active inhibitory activity in biofilm development at a dosage of 0.5 μg/mL against *P. aeruginosa* biofilm and diminished pre-grown biofilms (Overhage *et al.*, 2008a). It was also revealed that these effects were reached by reducing the attachment of bacteria onto the surface, triggering twitching motility mediated by type IV pili, and supressed the genes associated with the QS system (Overhage *et al.*, 2008a).

1.3.2.5 AMP intracellular antibacterial targets

Other than the capacity to effectively lyse bacterial membranes, there are AMPs that can successfully cross the membrane barrier without disturbing it and display their antimicrobial potency through intracellular targeting. There is a collective suggestion stated in the literature for such AMPs. Human neutrophil peptide 1 (HNP1) and human-β defensin 3 (HBD3) have been stated to attach to lipid II, a bacterial cell wall foundation (Sass *et al.*, 2010). Human β-defensin 3 also influences electron transport in *S. aureus* (Sass *et al.*, 2008). Numerous AMPs possess immune-modulatory roles. For instance, human neutrophil peptides 1-3 that once released by invading granulocytes induce discharge of tumour necrosis factor (TNF-α) and interferon-gamma (IFN-γ) from tissue macrophages. This increases the eradication of bacteria as examined in a murine *in vivo* model (Soehnlein *et al.*, 2008). Human β-defensin 3 also triggers particular antigen carrying cells such as monocytes therefore inducing the adaptive immune system

(Funderburg *et al.*, 2007). Buforin II, a 21-amino acid peptide (TRSSRAGLQFPVGRVHRLLRK) (Park *et al.*, 1996) with active wide spectrum antimicrobial potency, is also capable of crossing the cell membrane and reducing cellular metabolism by attaching to DNA and RNA of the cells, leading to prompt cell death (Park *et al.*, 1996). Lately, it has been shown that indolicidin attaches DNA, thus reducing DNA replication and transcription (Ghosh *et al.*, 2014)in addition to bind to DnaK (Li *et al.*, 2006).

1.3.3 Bacterial Resistance to AMPs

Applying antibiotic treatments to fight numerous infections has significantly contributed to improve human life expectancy through the decades. Nevertheless, only a year after the discovery and the global use of penicillin, resistant strains of bacteria were already identified owing to the extreme selection pressure and fast progress of resistance. It is a similar picture with other antibiotics against which numerous bacteria have advanced resistance; therefore there are numerous infections currently, which do not have treatment options. AMPs are possible good candidates for being part of novel anti-infective strategies. The predominant part of the peptides act on the microbial membrane and not on particular intracellular targets, with a mode less inclined to the formation of resistance. Nevertheless, to resist the effect of AMPs, bacteria have developed numerous strategies. These comprise, proteolytic degradation, shielding bacterial cell surface, alteration of superficial membrane structures, dynamic efflux and suppression of AMP expression (Gruenheid & Le Moual, 2012). Proteolytic denaturation has been described for LL-37 and β-defensin 2, by proteases from *P. mirabilis* (Belas *et al.*, 2004) on top of numerous proteases discharged by bacteria that slice peptides on particular amino acid units. Amongst Gram-negative bacteria, particularly, various proteases that deactivate AMPs are identified at the outer membrane (Hritonenko & Stathopoulos, 2007). Extracellular constituents such as capsule polysaccharides, fimbriae, exopolysaccharides and *O*-polysaccharide of LPS (Figure1.10) assist bacteria by attaching AMPs and thus diminishing the quantity of peptide reaching the bacterial membrane (Ilg *et al.*, 2009). Reduced overall negative charge has also been documented as a resistance strategy for cationic peptides (Hein-Kristensen *et al.*, 2013). Gram-positive bacteria also resist the AMPs by enzymatic mechanism of their extracellular proteases (Nawrocki *et al.*, 2014) as well as cell wall and membrane modifications. The last is predominantly by deacetylation

of *N*-acetylglucosamine or *O*-acetylation of *N*-acetylmuramyl units in the cell wall and modification of charge by d-analynation of the lipoteichoic acids (Bernard *et al.*, 2011). An alternative tactic to resist AMP activity is to pump these antimicrobials out of the bacterial cytoplasm. Bacteria utilize membrane pumps for the transportation of diverse molecules through the bacterial membrane. When bacterial strains with deleted or inactive pumps were tested, it was shown that these mutant strains were much more susceptible to AMP action (Shafer *et al.*, 1998). At the same time as regulation of their defence system, numerous bacteria also generate toxins that influence host identification of bacteria. For instance, the exotoxins of *Vibrio cholerae* and enterotoxigenic *E. coli* were found to be involved in down-regulation of expression of AMP such as LL-37 and hBD1, by host cells (Chakraborty *et al.*, 2008).

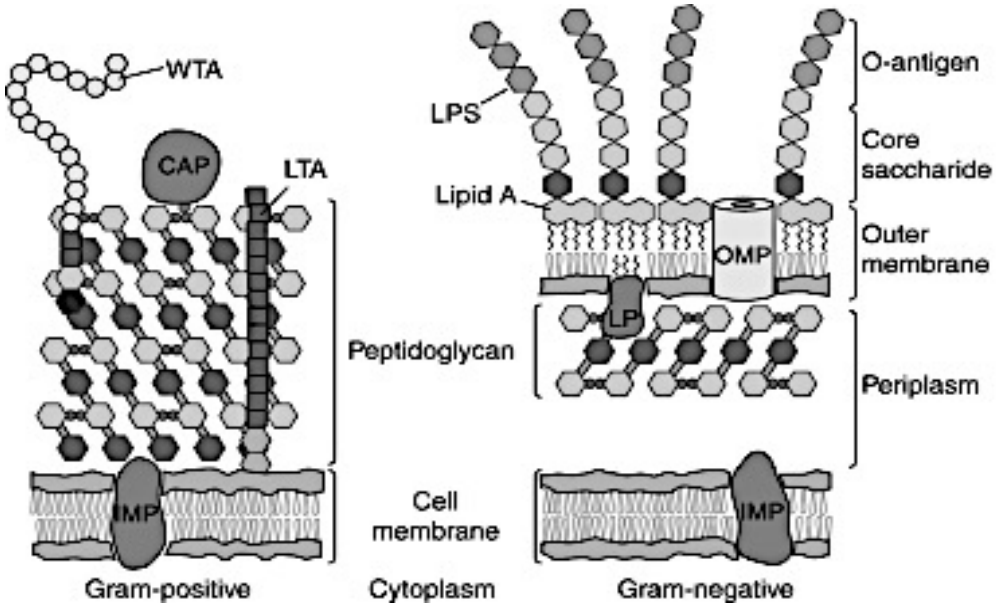


Figure 1.10 Depiction of Gram-positive and Gram-negative cell envelopes: CAP = covalently attached protein; IMP, integral membrane protein; LP, lipoprotein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; OMP, outer membrane protein; WTA, wall teichoic acid (Silhavy *et al*., 2010).

1.3.4 Mammals as a source of AMPs

Amongst mammals, AMPs have been found from diverse sources such as the granules of neutrophils, Paneth cells, mucosal secretions from epithelial cells and as protein degradation products (Boman, 1995). Three classes of AMPs, identified in abundance in neutrophils, defensins, cathelicidins and histatins (Selsted & Ouellette, 2005) have been researched comprehensively.

Defensins, discovered in human neutrophils, are 18-45 amino acids long cationic molecules which were identified from mast cells and tissues encompassed in host defence (Ganz, 2003). They have been categorised as α-, β and θ-defensins according to structural variances. All three categories are rich in cysteine and arginine residues. The variance amongst the classes lies in the site of disulphide linked cysteine residues. Herein, the first class of defensins (α-defensins), have disulphide connections between cysteine residues 1-6, 2- and 3-5, while β-defensins disulphide bridges are situated between cysteine residues 1-5, 2-4 and 3-6 (Bowdish *et al.*, 2006). Furthermore, human α-defensins are less cationic, shorter and more hydrophobic than human β-defensins (Pazgier *et al.*, 2006). Human neutrophil peptides (HNP1-HNP4) and human defensins (HD-5 and HD-6) are six diverse human α-defensins identified in monocytes, NK cells, B and T cells, neutrophils and Paneth cells, respectively (Ouellette & Selsted, 1996). Findings demonstrate that HNPs display antimicrobial activity against both Gram-negative and Gram-positive bacteria (Ericksen *et al.*, 2005). Human defensin 5 expresses its antimicrobial potency against numerous bacteria such as *E. coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *S. aureus* and *Vibrio cholerae* (Porter *et al.*, 1997). Dissimilar to α-defensins, four human β-defensins (HBD 1-4) have been identified from leukocytes and epithelial cells. The β-defensins also demonstrate microbicidal potency against a range of bacteria, with HBD-4 being the most active against *P. aeruginosa* (Xu *et al.*, 2006). It has been shown that epithelial human β-defensins are over-expressed in patients suffering from psoriasis, a chronic skin inflammation, with specific skin lesions cleared of infection. Contrariwise in atopic dermatitis where the expression of HBDs is inhibited, the lesions are infection-prone (Nomura *et al.*, 2003), clarifying the significance of these AMPs in host defence.

The cathelicidins are another class of AMPs which have been isolated in various vertebrates such as fish, bird, cow, pig, rabbit, sheep, mouse, monkey, horse and human (Scocchi *et al.*, 2009). They are predominantly manufactured in epithelial cells,

neutrophils and macrophages. Indolicidin and human cathelicidin LL-37 are two of the most investigated members of this category of peptides. LL-37 is the only member of this category of peptides identified in man. The prominence of these peptides is revealed in findings where increased susceptibility to infections is observed in patients with deficiencies in neutrophil manufacture of LL-37, the foremost source of human AMPs (Pütsep *et al.*, 2002).

Indolicidin is a short antimicrobial peptide with 13 amino acids identified from bovine neutrophils. It is rich in tryptophan (39%) and arginine (23%) residues, and it is amidated at the *C*-terminal arginine (Falla *et al.*, 1996). The antimicrobial potency towards both Gram-negative and Gram-positive bacteria, fungi and protozoa is most possibly not an outcome of any confined secondary structure, as nuclear magnetic resonance spectroscopy investigations failed to report distinct secondary or amphipathic structure which is specific for the majority of AMPs (Hsu *et al.*, 2005).

Human cathelicidin LL-37 is a broadly researched representative of the cathelicidin family of AMPs, and is also the only cathelicidin identified in humans. It is most identifiable in the granules of neutrophils. Upon infection and inflammation, LL-37 is discharged in high intensities at neutrophil accumulation sites. Human cathelicidin LL-37 is also manufactured by lymphocytes and macrophages as well as diverse epithelial cells, and hence found in plasma, sweat and other body fluids (Zanetti, 2004). The family of cathelicidins are categorised in accordance to their highly conserved cathelicidin domain, which is lined by an *N*-terminal signal peptide and a *C*-terminal segment related to the active antimicrobial peptide.

Bacterial constituents are another mode of LL-37/hCAP-18 gene expression inducers. For instance, it has been shown that expression of LL-37/hCAP-18 considerably boosted in *Helicobacter pylori*-infected patients compared to patients with non-*Helicobacter pylori* induced inflammation (Hase *et al.*, 2003). Once expressed, LL-37 applies diverse biological activities; direct bactericidal activity against Gram-positive and Gram-negative bacteria detected *in vitro* (Travis *et al.*, 2000) and modulation of inflammation and immune response in host cells against various infections (Bowdish *et al.*, 2005).

1.3.5 AMPs isolated from Insects

Insects form the largest group of multicellular organisms in nature. Lacking an apparent adaptive immune system, they combat numerous pathogens by rapid innate cellular and humoral reactions. Therefore, AMPs play a significant role in the insect's defence system as they are discharged in the haemolymph as a consequence of the humoral defense mechanism upon microbial infections. AMPs isolated from diverse insect species are classified as cecropins, attacins, lysozymes, defensins and dipteracins. Moreover, in *Drosophila* two groups, drosomycin and the metchikowins, have been isolated and characterized (Bulet *et al.*, 1999).

Cecropins are the most widespread group of linear AMPs in insects. They lack cysteine residues and adopt most regularly an α-helical conformation in membrane mimetic environments. To demonstrate, cecropin A (KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH_2) is one of the first cecropins for which a solution confirmation for adaptation of a stable α-helix in hydrophobic environments has been established exploiting circular dichroism spectroscopy (Steiner, 1982).

Melittin is the most investigated α-helical, cationic and haemolytic antimicrobial peptide in the field, which comprises 50 % of the dry mass of bee venom (Raghuraman & Chattopadhyay, 2007). It is a 26-amino acids amphiphilic peptide (GIGAVLKVLTTGLPALISWIKRKRQQ), described by a hydrophilic *C*-terminal and mainly hydrophobic *N*-terminal region, and performs as a model peptide in many membrane-peptide interaction studies (Dempsey, 1990). Furthermore, structure-activity relationship investigations where diverse segments of melittin have undergone substitution or deletion, have established the fundamental elements required for the high haemolytic and antimicrobial potency of this peptide (Mojsoska & Jenssen, 2015).

1.3.6. AMPs isolated from Plants

Representatives of plants have also developed defence molecules to combat undesirable infections. AMPs identified in plants are positively charged and exhibit the characteristic structural patterns such as α-helices, β-sheets and loops. They are separated into five categories: lipid transfer proteins (Marion *et al.*, 2007), thionins, defensins (Stotz *et al.*, 2009), chitin-binding proteins (Beintema, 1994) and cyclotides (Burman *et al.*, 2014). Thionins and defensins were the first AMPs identified in plants.

Thionins comprise up to 48 amino acids, mainly arginine, lysine and cystein residues and include a few conserved disulphide bonds. As a constituent of the plant defence system, thionins act on diverse pathogens and apply toxic effects on bacteria, fungi, yeast and mammalian cells (Stec, 2006). A subgroup of the thionins group is the β-purothionins, which interact intensely with lipid bilayer, then insert into the hydrophobic core, causing cell lysis (Clifton *et al.*, 2011).

Viscotoxins, another class of plant thionins, were identified in the leaves and stems of the European mistletoe (*Viscum album*) in 1973 (Samuelsson & Jayawardene, 1974). Reports have shown their high conformational stability and their ability to disturb bacterial membranes (Giudici *et al.*, 2003).

1.3.7 Bacteria as a source of AMPs

AMPs manufactured by bacteria, initially referred to as colicins, are known currently as bacteriocins. Bacteriocins manufactured by the host bacteria have the property to selectively act against a broad spectrum of bacterial species without destroying the producer itself. This group of AMPs finds extensive application in the food industry to keep food safe and prevent contamination. Some bacteriocins are synthesized by lactic acid bacteria. Moreover, bacteriocins draw exceptional attention for their clinical potential for treatments of MRSA, enterococci, including Vancomycin-resistant enterococci (VRE), streptococci, *Clostridium botulinum* and *Propionibacterium acnes*. As an outcome of intensive studies over the last years there have lately been requests for the classification of bacteriocins to be modified, and the current grouping proposed by Cotter *et al.* includes Class I (lanthionine-containing), Class II (non-lanthionine containing) and bacteriolysins (non-bacteriocin lytic proteins) (Cotter *et al.*, 2005).

Nisin is a conventional exemplar of a Class I bacteriocin that has been successfully approved as an antimicrobial with marketable applicability in over 50 countries globally since its discovery in 1927 (Rogers, 1928). Found from *Lactoccocus lactis* it was not until 1971 that its multipart structure, comprising 34 amino acids containing some uncommon lanthionine residues, four β-methyllanthionines, didehydroalanine and didehydroaminobutyric acid was fully designated (Gross & Morell, 1971). Its potency against Gram-positive pathogenic bacteria is related to its interaction with the bacterial membrane-occasioning disturbance of the membrane's integrity. This happens through pore formation and so far two mechanisms are suggested. The first one depends on the

low-affinity pore construction of nisin alone, while the second one suggests nisin and lipid II interaction consequently contributing to the pore formation by this complex (Lipid II-dependent pathway) (Hasper *et al.*, 2006). Some structure-activity relationship (SAR) investigations have been described where the significance of diverse segments, particularly those comprising lanthionine rings, have been emphasised to be fundamental for the observed antimicrobial activity (Chan *et al.*, 1996).

Mutacin 1140 (Strother *et al.*, 2000) is another representative of the lanthionine-comprising bacteriocins which has been widely investigated. It is a 22 amino acid long peptide manufactured by *Streptococcus mutans* (Hillman *et al.*, 1998) with a type of action analogous to that of nisin, which associates with the capacity to inhibit peptidoglycan synthesis by binding to lipid II (Hasper *et al.*, 2006). Mutacin 1440 has a broad spectrum of potency against clinically significant bacteria with time of eradication profiles comparable with that of vancomycin, an antibiotic licensed for treatment of severe infections induced by Gram-positive bacteria. This peptide is presently under examination for its pharmacological application and has been submitted for preclinical trials (Fox, 2013).

Lysostaphin is a bacteriolysin. It hydrolyses the cell wall of the target bacteria. Currently, it is being examined for its potency in food and nutritional microbiology. Even though bacteriolysins are structurally much greater than the conventional bacteriocins and AMPs, their application as peptide antibiotics possess massive potential. Lysostaphin (27 kDa) for instance, is capable of splitting the pentaglycine cross-links in the cell wall of *Staphylococci* and consequently lyse the pathogenic bacteria in entirely metabolic conditions. The therapeutic effectiveness of this bacteriolysin against clinical methicillin-resistant *S. aureus* isolate has been stated in animal models and matched to that of vancomycin. The recombinant lysostaphin, showed better *in vitro* and *in vivo* antibacterial potency against methicillin-resistant *S. aureus* compared to vancomycin, as well as having low toxicity, thus it is a possible main candidate for advanced structural optimization investigations (Chen *et al.*, 2014).

1.3.8 AMPs from Frog Skin

Amphibian genomes have developed the ability to respond to environmental challenges with the vastly concentrated genetic alterations (Caporale, 1999). The dermal glands of anuran frogs not only manufacture colossal quantities of biologically active

peptides which are very like mammalian neuropeptides as hormones (Severini *et al.*, 2002), but they also comprise a rich resource for broad spectrum, cytosolic AMPs (Simmaco *et al.*, 1998). Many of these peptides display antibiotic, fungicidal, anti-viral and anti-tumour potencies with a low cytotoxicity on mammalian cells. These peptides are synthesized as prepropeptides that are managed by the elimination of the signal peptide to proform and they are deposited in the large granules of the glands (Lacombe *et al.*, 2000). Glands may discharge their peptide content onto the skin surface by holocrine mechanisms to deliver an efficient and rapid-acting defence against pernicious microorganisms (Lacombe *et al.*, 2000). The AMPs are short, 10–50 amino acid residues long, cationic and function in a range of modes, though permeabilising and disturbing the target cell membrane is the most common mechanism (Reddy *et al.*, 2004).

The sequences of these AMPs diverge significantly from one amphibian to another. The skin of a frog may have 10–20 AMPs with diverse size, sequences, charges, hydrophobicity, tri-dimensional constructions and spectrum of potential, and this arsenal differs amongst frogs belonging to diverse families, genera, species or even subspecies, consequently, no two species with the similar arsenal of peptide antibiotics has been discovered (Ong *et al.*, 2002).

This remarkable variance amongst and within species means that there may be as many as 100 000 various peptides manufactured by the skin glands of the 5000 anuran amphibians (Jacob & Zasloff, 1994). The outstanding variants in the peptide sequence of frog skin AMPs may be manipulated for finding novel molecules and structural patterns targeting precise microorganisms for which the therapeutic armamentarium is limited.

Magainins, also identified as peptide glycine serine (PGS) (Giovannini *et al.*, 1987), are 23 amino acid long peptides isolated from the skin of the African clawed frog *Xenopus laevis* (Zasloff, 1987a). Magainins are representatives of a large family of amphibian amphipathic α-helical AMPs. These peptides have been stated to have an extensive spectrum of antimicrobial potencies against Gram-positive and Gram-negative bacteria and fungi. They are also acknowledged to accelerate wound closure and decrease inflammation (Sai *et al.*, 1995). Analogs of the peptide representing increased antimicrobial potency have been synthesized (Zasloff, 1987a). Arhana *et al*. revealed that magainin-A reduces the development of various strains of STI-causing pathogens, and the effects were established to be dose-dependant. The minimum inhibitory concentrations (MICs) for typical strains of *E. coli, S. aureus*, *Candida albicans* and *P. aeruginosa* were

fluctuating from 50 μg/mL to 150 μg/mL. It is important to underline that this peptide did not induce red blood cells (RBC) haemolysis up to 300 μg/mL (Clara *et al.*, 2004). The antimicrobial efficacy might be related to the disturbance of the bacterial membrane. Because of the somewhat non-specific mechanism on the bacterial membrane (Albiol Matanic & Castilla, 2004), magainin-A is potent against a broad spectrum of microbes.

1.3.9 Developing novel AMPs

The foremost goal of all research studies in the area of AMPs is to contribute to the AMP range with novel strategies for the advancement of pharmacologically applicable AMP configurations. The production of *de novo* constructions is not typically precise and it requires broad experimental investigation. To achieve this objective and assist the discovery of *de novo* peptides, numerous computer-based approaches have been engaged. Most include constructing quantitative structure-activity relationship parameters for computer-assisted design of AMPs. The overall concept is to relate chemical sequence and configuration of AMPs, defined with physiochemical parameters and associate them with their corresponding biological activities exploiting mathematical models (Juretić *et al.*, 2009). One of the mathematical models exploited for prediction of novel polypeptide sequences with potentially upgraded biological potencies is the Designer algorithm (Ilić *et al.*, 2013). Operating the Designer algorithm the latest study of Ilic *et al.* reveals on a number of peptide sequences with high antibacterial potency against Gram-negative bacteria (0.5–4 μM) and low hemolytic properties (HC_{50} > 400 μM) (Fjell *et al.*, 2009). Fjell *et al.* have also generated a software system that could predict peptides with antibacterial potency with up to 94% accuracy (Fjell *et al.*, 2011).

AMPs can be the next generation cohort of antimicrobial agents for struggling with multi-drug resistant and biofilm forming bacterial infections. These cationic peptides possess a prospective for employment as Nano-films or other coating constituents for surgical devices, involving catheters. Despite obstacles in AMP properties to the exploitation of peptides as therapeutics, such as low bioavailability and great cost, these difficulties may be overcome. There is enormous effort to avoid the complications associated with diverse approaches comprising the utilizing of d- or unnatural amino acid, formulation, and recombinant DNA expression of peptides, binding of fatty acyl chains to short peptides. Consequently, it is probable that AMPs will become the optimal drugs for emerging bacterial infections in the following decades.

1.4 Aim and objectives

Bacterial pathogens are the main causes of morbidity and mortality among CF patients. The thick mucus and impaired mucociliary clearance result in accumulation of bacterial pathogens, the formation of biofilms, and compromised immunity. Treatment is prolonged, problematic and re-infection is quite common. Along with bacteria, CF lungs can be colonized with protozoan microorganisms such as *Acanthamoeba, which* feed on bacteria. The widespread distribution of *Acanthamoeba* in the environment and its role as a "Trojan horse" for various bacterial strains suggest that these organisms may play a critical role in CF lung infections. *Acanthamoeba* could be acting as a protector of intracellular bacteria against drug treatment (Figure 1.11). Moreover, occurrences of bacterial re-infections and longer therapeutic periods could be related to the presence of *Acanthamoeba* in lower respiratory airways. However, there is no single report showing the presence of *Acanthamoeba* in human lower respiratory tract. Despite all the attempts to get clinical sputum samples, it was problematic to acquire Ethical approval within time of study. The major questions of the current thesis are: is the antimicrobial peptide magainin II effective against bacterial pathogens associated with CF and if *Acanthamoeba* occurs in human lower respiratory airways, does it have any role in bacterial infections in CF lung?

To this end, several aspects and objectives have been examined. First, can bacteria interact with and survive inside a lung adenocarcinoma cell line and in an *Acanthamoeba* strain. Secondly, to investigate new alternative therapies to combat pathogenic bacteria associated with CF pulmonary infections. Thirdly, to study the effect of an environment resembling CF conditions on the growth of bacteria and on the effectiveness of alternative therapy. Finally, to investigate if magainin II has the ability to prevent bacterial pathogens forming biofilms (Figure 1.12).

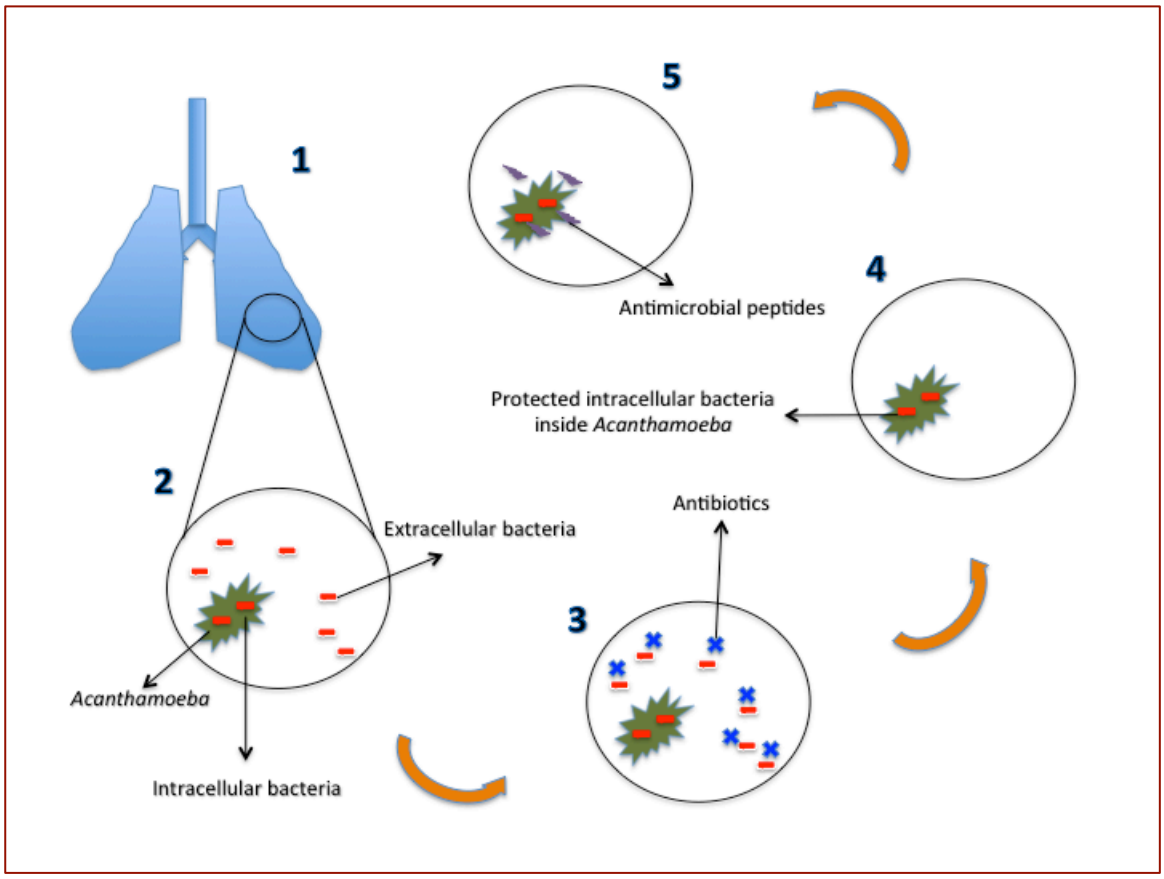


Figure 1.11 Using antimicrobial peptides in treatment of bacteria that are protected inside *Acanthamoeba*. 1-Lung of CF patient containing pathogenic bacteria. 2- *Acanthamoeba* engulfs bacteria. Some of them are digested and some of them survive. 3-Antibiotic treatment kills extracellular bacteria. 4-Intracellular bacteria remain intact. These bacteria survive, multiply and burst *Acanthamoeba*, causing reinfections in CF lung. 5- Antimicrobial peptides are used to target intracellular pathogenic bacteria along with eradicating *Acanthamoeba*.

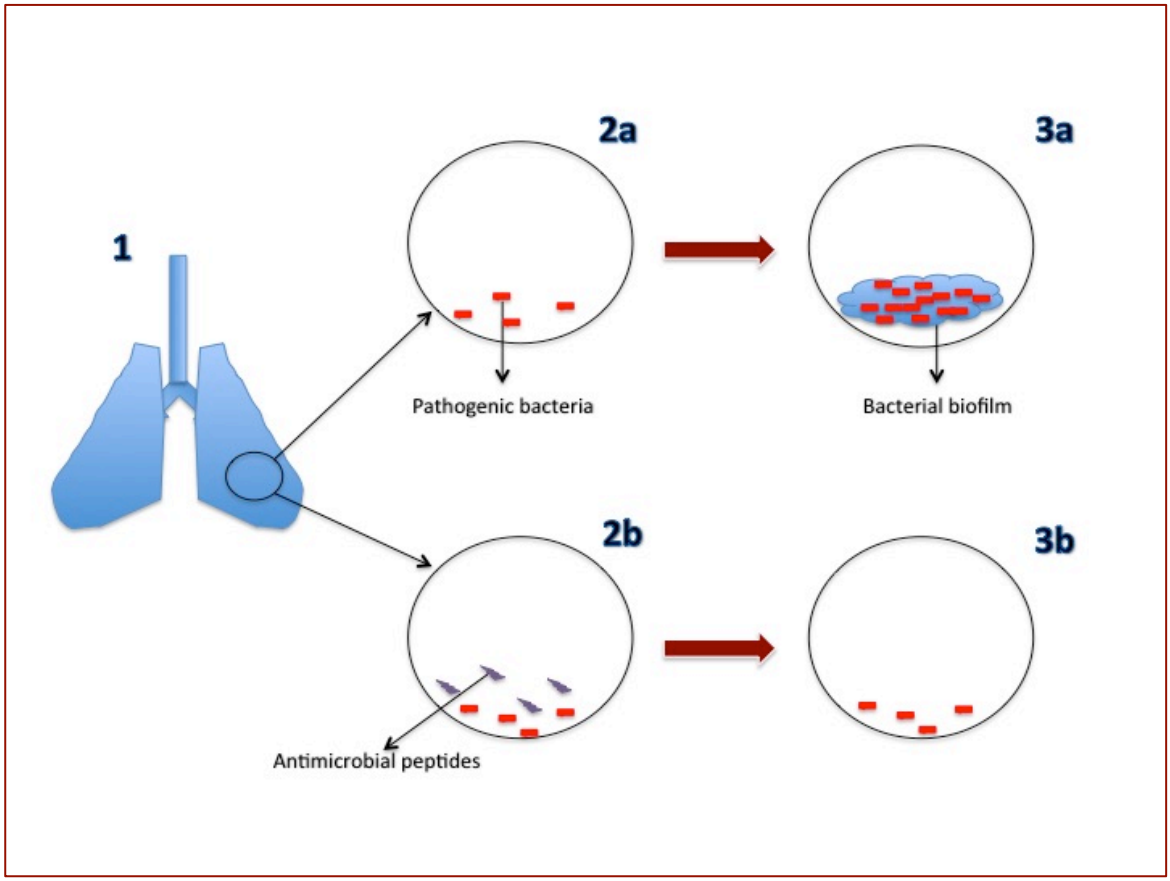


Figure 1.12 Antibiofilm activity of antimicrobial peptides. 1-Lung of CF patient with bacteria that are capable of forming biofilms. 2a-Pathogenic bacteria accumulation. 3a-Biofilm formation. 2b-Treatment with antimicrobial peptide. 3b-No biofilm formation.

Chapter 2

"The interactions of bacteria related to Cystic Fibrosis with *Acanthamoeba castellanii* and Human epithelial lung carcinoma (A549) cell line"

2.1 Background

CF is a recessive genetic disorder characterized by thickening of the airway mucus and weakened self-clearing mechanism of the bronchi. As a consequence, patients with the disease have a difficulty clearing pathogens from the respiratory tract and undergo chronic respiratory infections and inflammation. Generally respiratory insufficiency eventually results in death (Emerson *et al*., 2002).

Recently introduced therapies and continuous management of the lung disease have resulted in great enhancements in length and quality of life, with the outcome that the average estimated survival age has risen to 36 years. Yet, as the number of treatments broadens, the medical regimen is getting even more onerous in time, money, and health resources. Therefore, it is important that treatments should be recommended on the basis of available proof of effectiveness and safety (Flume *et al*., 2007).

CF is believed to be inherited as an autosomal recessive trait, and to be highly inconstant in its severity, duration, and range of symptoms (Minear *et al*., 2013). There are about 70,000 people suffering from CF worldwide (http://www.cff.org, 2012). About 1 in every 2,500 children born in the UK is born with CF and there are more than 9,000 people living with the condition in this country (NHS, 2012).

CF is a result of mutations in the cystic fibrosis trans-membrane conductance regulator (*CFTR*) gene on chromosome 7, which encodes a chloride ion channel. Such mutations influence a huge protein pore functioning as a conductor of negatively charged chloride atoms through the plasma membrane. Genetically changed CFTR protein results in an accumulation of dense, gluey mucus in the lungs, digestive tract and reproductive system. This kind of mucus makes it problematic for patients to clear lung infections, which are the prominent reason of death in CF (http://www.lung.org, 2012). Compromised mucociliary clearance in lung makes CF patients susceptible to opportunistic infections (De Soyza *et al*., 2013).

Recent records from nonculture-based techniques put forward that the airway microbiome in CF is polymicrobial with numerous organisms existent (Blainey *et al*., 2012). The pathogens isolated by culture are mainly *Staphylococcus aureus, Haemophilus influenzae,* and *Pseudomonas aeruginosa*. Fewer microorganisms from the *Burkholderia cepacia* complex (Bcc) and other pathogens are also found (Davies & Rubin, 2007).

2.1.1 *Acanthamoeba*

Parts of the human respiratory tract also can be infected with protozoans such as *Acanthamoeba castellanii (A. castellanii)*, which cause eye and central nervous system diseases. *Acanthamoeba* are abundant in surroundings and identified in nasal cavity (Cerva *et al*., 1973), and pharynx of healthy individuals (Wang, 1961). The *Acanthamoeba* cysts are able to stand drying, which allows them to be airborne. (Im & Kim, 1998). On average, each human breathes in two cysts of *Acanthamoeba* per day (Kingston & Warhurst, 1969). Moreover, it is largely accepted that the routes of entrance for *Acanthamoeba* include the respiratory tract, enabling amoebic invasion of the alveolar capillaries, followed by distribution in the blood (Marciano-Cabral & Cabral, 2003).

2.1.2 *Acanthamoeba*-bacteria interactions

Acanthamoeba is the opportunistic representative of protists group which has been distinguished from various environments (De Jonckheere, 1991). *Acanthamoeba* living in water and soil feeds on bacteria living in the same ecosystem by engulfing and at the same time has essential ecological role, which is maintaining of number of bacterial populations (Khan, 2012).

Some bacterial strains have evolved strategies to defend themselves from *Acanthamoeba* phagocytosis by secreting soluble anti-Acanthamoebic materials in the medium (Jousset *et al*., 2010), or straight inserting such materials into the protoplasm of *Acanthamoeba* via the type III secretory system (Matz *et al*., 2008). Moreover, some bacteria mainly pathogenic ones, have evolved approaches to slip away the microbicidal strategies of *Acanthamoeba* and furthermore exploit the amoeba as a "Trojan horse" (Greub & Raoult, 2004). *Burkholderia cepacia* was shown to be able to survive in free-living amebae (Marolda *et al.*, 1999), as well as in *Acanthamoeba polyphaga* (Landers *et al.*, 2000). In another study it was revealed that *Burkholderia cepacia* survive intracellularly without replication within acidic vacuoles of *Acanthamoeba polyphaga* (Lamothe *et al.*, 2004). *P. aeruginosa* is another bacteria to demonstrate ability to survive within *Acanthamoeba* (José Maschio *et al.*, 2015).

These facts propose that *Acanthamoeba* and bacteria interact in the ecosystem constantly. However, what are the strategies and mechanisms of *Acanthamoeba* survival among overcrowded bacterial population keeps staying insufficiently conceived.

Accordingly, it is rational to assume that, bacteria are not only organisms to have antibiotics, *Acanthamoeba* also holds antibiotics to fight bacterial onslaught. The majority of antibiotics used nowadays have been developed according to these targeting strategies of microorganisms (Iqbal *et al*., 2014). The attacking of each other of these microorganisms is of great importance in finding new antibiotics where identification experiments of antibacterial potency of *Acanthamoeba* and anti-Acanthamoebic properties of employed bacteria were carried out.

In this project, the interaction patterns of bacteria such as MSSA, *H. influenzae, P. aeuriginosa, Str. pneumoniae, E. coli* and *Staphylococcus epidermidis* with human epithelial adenocarcinoma cell line (A549) and *Acanthamoeba* trophozoites have been studied. Furthermore, conditioned medium of *Acanthamoeba* was tested for its bacteriostatic and bactericidal potency; and conditioned media of employed bacteria were investigated to possess amoebistatic and amoebicidal activities.

2.2 Materials and Methods

All chemicals were purchased from Sigma-Aldrich (UK), unless otherwise stated.

2.2.1 *Acanthamoeba* culture and conditioned medium

A clinical isolate of *A. castellanii* (ATCC 50492) belonging to the T4 genotype, isolated from a keratitis patient, was kindly provided by Dr S. Maciver (University of Edinburgh, UK). *Acanthamoeba* was grown axenically without shaking in 10 mL of sterile PYG medium [proteose peptone 0.75 % (w/v), yeast extract 0.75 % (w/v) and glucose 1.5 % (w/v)] in T-75 culture flasks at 30 °C and medium refreshed 17-20 h prior experiments (Alsam *et al*., 2003). *Acanthamoeba* was maintained by weekly sub-culturing 5 $\times 10^5$ amoebae into 10 mL of PYG medium in T-75 tissue culture flasks. *A. castellanii* conditioned medium (ACM) was prepared by incubating confluent cultures ($\sim 2\times 10^6$ trophozoites/mL) for 48 h in PYG. Given confluent cultures, amoebae number increased slightly to $\sim 3\times 10^6$ trophozoites/mL. Following this, cell-free medium was collected by centrifugation (1000 × *g* for 5 min) and filtered using 0.22 µM pore size filters.

2.2.2 Bacterial cultures and conditioned media

Clinical bacterial strains such as MSSA, *Str. pneumoniae*, *P. aeruginosa*, *H. influenzae* *E. coli* and *S. epidermidis* were used in this study. All strains were kindly provided by Dr. Tony Elston from Colchester General Hospital. MSSA, *P. aeruginosa*, *E. coli* and *S. epidermidis* strains were streaked onto Cysteine Lactose-Electrolyte-Deficient (CLED) agar and incubated in 37 °C in air, and refreshed every week. One or two single colonies of MSSA, *P. aeruginosa*, *E. coli* and *S. epidermidis* were grown in Luria-Bertani broth (LB) and incubated overnight at 37 °C. The MSSA, *P. aeruginosa*, and *E. coli* optical densities (OD) were measured by spectrophotometer with wavelength 595 nM and optical density of 0.22 (OD595 0.22= $\sim 10^8$ CFU/ml), *S. epidermidis* with OD 0.35 (OD595 0.35= $\sim 10^8$ CFU/ml). *Str. pneumoniae* strains were streaked onto Blood agar, *H. influenzae* onto Chocolate agar and incubated in 37 °C in a 5 % CO_2-enriched incubator. One or two single colonies of *Str. pneumoniae* were grown in Brain Heart Infusion (BHI) and *H. influenzae* were grown in BHI Broth with Fildes Enrichment and incubated overnight at 37 °C in CO_2 incubator. OD of *Str. pneumoniae* and *H. influenzae* were measured by spectrophotometer with wavelength 595 and optical density of 1.06 (OD595 1.06= $\sim 10^8$ CFU/ml) and OD 0.22 (OD595 0.22= $\sim 10^8$ CFU/ml) respectively. Bacterial conditioned media (BCM) were

prepared by culturing single colonies (grown on nutrient agar plates) in 100 mL PYG medium and incubating for 24h at 37 °C aerobically, with shaking. These conditions resulted in growth of all bacterial cultures to stationary phase of their growth. The bacterial cultures were centrifuged at 4000 × *g* at 4 °C for 20 min. The supernatants were collected and filter sterilized using 0.22 μM pore size membrane filters.

2.2.3 Cell line and cell culture

Human epithelial lung carcinoma (A549) cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FCS), 1 % nonessential amino acids. A549 cell line was provided as proliferating cells in T-75 flask. Cells were passaged into three T-75 flasks and incubated at 37 °C in humidified 95 % air, 5 % CO_2. Theoretically, cells become confluent within 2 days (~$3x10^6$ cells). All passages which used in experiments were between passage numbers 7 to 13.

2.2.3.1 Passage cells into T-75 flasks

The growth medium was aspirated off from the confluent flask. Growing cells were washed off with phosphate-buffered saline (PBS) once, and followed by adding into the tissue culture flask 1 mL of accutase to detach cells. They were rocked for 1-2 minutes, and then the flask was tapped. Once the cells detached from the tissue culture flask, accutase was immediately inactivated by adding 9 mL of growth medium. The flask contents were transferred to 15 mL centrifuge tube. The sample was centrifuged for 5 min at 300 × *g*. The supernatant was discarded and the pellet was re-suspended in warm growth medium. In turn, suspended cells were passaged to three other flasks and incubated at 37 °C in humidified 95 % air, 5 % CO_2 incubator.

2.2.4 Association assays

To determine the interaction of bacterial strains with the A549 cell line and *Acanthamoeba* association assays were performed (Figure 2.1). In brief, A549 cells or *Acanthamoeba* were cultured and grown in 96-well plates in growth medium [DMEM for cell line and PYG for *Acanthamoeba*](~$2×10^5$ cells per 0.2 mL in each well). Plates were incubated till confluence. Growth medium was discarded, and Roswell Park Memorial Institute medium 1640 (RPMI 1640) (with Stable Glutamine and Phenol Red) was added for *Acanthamoeba*, DMEM for A549 cell line. Bacterial strains (~$2×10^6$ CFU/well) were

added onto cells with medium and incubated for 1h at room temperature. Supernatants were discarded and washed with PBS once to remove non-adherent bacteria. A549 cells and *Acanthamoeba* were lysed by adding 0.2 mL Cell Lytic M for 10 and 5 min, respectively. Finally, 20 µL of lysed solution were cultured onto agar (CLED, blood and chocolate), and incubated overnight at 37 °C O_2 incubator.

2.2.5 Invasion assays

Invasion assays were performed to determine the ability of bacteria to invade or be taken up by A549 cell line or *A. castellanii* (Figure 2.1). Briefly, A549 cells or *Acanthamoeba* were grown until confluent in 96-well plates as described above. After 1h of incubation, wells were washed with PBS, and then antibiotics were added to kill extracellular bacteria (Vancomycin with final concentration of 100 µg/mL for 45 min against MSSA, *Str. pneumoniae* and *S. epidermidis* and Ciprofloxacin with final concentration of 75 µg/mL against *E. coli, P. aeruginosa* and *H. influenzae* strains for 45 min). Finally, cells were lysed and bacteria cultured onto agar.

2.2.6 Intracellular survival assays

To determine the long-term fate of bacteria inside A549 cells or *Acanthamoeba,* intracellular survival assays were performed (Figure 2.1). Briefly, A549 cells or *Acanthamoeba* were incubated with bacteria, followed by the addition of antibiotic for 45 min, as described above. After incubation, wells were washed with PBS. A549 cells were incubated in 0.2 mL of DMEM, while *Acanthamoeba* were incubated in 0.2 mL RPMI 1640 for 24h at 30 °C. Finally, cells were lysed and cultured onto agar and counted the number of bacteria survived inside cells.

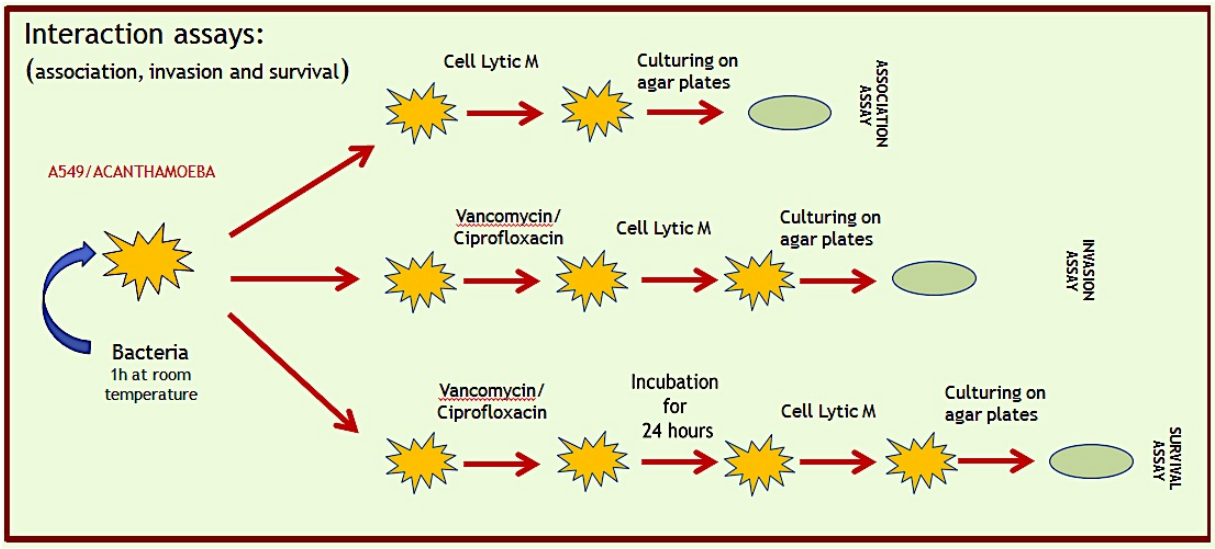


Figure 2.1 Steps of bacterial interaction assays (association, invasion and survival) with host cells (A549 cell line and *Acanthamoeba*).

2.2.7 Intracellular localization of MSSA and *P. aeruginosa*

To visualize MSSA and *P. aeruginosa* inside *A. castellanii* and A549 cell line, Rose Bengal stain was used. Briefly, a few colonies of each bacterium were inoculated into 10 mL LB medium and incubated at 37 °C overnight. Next day, bacteria were centrifuged at 300 x *g* for 10 min and the pellets were washed twice with PBS. Following centrifugation and washing, the pellets were suspended in Rose Bengal (1 mM final concentration in PBS) and incubated at 37 °C for 20 min. After incubation, the pellets were washed three times with PBS and bacterial fluorescence and viability were verified every 30 min for up to 3 h. The bacterial viability was determined by inoculation onto nutrient agar plates and examined under Nikon Optiphot fluorescence microscope. Next, 10^6 cells/mL *Acanthamoeba* trophozoites or A549 cells were detached, centrifuged (300 x *g* for 3 min) and washed twice with PBS. Then, 10 000 bacteria per cell were added in a final volume of 500 µL RPMI-1640 medium and incubated for 1h at 30 °C for amoebae and 37 °C in 5 % CO_2 for A549 cells. Following 1h of co-culture, cells were harvested by centrifugation (300 x *g* for 3 min) and pellets were re-suspended in 100 nM Lyso Tracker Green (Molecular Probes) for 20 min at 30 °C. Lyso Tracker Green stains acidic organelles green. Cell suspensions with intracellular bacteria were pipetted on slides followed by coverslip application for 5 min at room temperature to allow the infected cells to settle and form a monolayer. The intracellular localization of bacteria was assessed using the fluorescence microscope.

2.2.8 Transmission electron microscopy

Images were obtained in collaboration with Dr. Matthew Hannah, Lead Electron Microscopist in the Virus Reference Department of National Infection Service, Public Health England in London. All samples were prepared in Medical Microbiology laboratory, School of Biological Sciences, Essex University. Afterwards, samples were taken to Dr. Matthew Hannah for getting micrographs using Electron Microscope.

Bacteria were centrifuged (10 min at 300 x *g*) and washed three times. The pellets obtained were fixed in 2.5 % glutaraldehyde (v/v) (in 0.1 M sodium cacodylate buffer, pH = 7.2) and incubated for 60 min at room temperature. Next, the samples were centrifuged (10 min at 300 x *g*) and re-suspended in 0.1 M sodium cacodylate buffer. This step was repeated twice to remove the fixative agent. Then, the pellets were washed again in 0.1 M sodium cacodylate buffer as mentioned above and embedded in 3 % Nobel agar prior to

being cut into section of 1-2 mm cubes. Bacteria were dehydrated using graded series of ethanol. Afterwards, the pellets were infiltrated with London Resin (LR) white resin and polymerized at 67 °C for 24 h. Sections of 80-100 nm were cut using a NOVA ultramicrotone, stained with lead citrate for 20 min, rinsed with distilled water and stained with uranyl acetate for 5 min. After drying, the grids were imaged in a JEOL JEM-1400 TEM operating at 120 kV. Images were acquired using an AMT XR60 digital camera.

2.2.9 PCR-based studies for capsular gene identification

2.2.9.1 Identification of capsular gene *bexB* in *H. influenzae*

The design of PCR primers (Table 2.1) targeting a 567- or 760-bp region of *H. influenzae bexB* was based on sequence data available in GenBank. Primers targeting *bexA*, *pepN* (which served as a DNA positive control), and serotype-specific capsule genes have been described (McCrea *et al.*, 2010). Whole-cell lysate, i.e., crude lysate, served as the DNA template source for PCRs. It was prepared by suspending several bacterial colonies, from a fresh plate into Tris-EDTA (TE; pH 7.5), heating them for 10 min at 100 °C in a thermocycler, centrifuging them for 10 min. Next, the supernatant was removed and the rest was stored at −20 °C until it was used for PCR. PCR was performed with *Taq* polymerase. Each 20 μL PCR mixture consisted of 1× ThermoPol reaction buffer, a 0.2 mM concentration of each deoxynucleoside triphosphate (dNTP), a 0.2 μM concentration of each primer, 2.0 U *Taq*, and 1 μL crude lysate (template). Reaction mixtures were subjected to an initial denaturation step of 2 min at 95 °C followed by 30 amplification cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 45 s. Amplicons were separated in 2 % agarose gels in 1× Tris-acetate-EDTA (TAE) buffer. Gels were stained with Safeview dye (5 μL), and gel images were recorded. The sizes of the PCR products were determined by comparison with the molecular size standard (1 Kb DNA ladder).

2.2.9.2 Identification of capsular gene *cpsA* in *Str. pneumoniae*

Pneumococcal isolates were retrieved from storage by subculture on blood agar plates (blood agar base supplement with 5 % sheep blood) and incubated overnight at 37 °C in 5 % CO_2. Bacterial cells were suspended in 250 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The suspension was heated at 100 °C for 5 min (Lawrence *et al.*, 2003)

and immediately frozen at −20 °C for 5 min. These lysates were stored at −20 °C until further use. The PCRs were performed in 25 µL volumes, with each reaction mixture containing the following: 1× PCR buffer (Solution, 100 mM Tris-HCl, 500 mM KCl), 200 µM of each deoxynucleoside triphosphate, 2.5 mM of $MgCl_2$, 2.0 U of *Taq* DNA polymerase, and primers (Table 2.2). Crude extract (2.5 µL) was used as the DNA template for each PCR. Thermal cycling was performed under the following conditions: 94 °C for 4 min followed by 30 amplification cycles of 94 °C for 45 s, 54 °C for 45 s, and 65 °C for 2 min 30 s. The PCR products were analyzed by gel electrophoresis on 2 % agarose gels in 1× TAE buffer (40 mM Tris, 20 mM of glacial acetic acid, 1 mM EDTA, pH 8.0) at 120 V for 45 min. Gels were stained with Safeview dye (5 µL), and gel images were taken. The sizes of the PCR products were determined by comparison with the molecular size standard (1 Kb DNA ladder).

2.2.10 Inhibition of *Acanthamoeba* growth by bacterial conditioned media

To determine amoebistatic activity of BCM, *A. castellanii* trophozoites (10^5 amoebae/0.2 mL/well) were incubated in PYG with different amounts of BCM (in 96 well plates at 30 °C for 48 h). After this incubation, amoebae were counted using a haemocytometer. In controls, *A. castellanii* trophozoites were incubated in PBS and in PYG

2.2.11 Amoebicidal effects of bacterial conditioned media

For amoebicidal activity of BCM, *A. castellanii* trophozoites (10^6 amoebae/0.2 mL/well) were incubated in PYG with different amounts of BCM in 96 well plates at 30 °C for 48 h. Amoebae viability was determined by adding 0.1 % Trypan blue and number of live (non-stained) and dead (stained) *A. castellanii* were enumerated using a haemocytometer.

Table 2.1 PCR primers used for *H. influenzae* capsule identification

Target	Primer	Primer sequence (5' to 3')	Expected size (bp)	Reference
bexB	bexB.1F	GGTGATTAACGCGTTGCTTATGCG	567	(Davis *et al.*, 2011)
	bexB.1R	TTGTGCCTGTGCTGGAAGGTTATG		
	bexB.FLF	TCATTGTGGCTCAACTCCTTTACT	760	(Davis *et al.*, 2011)
	bexB.FLR	AGCTATTCAAGGACGGGTGATTAACGC		
pepN	pepN_F	GATGGTCGCCATTGGGTGG	918	(Ecevit *et al.*, 2004)
	pepN_R	GATCTGCGGTTGGCGGTGTGG		

Table 2.2 PCR primers used for *Str. pneumoniae* capsule identification

Target	Primer	Primer sequence (5' to 3')	Expected size (bp)	Reference
cpsA	cpsA-f	GCAGTACAGCAGTTTGTTGGACTGACC	160	(Pai *et al.*, 2006)
	cpsA-r	GAATATTTTCATTATCAGTCCCAGTC		

2.2.12 Cytotoxic effects of ACM and BCM on A549 cells.

The conditioned media (100 µL) or PYG medium (100 µL) were added in 100 µL RPMI-1640. Plates were incubated at 37 °C in a 5 % CO_2 incubator and monitored for monolayer disruptions over the period of 24 h. After this incubation, the supernatants were collected from each well, centrifuged to remove cellular debris and then cytotoxic effects were determined by estimating the amount of lactate dehydrogenase released from A549 cells using a Cytotoxicity Detection kit (Roche Applied Sciences). The cytotoxicity percentage was calculated as follows: % cytotoxicity = (sample value – control value) / (total LDH release – control value) × 100 (Khan, 2007). Control values were determined by incubating A549 cells in RPMI 1640 alone and total LDH release was obtained by completely lysing the A549 cells using 1 % Triton X-100.

2.2.13 Bactericidal effects of Acanthamoebic conditioned media

To determine the effects of ACM on bacteria, clinical isolates of bacteria were used. Approximately 10^6 colony forming units (suspended in 20 µL) were incubated with 180 µL of ACM at 37 °C for 18 h. After incubation, cultures were serially diluted in PBS and plated onto agar plates. Plates were incubated at 37 °C, overnight and bacterial C.F.U. were enumerated (Lee *et al.*, 2011). For controls, bacteria were incubated in PYG alone and PBS alone.

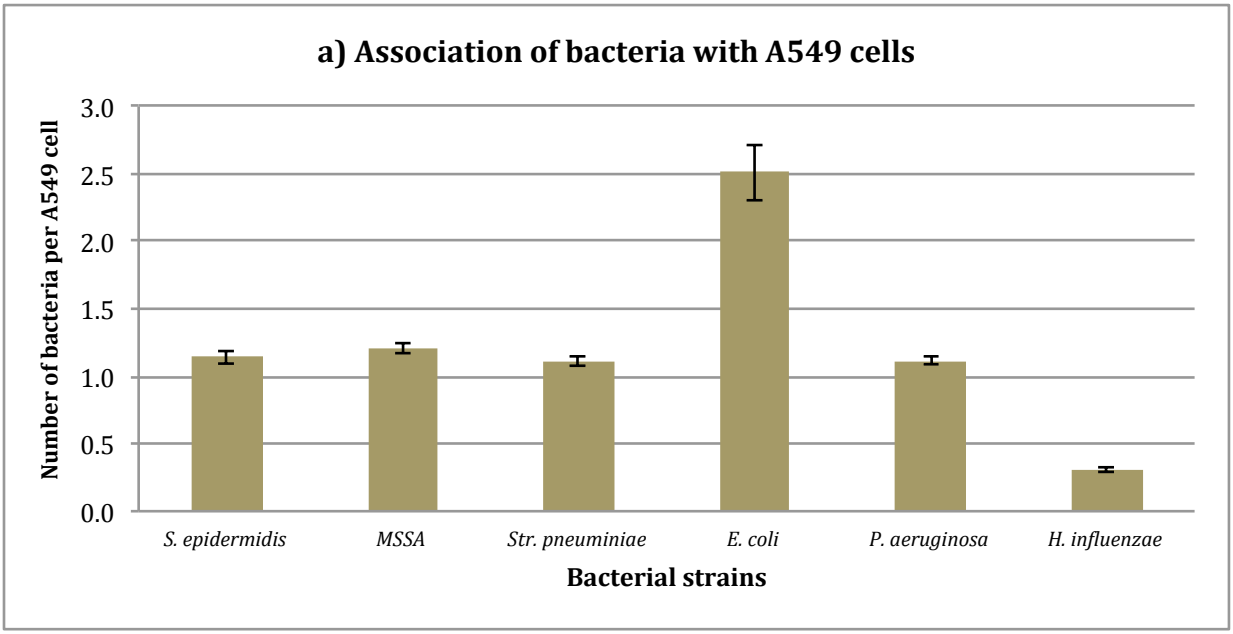
3.2.14 Presentation of results and statistical analysis.

The results shown in this work are representative of three independent experiments performed in triplicate by identical methods. The results are expressed as means ± 1 standard deviation. Differences were considered statistically significant if the *P* value was <0.05 by Student's *t* test.

2.3. Results

2.3.1 Interaction assays

Association assays were performed to determine the ability of bacteria employed in these experiments to associate with the A549 cell line and *A. castellanii*. Our findings show that *H. influenzae* strains exhibited the least association with both A549 cells and *A. castellanii,* (0,4 and 0,3 bacteria per host cell, respectively). A significant association with *Acanthamoeba* was with MSSA, and for A549 cell line with *E. coli* (control for Gram-negative bacteria) with indices of 7 and 2.5 bacteria per cell respectively. However *S. epidermidis* (control for Gram-positive bacteria) showed a similar association with A549 cells, compared to pathogenic Gram-positive bacteria (Figure 2.2). Association of *E. coli* with *Acanthamoeba* was significantly higher than other pathogenic Gram-positive bacteria (one tail *t-test P<0.05*). The number of associated bacteria per *Acanthamoeba* was: 1.5; 0.5; 2.9; and 1.5 for *P. aeruginosa*; *Str. pneumoniae*; *E. coli*; and *S. epidermidis,* respectively. And associated bacteria per A549 cell was: MSSA 1.2; *P. aeruginosa* 1.1; *Str. pneumoniae* 1.1; and *S. epidermidis* 1.1.



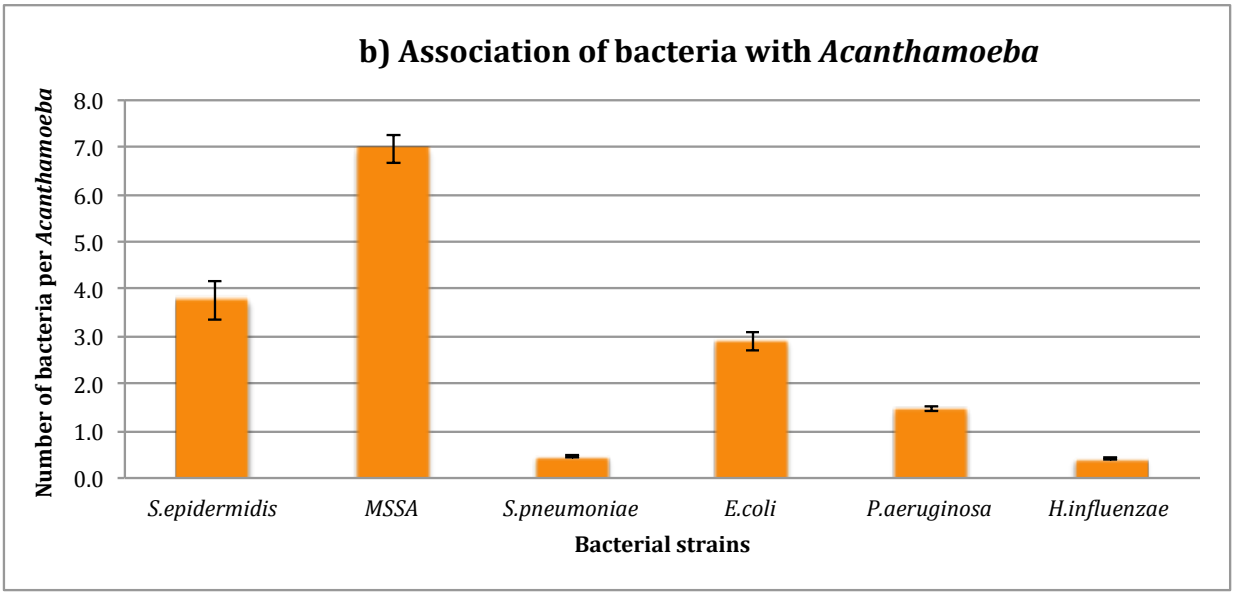


Figure 2.2 The number of bacteria associated per A549 cell (a) and *A. castellanii*. (b). Results are the mean of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

To define whether bacteria entered A549 cells and *Acanthamoeba*, invasion assays were carried out. All bacterial strains could be recovered from *Acanthamoeba,* but not from A549 cells. From *Acanthamoeba*, MSSA, *S. epidermidis*, *E. coli and P. aeruginosa* showed the highest recovery (1.99; 1.43; 1.18; and 0.82 bacteria per *Acanthamoeba*, respectively) compared with *H. influenzae* and *Str. pneumoniae*. The number of invaded *H. influenzae* and *Str. pneumoniae* were 0.02 and 0.01 per *Acanthamoeba*. The highest number of bacteria invading each A549 cell was exhibited by *E. coli* 1.84. MSSA, *H. influenzae*, *Str. pneumoniae* and *S. epidermidis* had lower ability of recovery with indices 0.409; 0.029; 0.002; and 0.166 per cell (Figure 2.3).

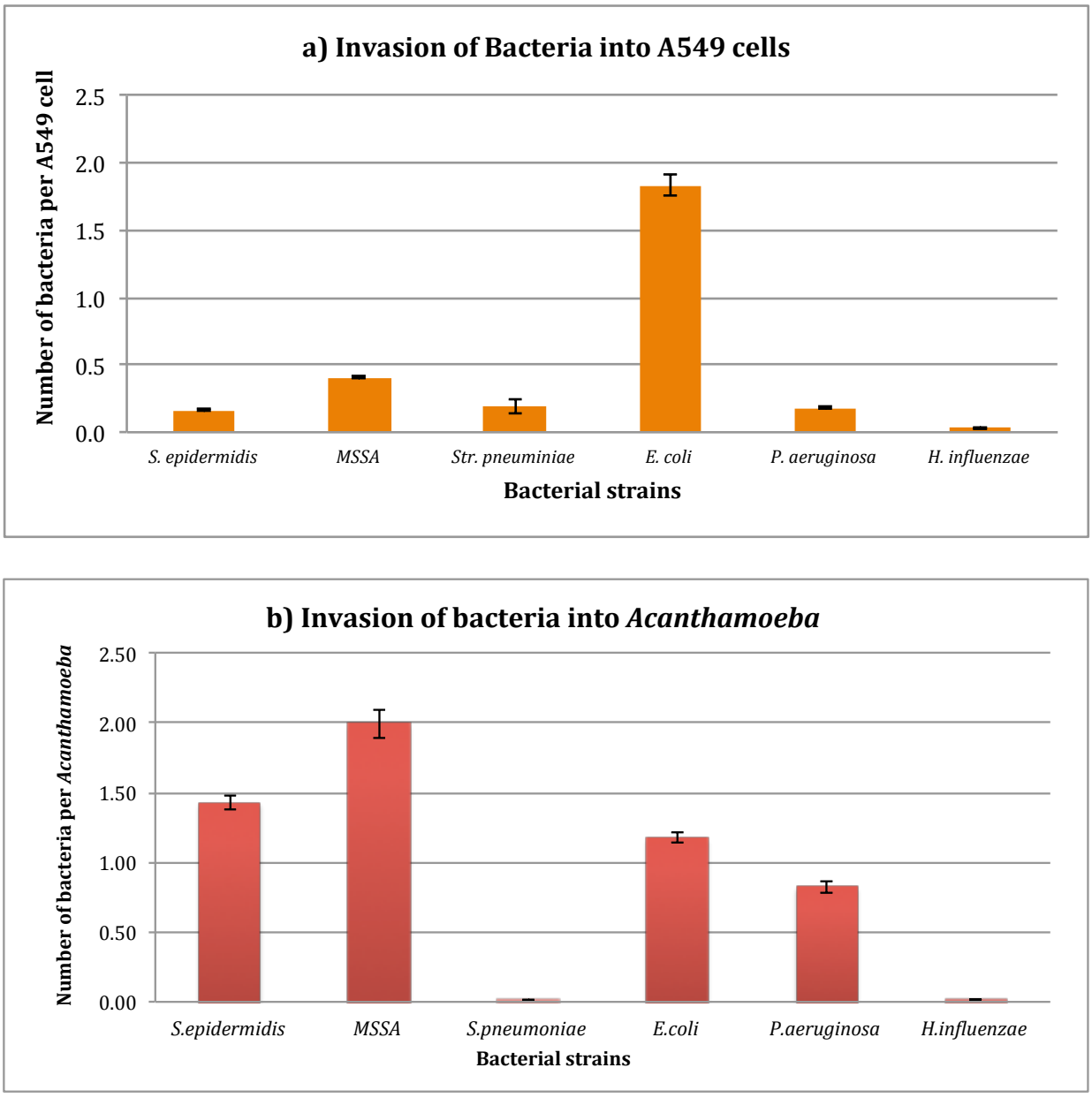


Figure 2.3 The number of bacteria invading each A549 cell (a) and *A. castellanii*. (b). Results are the mean of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

To visualize MSSA and *P. aeruginosa* inside *Acanthamoeba* and A549 cells, bacteria were stained with Rose Bengal and *Acanthamoeba* with lysotracker. Following 1h co-culture at a multiplicity of infection of one cell: 10 000 bacteria, fluorescence microscopy showed that amoebae and A549 cells were infected with MSSA or *P. aeruginosa*. Microscopy showed that most of host cells contained bacteria within phagolysosomes. Bacteria were able to invade or be engulfed by amoebae and A549 cells (Figure 2.4) within 1h of incubation and withstand acidic conditions. The infected A549 cells and amoebae also stain green throughout the cell suggesting that perhaps phagolysosomal fusion does occur before the bacterium escapes the phagolysosome.

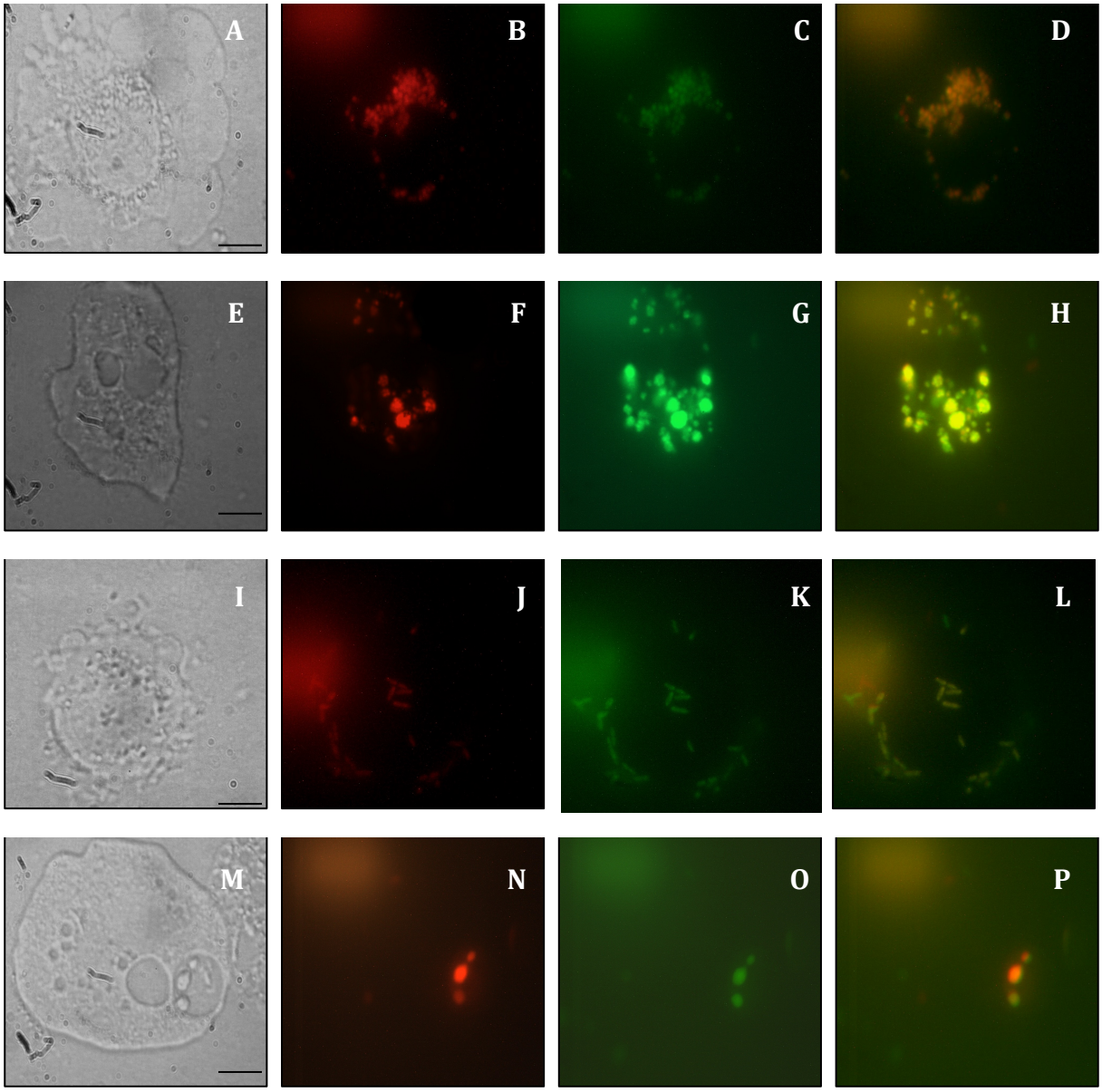
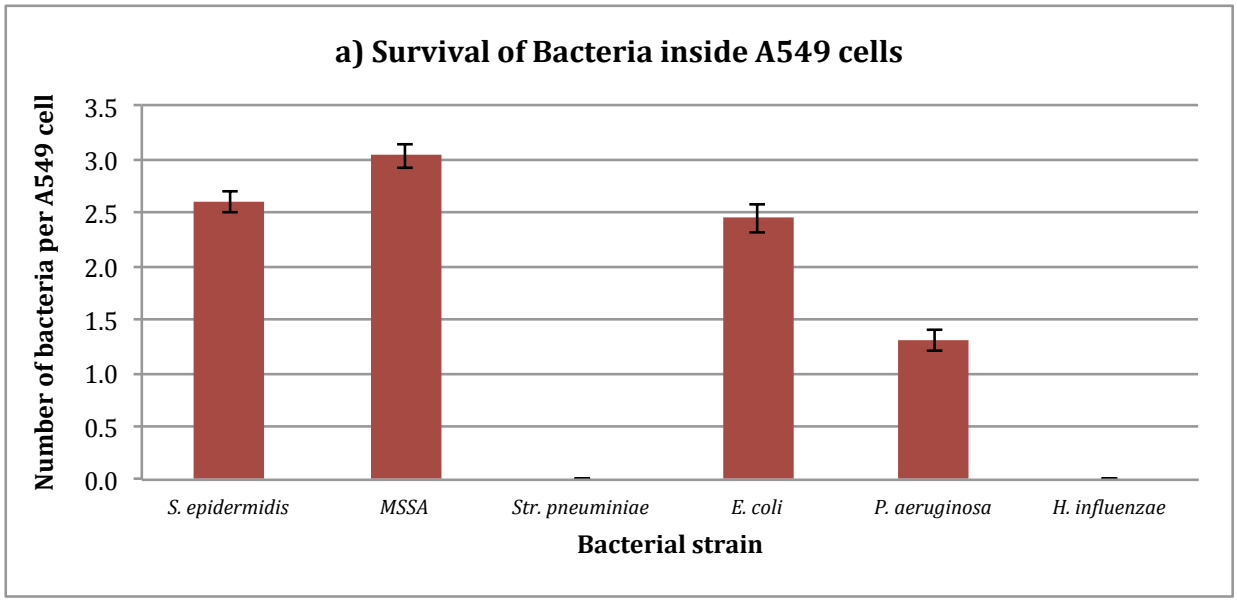


Figure 2.4 Localization of bacteria within A549 cell, and *Acanthamoeba* trophozoite. Fluorescence microscopy images of MSSA in A549 cell (A-D); MSSA in *A. castellanii* (E-H); *P. aeruginosa* in A549 cell (I-L); and *P. aeruginosa* in *A. castellanii*. MSSA (B, F), *P. aeruginosa* (J, N) are stained red, and acidic vacuoles are stained green (C, G, K & O) with the corresponding overlay, which is yellow/orange (D, H, L & P). Bar = 10 µm.

To determine the number of bacteria inside A549 cells and *Acanthamoeba*, survival assays were carried out, by incubating A549 cells or *Acanthamoeba,* with internalized bacteria, for 24 h. Our results indicated that *H. influenzae* and *Str. pneumoniae* were not able to survive and multiply in A549 cells or *Acanthamoeba*, while *P. aeruginosa* did not survive in A549 cells. The number of MSSA, *E. coli* and *S. epidermidis* per A549 cell was 3.7; 2.9; and 3.9 respectively. Interestingly, the number of bacteria inside each *Acanthamoeba* for MSSA, *P. aeruginosa*, *E. coli* and *S. epidermidis* was 967, 1390, 753 and 1553 respectively (Figure 2.5).



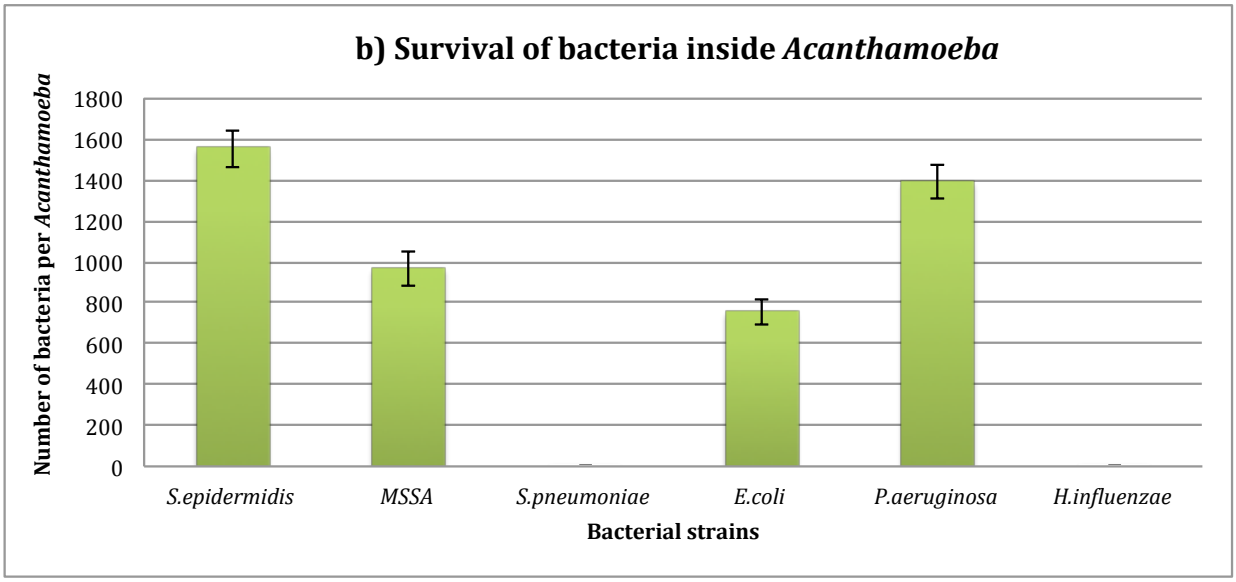


Figure 2.5 The number of bacteria surviving inside A549 cells (a) and *A. castellanii* (b) after 24 h of incubation. Results are the mean of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

To determine if low survival of *Str. pneumoniae* and *H. influenzae* was due to capsule presence, Electron Microscopy studies were carried out. Electron micrographs demonstrated capsular polysaccharides (Figure 2.6).

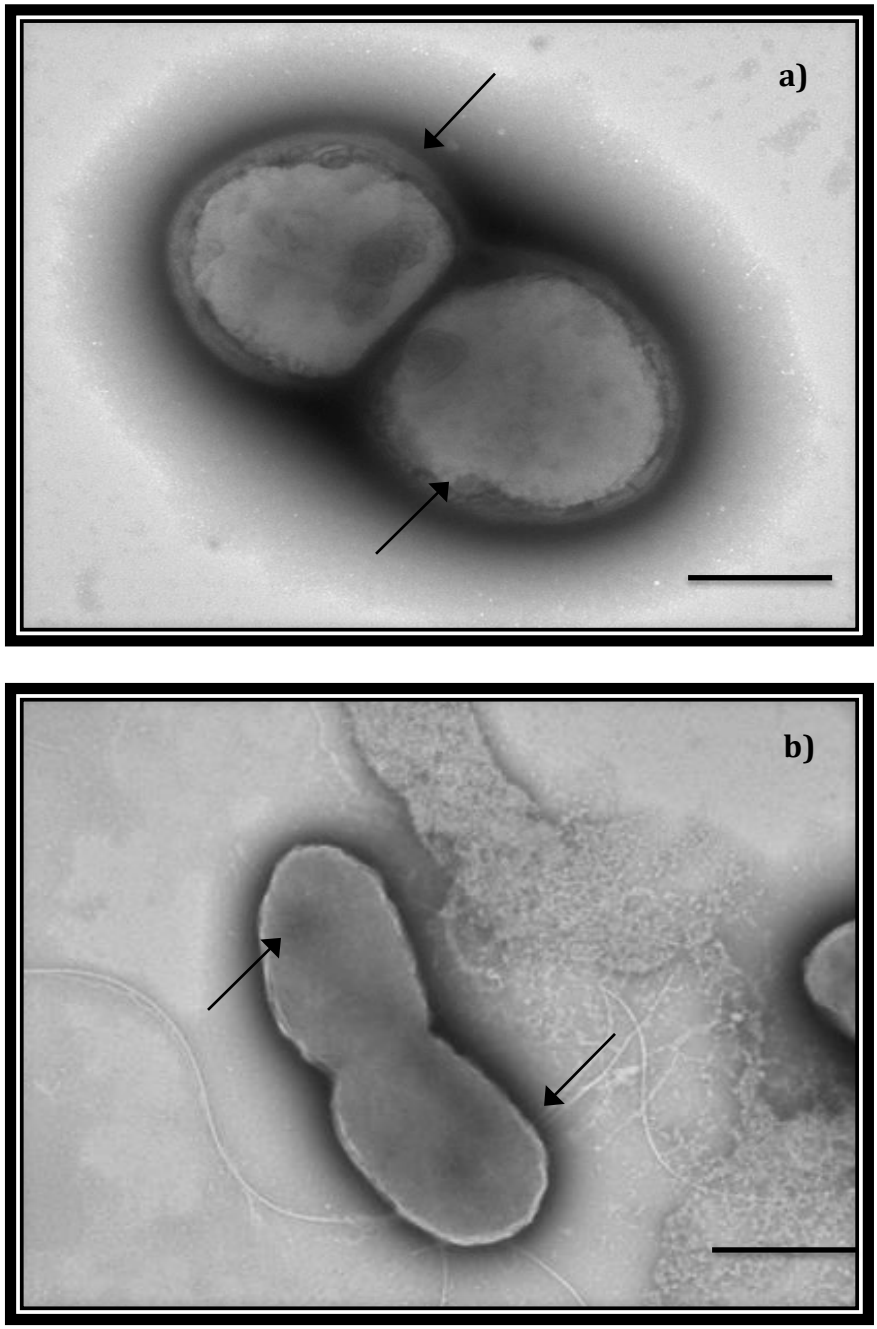


Figure 2.6 Identification of bacterial capsules by Electron Microscopy. Electron micrographs of *Str. pneumoniae* (a); and *H. influenzae* (b). Black arrows indicate polysaccharide capsules. Bars, 200 nm.

To further analyse if *Str. pneumoniae* and *H. influenzae* are encapsulated PCR-based studies were carried out. Capsular gene loci were targeted in both bacterial genotypes. Results revealed the presence of capsular genes (Figure 2.7 for *Str. pneumoniae* and 2.8 for *H. influenzae*).

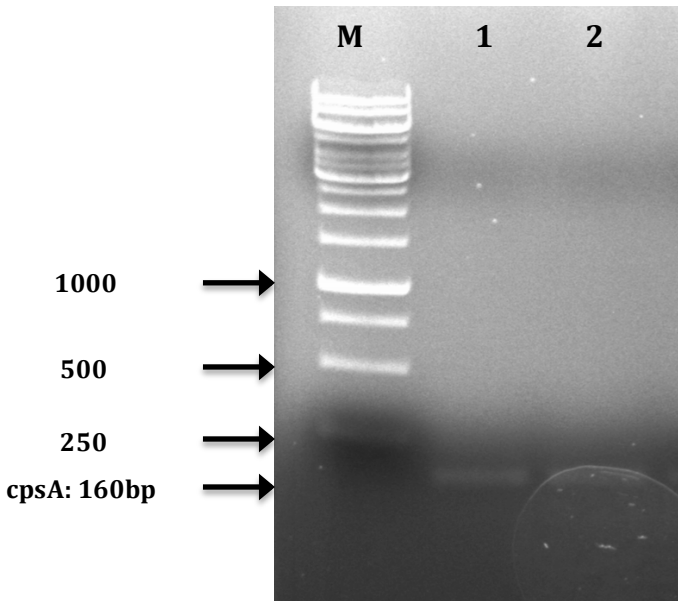


Figure 2.7 Molecular identification of *Str. pneumoniae* capsules by detection of *cpsA* gene. Lane M, 1 kbp ladder; Lane 1, Control strain (*Str. pneumoniae* ATCC6305); Lane 2, alpha-hemolytic isolate detected as encapsulated *S.pneumoniae*.

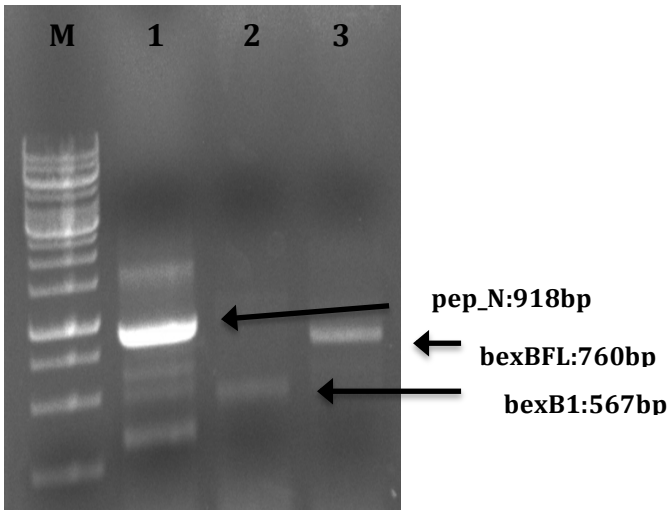


Figure 2.8 Molecular identification of *H. influenzae* capsules by detection of *bexB* gene. Lane M, 1 kbp ladder; Lane 1, Primer (pep_N) targeting *pepN* gene (which served as a DNA positive control); Lane 2, Primer (bexB.1) targeting *bexB* gene; Lane 3, Primer (bexB.FL) targeting *bexB* gene.

2.3.2 Inhibition of *Acanthamoeba* growth by bacterial conditioned media

Among the various bacterial isolates investigated, *Str. pneumoniae, P. aeruginosa* and *H. influenzae* BCM inhibited *Acanthamoeba* growth (75 %, 82 % and 80 % amoebistatic effects respectively). To identify whether amoebistatic effects of BCM were because of nutrient-depletion, *A. castellanii* trophozoites (10^5 amoebae in 100 µL PYG) were incubated with 100 µL of PBS in a 1:1 ratio. The results showed amoebae growth profiles, in 50 % PBS, similar to amoebae incubated in neat PYG (Figure 2.9).

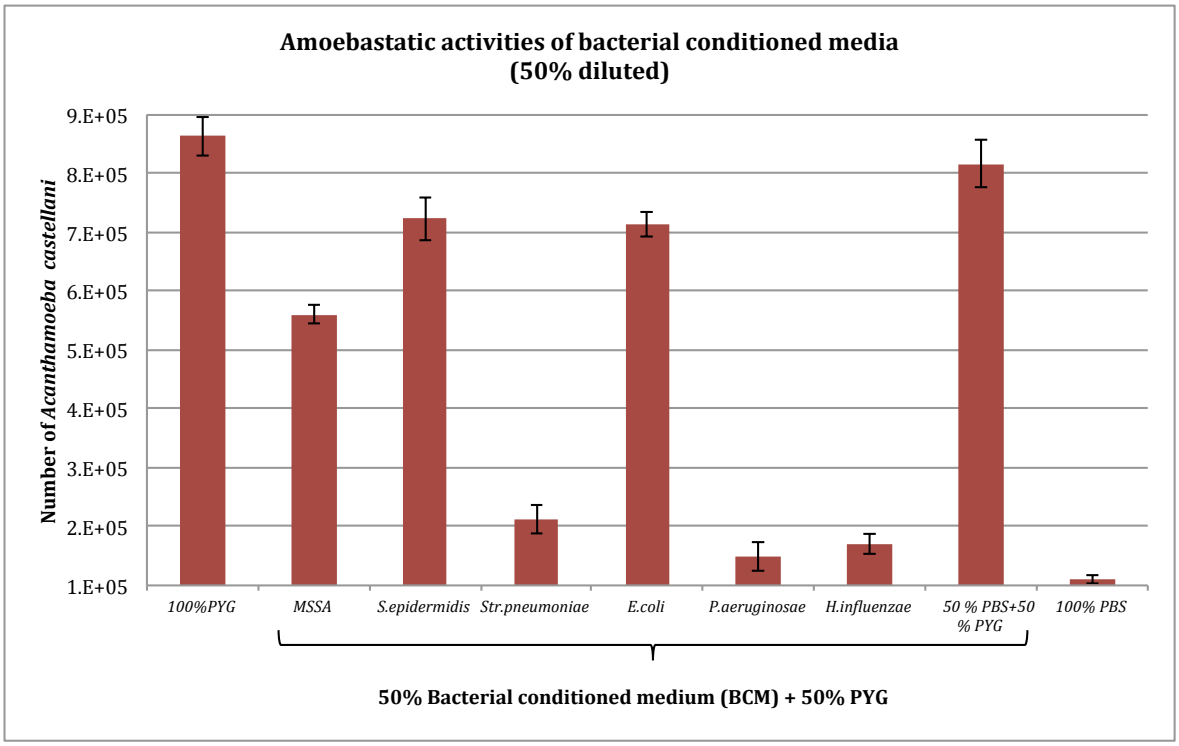


Figure 2.9 Amoebastatic activities of bacterial conditioned media. Results are the mean of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

Among other bacteria, BCM of MSSA, *S. epidermidis* and *E. coli* also inhibited *A. castellanii* growth (35 %, 16 % and 18 % amoebistatic effects respectively). In order to address the possibility of conversion of *Acanthamoeba* trophozoites into cysts after BCM treatment, BCM-treated *Acanthamoeba* trophozoites were incubated with 0.5 % SDS for 10 min, which is known to selectively lyse trophozoites. Post-SDS treatment, none of the BCM-treated *Acanthamoeba* exhibited cyst presence. To identify the activity of BCM of bacteria employed in this study, additionally 25 % diluted BCM was prepared and used against *A. castellanii*. The results demonstrated that BCM of *Str. pneumoniae, P. aeruginosa* and *H. influenzae* exhibited amoebistatic effects in a concentration-dependent manner (Figure 2.10).

2.3.3 Amoebicidal effects of bacterial conditioned media

Diluted at 1/2 (50 % concentration), *Str. pneumoniae, P. aeruginosa* and *H. influenzae* BCM were ineffective against *A. castellanii* viability (0.3 %, 0.8 and 0.7 % amoebicidal effect respectively), while undiluted (at 100 % concentration), BCM of *Str. pneumoniae, P. aeruginosa* and *H. influenzae* displayed more than 90 % amoebicidal effects (Figure 2.11).

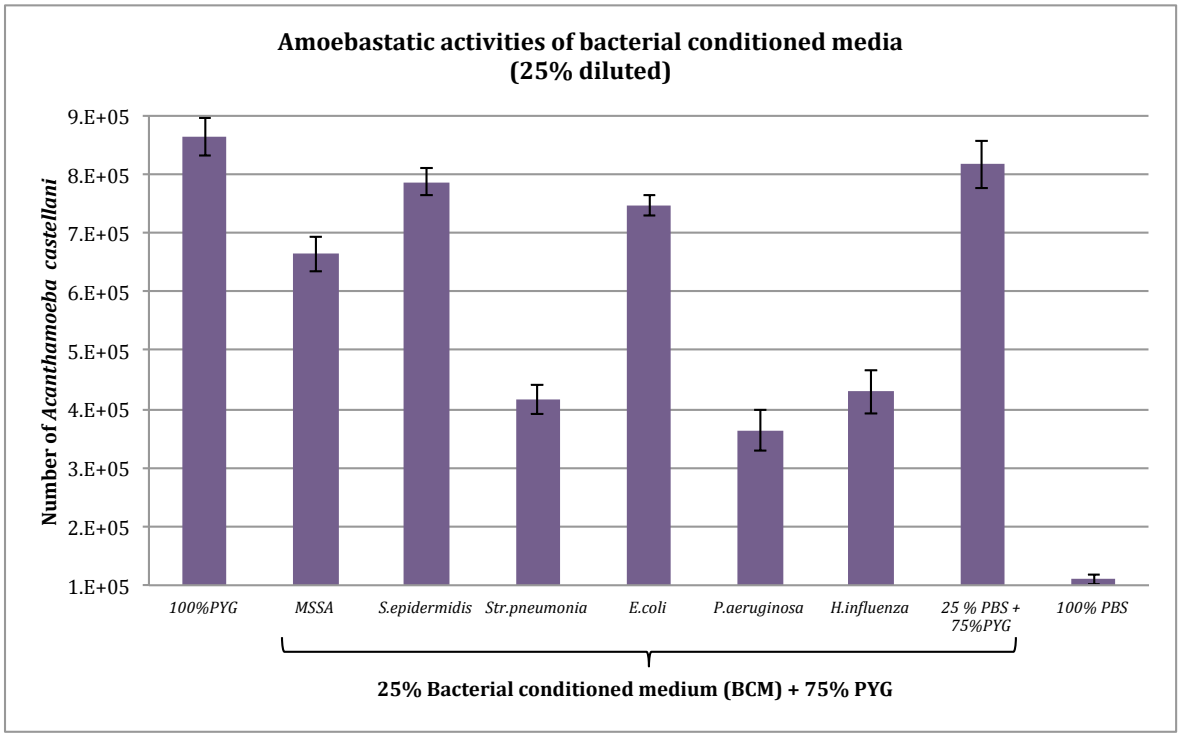


Figure 2.10 Amoebastatic activities of bacterial conditioned media. Results are the mean of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

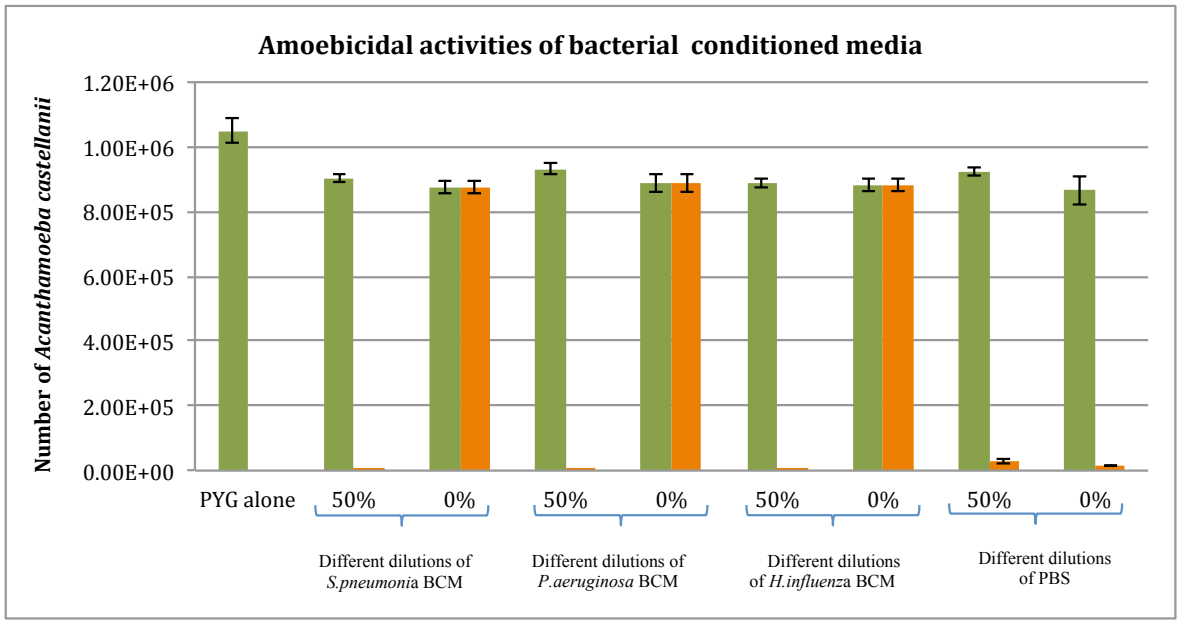


Figure 2.11 Amoebicidal activities of bacterial conditioned media. Green bars indicate the total number of *Acanthamoeba* in PYG only. The orange bars represent the number of dead *Acanthamoeba* in various dilutions of BCM (50 % diluted and non-diluted BCM) with either PYG or PBS. Results are the mean of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

2.3.4 Cytotoxic effects of Acanthamoebic and bacterial conditioned media on A549 cells.

Cytotoxicity assays were performed to define the activities of the *Acanthamoebic* and bacterial (MSSA, *S. epidermidis, Str. pneumoniae, E. coli, P. aeruginosa and H. influenzae*) conditioned media on A549 cells. Neither ACM nor BCM exhibited significant host cell cytotoxicity (9.3 %, 5.3 %, 4.5 %, 6.2 %, 9 %, 6.5 % and 7.1 % host cell death respectively). As BCM was prepared using PYG, similar volumes of PYG alone (100 µL) were tested as controls. The results showed that PYG alone exhibited no cytotoxicity on A549 cells (1.2 %) (Figure 2.12).

2.3.5 Bactericidal effects of Acanthamoebic conditioned media

Among the various bacterial strains investigated, ACM inhibited 100 % growth of *Str. pneumoniae* and slight bactericidal effects against MSSA, *S. epidermidis, E. coli, P. aeruginosa* and *H. influenzae* (10.6 %, 3.4 %, 6.1 %, 11.1 % and 2.5 %, respectively) (Figure 2.13).

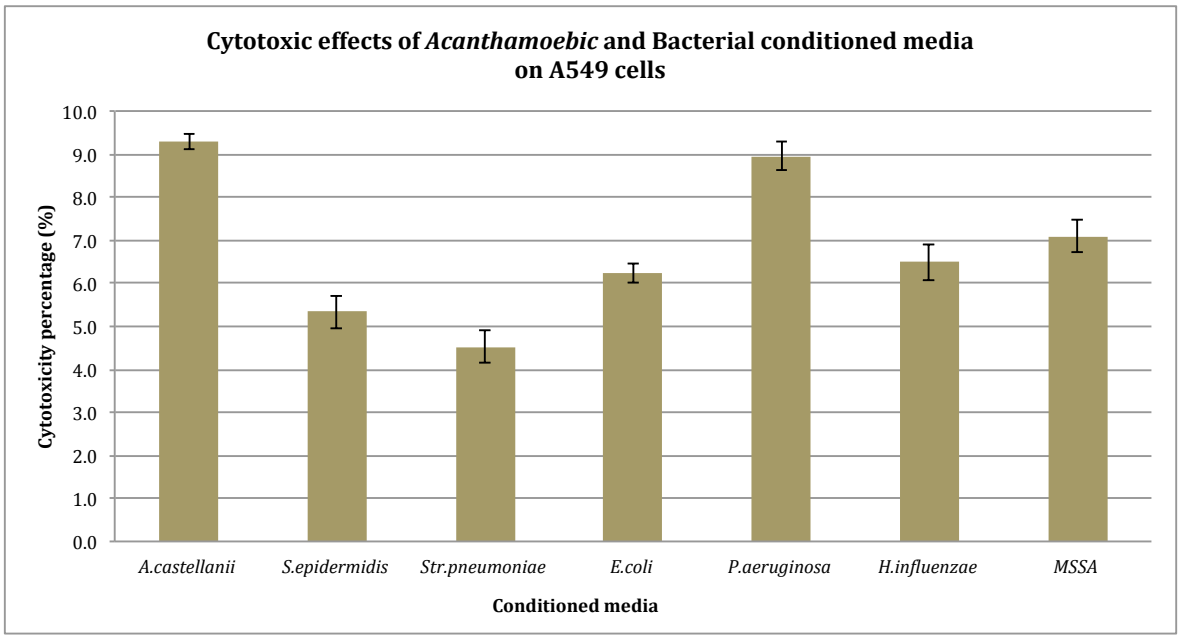


Figure 2.12 Cytotoxic effects of *Acanthamoebic* and bacterial conditioned media on A549 cells. Results are the mean of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

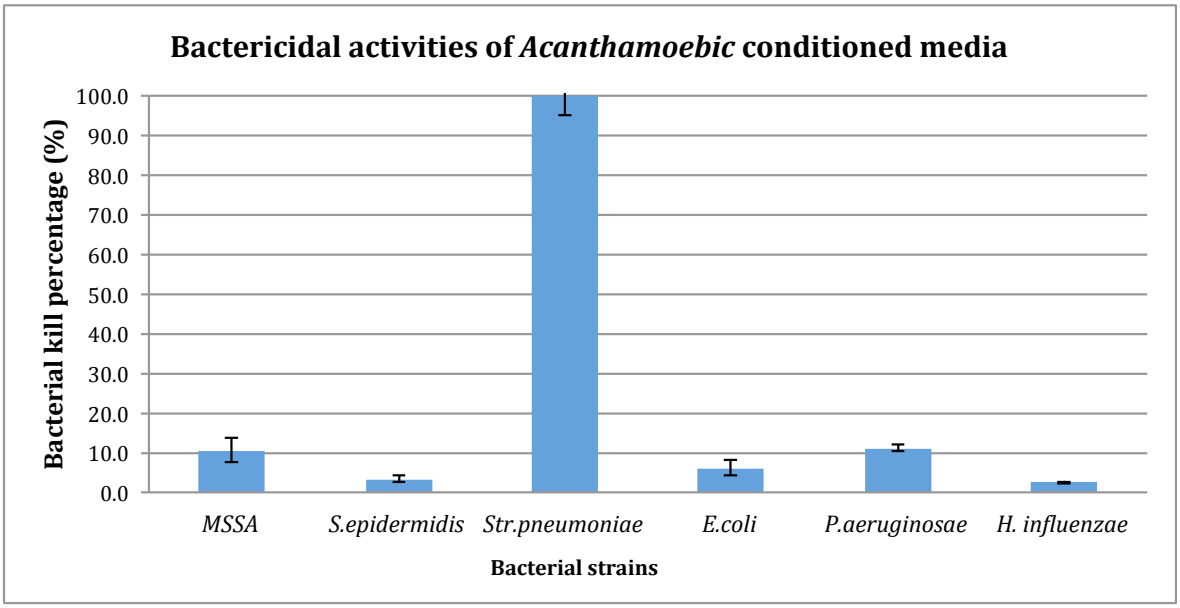


Figure 2.13 Bactericidal activities of *Acanthamoebic* conditioned media. Results are the mean of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

2.4. Discussion

It is well known that several species of bacterial pathogens are associated with CF and that they are major causes of morbidity and mortality among patients. However, knowledge of pathogenesis and pathophysiology of these infections as well as the number of effective treatments remain insufficient. A fundamental question is how bacteria interact with epithelial cells of the respiratory tract, causing at the same time infections. Moreover, the presence of *Acanthamoeba* in the respiratory tract could contribute in exacerbation of pulmonary infections. For this reason, in this project, human epithelial lung carcinoma (A549) cell line, *Acanthamoeba* trophozoites and six types of bacteria (pathogenic MSSA*, H. influenzae, Str. pneumoniae, P. aeruginosa, nonpathogenic S. epidermidis* and *E. coli*) isolated from CF patients were studied. To identify if bacteria interact with A549 cells or *Acanthamoeba*, interaction assays were carried out. The results clearly showed that all bacteria used have the ability to associate with host cells. However, *H. influenzae and Str. pneumoniae* exhibited very low ability to invade and no ability to survive within A549 cells and *Acanthamoeba* trophozoites.

In vitro interaction assays showed that, *S. aureus* and *P. aeruginosa* possess the capability to adhere, invade and survive within epithelial cells (Ichikawa *et al.*, 2000). Virulence factors of *S. aureus*, such as protein A, elastin-binding protein, collagen-binding protein, fibronectin-binding protein and clumping factor, are associated with adherence and invasion of epithelial cells (Gordon & Lowy, 2008). Pili and LPS of *P. aeruginosa* have a role in binding to and invading epithelial cells (Ben Haj Khalifa *et al.*). Several lines of evidence have indicated that amoeba serve as a host to a number of human pathogens, including *Mycobacterium avium* (Steinert *et al.*, 1998), *Legionella pneumophila* (Kilvington & Price, 1990), *Escherichia coli* (Alsam *et al.*, 2006), *Enterobacter aerogenes*, *Aeromonas hydrophila* (Yousuf *et al.*, 2013), *P. aeruginosa* (Michel *et al.*, 1995) and MRSA (Cardas *et al.*, 2012a) and are able to accommodate bacterial intracellular replication and augment their virulence properties pertinent to susceptible hosts (Abd *et al.*, 2010).

Although a lot is known about bacterial strains employed in this project, performing further characterization assays could help to conduct broader investigations.

As it has been shown in this project, *Acanthamoeba* takes up bacteria through phagocytosis, and employs phagolysosomes for lysing. Although *Acanthamoeba* feeds on

both Gram-positive and Gram-negative bacteria, they prefer Gram-negative bacteria, which are widely used as a food source to isolate *Acanthamoeba* (Khan, 2006). Proteases produced by *Acanthamoeba* are used as significant markers to differentiate between pathogenic and non-pathogenic *Acanthamoeba* species (Khan *et al.*, 2000; Sissons *et al.*, 2006; Dudley *et al.*, 2008). *Acanthamoeba* proteases have antimicrobial effects against different types of bacteria including MRSA and *P. aeruginosa* (Iqbal *et al.*, 2014). Our findings indicate that *Acanthamoeba* (genotype T4), was able to host *S. epidermidis*, MSSA, *E. coli* and *P. aeruginosa*. This is interesting as this could play role in infections associated with CF. Moreover, bacteria can remain and multiply inside *Acanthamoeba* within a harsh environment, where they can turn to cysts that may help transmit bacterial pathogens to susceptible hosts and also to cause reinfection.

Using PCR method in present study it was demonstrated that *Str. pneumoniae* and *H. influenzae* are encapsulated. Bacterial capsules play important roles in virulence, including preventing phagocytosis (Merino & Tomás, 2015). Thus, *H. influenzae* capsule inhibits ingestion by macrophages (Noel *et al.*, 1992). Encapsulated *H. influenzae* are more virulent than non-encapsulated *H. influenzae* (NTHi) strains (Hallström & Riesbeck, 2010). Data obtained in a recent study have shown that non-encapsulated *Str. pneumoniae* is susceptible to phagocytosis by neutrophils, whereas polysaccharide capsules were able to inhibit complement activity, which resulted in resistance to phagocytosis (Hyams *et al.*, 2010). Current project demonstrated that the outcome of bacteria interactions with *A. castellanii* and an epithelial cell line, are related to the virulence of bacteria.

The existence of antimicrobial substances in microorganisms is not an unusual fact and they are accepted to be an ancient defence system contrary to the progression of other microorganisms. From the time when penicillin was discovered in nature produced by mould, *Penicillium* in 1928 (Fleming, 1946), a great quantity of microorganisms have been checked for the isolation of hypothetically advantageous antimicrobials, while there has been effort in searching for new antibiotics from other sources like Actinomycetes, cyanobacteria and uncultivable bacteria, in addition to acknowledged sources like Streptomycetes (Iqbal *et al.*, 2014). The capability of bacteria to prosper at high population masses and the predatory role of *Acanthamoeba* in the regulation of bacterial populations in the ecosystem as well as the capability of the amoeba to perform as a "Trojan horse" of the microbial world suggests that bacteria-amoeba are involved in

complex communications (Jousset *et al*., 2010). The exact nature of such complicated interactions remains unclear, but it is identified in this project that both bacteria and amoebae are competent to counter-attack one another and the overwhelming micro organismal populations by discharging antimicrobial substances. Among six bacteria tested, it is attention-grabbing that ACM exhibited selective and effective activity against *Str. pneumoniae,* which is a pathogen of acute infections, many of which may possess strong epidemiological consequences, such as meningitis, otitis media, pneumonia, and sinusitis. Simultaneously, some clones of *Str. pneumoniae* may be implicated in initial stages of lung inflammation in CF patients (Rosenfeld *et al*., 2003). It is possible that bacteria resistant to ACM and *Acanthamoeba* are involved in multipart communications where the bacteria develop mechanisms to safeguard themselves and stand amoebic attack (Pantosti & Venditti, 2009). Yet to come are projects where ACM will be investigated against a great panel of various bacterial pathogens to define broad-spectrum or species-specific antibacterial characteristics. The detection of antibacterial compounds from *Acanthamoeba* will perhaps lead to ideas for future investigations in other free-living protists as a prospective source of antibiotics for the treatment of bacterial pathogens. Due to emerging tendencies in antibiotic resistance in various pathogenic bacteria (Alanis, 2005), the current findings in this study show some promise in our future ability to fight infections caused by bacteria.

Among various bacterial strains studied, BCM of *Str. pneumoniae, P. aeruginosa and H. influenzae* displayed quantifiable amoebistatic and amoebicidal activities. Future investigations will determine the fact of whether these BCM possess selective effects on *Acanthamoeba* or possess broad-spectrum activities on diverse protist pathogens. Amazingly, neither ACM nor BCM were significantly cytotoxic to adenocarcinomic human alveolar basal epithelial cells *in vitro*.

The current findings are encouraging in our pursuit to find complementary sources of antimicrobials with novel modes of action to challenge this threat. As antimicrobial resistance of pathogens rises, discovery of novel non-toxic to human cells antimicrobial substances from a range of pathogens ubiquitous in clinical settings is fundamental. It is expected that these molecules will sooner or later be technologically advanced into treatments for bacterial and amoebic infections, which are progressively resistant to presently existing drugs.

Chapter 3

"Effects of magainin II on *Acanthamoeba* and bacteria in both extracellular and intracellular conditions"

3.1 Background

Despite aggressive antimicrobial therapies, respiratory pathogens such as *S. aureus*, *H. influenzae* and *P. aeruginosa* persist in the lung, underpinning the chronic inflammation and eventual lung decline that are characteristics of respiratory disease among CF patients (Reen *et al.*, 2016). Moreover, emerging multidrug-resistant CF pathogens contribute to this concerning scenario (López-Causapé *et al.*, 2015). Therefore, there is an urgent need to develop new therapies to overcome the drug resistance and eradicate pathogenic bacteria.

Drug combinations have been broadly used to overcome drug resistance and treat complicated infectious diseases (Chen *et al.*, 2016). Drug combinational treatment could inhibit new multiple targets and consequently provide the opportunity for surmounting drug resistance of infectious bacteria (Zimmermann *et al.*, 2007). The prospective molecular mechanism underlying this is that biological systems are less capable of compensating for the simultaneous activity of two or more drugs (Levy & Marshall, 2004). There is growing interest over the development of synergistic drug combinations in academia and the pharmaceutical industry (Iwata *et al.*, 2015). The use of synergistic drug combinations can increase treatment efficacy and decrease drug dosage to prevent toxicity. Additionally, "off-target" effects could be overcome by drug combinations (Lehár *et al.*, 2009). These advantages have progressively attracted researchers towards the search for safe and effective combinatorial drugs (Jansen *et al.*, 2009).

3.1.1 Cationic antimicrobial peptides (AMPs)

Cationic antimicrobial peptides which target the microbial membrane directly have long been a favorable treatment alternative (Easton *et al.*, 2009) for direct use, or for use in combination with other antimicrobial agents. However, their potential is still predominantly not understood. The potential advantages of membrane permeabilizing antimicrobial peptides are important. It was demonstrated that antimicrobial peptide, pleurocidin (Mason *et al.*, 2006a), and designed cationic helical peptides (Mason, 2005; Mason *et al.*, 2006b) cause strong reductions in lipid acyl chain order in the anionic but not the zwitterionic lipid component in mixed membranes, specifying that the peptide has a strong effect on the lipids related to it but not on other lipids in the membrane. They have broad-spectrum antibacterial potency against number of Gram-negative and Gram-

positive bacteria, *in vitro*, including drug-resistant strains (Huang *et al.*, 2012) and (Wiradharma *et al.*, 2011). Moreover, resistance to AMPs is rare and is not easily selectable or inducible (Pollard *et al.*, 2012) and (Daniels, 2011), unlike the case for majority of chemical antibiotics. It could be because AMPs act on bacterial membranes comprehensively, and not with a particular macromolecular component of the cell. The necessity for bacteria to adjust the architecture of the entire membrane to achieve resistance likely increases the overall fitness cost.

At present multidrug combinations are occasionally used for the treatment of routine bacterial infections (Tängdén, 2014) as well as tuberculosis (Zumla *et al.*, 2014). Therefore it is rational to propose a combination therapy utilizing AMPs and chemical antibiotics since the mechanism of action of membrane permeabilising cationic peptides is dramatically different from the chemical antibiotics. It has been documented in the literature that AMPs could synergise with antibiotics increasing the permeability of bacterial membranes (Giacometti *et al.*, 2000a). According to another study AMPs could enhance the activity of bacterial murein hydrolases (LeBel *et al.*, 2013) or other enzymes which decrease the integrity of the peptidoglycan layer, allowing antibiotics to act more effectively.

3.1.1.1 Magainin II

Magainin II is an antimicrobial cationic peptide glycine serine (Giovannini *et al.*, 1987), which consists of 23 amino acids (GIGKFLHSAKKFGKAFVGEIMNS). It was isolated from the skin of the African clawed frog *Xenopus laevis* (Zasloff, 1987). It is believed that peptides are secreted into the skin mucus following discharge of the contents of dermal granular glands, where it serves as a first line of defense against potential pathogens that may be present at the skin surface of amphibia (Rollins-Smith, 2009). Magainin II is representative of a large family of amphibian amphipathic *α*-helical antimicrobial peptides. Magainin was reported to possess antimicrobial potency against Gram-positive and Gram-negative bacteria (Sai *et al.*, 1995). The *in vitro* activity of magainin II alone and in combination with clinically used antibiotics was investigated against multidrug-resistant bacterial strains. Synergy occurred when magainin II was combined with β-lactam antibiotics (Giacometti *et al.*, 2000b). In another study, magainin in combination with silver nitrate showed synergistic effects against *Acanthamoeba polyphaga* (Schuster & Jacob, 1992a).

In AMP databases, more than 100 AMPs have been described as having prospective anti-tumor activity (Wang & Wang, 2004). AMPs are able to provoke death in cancer cells through two common mechanisms: apoptosis or necrosis. A necrotic death pathway could be the outcome of AMPs targeting negatively charged molecules on the cell membrane of cancerous cells, resulting in cell lysis, whereas apoptosis could be the consequence of mitochondrial membrane disarrangement (Boohaker *et al.*, 2012).

In this study the *in vitro* activity of magainin II alone and in combination with three clinically used antimicrobial agents against bacteria (MSSA, *S. epidermidis*, *E. coli, P. aeruginosa* and *H. influenzae*), *A. castellanii* trophozoites and human lung adenocarcinoma cell line was investigated.

3.2 Materials and Methods

All chemicals were purchased from Sigma-Aldrich (UK), unless otherwise stated.

3.2.1 *Acanthamoeba* culture

Acanthamoeba (T4 genotype) were grown and cultured as described in section 2.2.1.

3.2.2 Bacterial cultures

Six clinical bacterial strains were employed (MSSA, *Str. pneuminae*, *S. epidermidis*, *P. aeruginosa*, *H. influenzae* and *E. coli*). All strains were cultured, inoculated and optical densities (OD) were measured by spectrophotometer as described in section 2.2.2.

3.2.3 Cell line and cell culture

Human epithelial lung carcinoma (A549) cell line was grown and cultured as described in section 2.2.3.

3.2.4 Preparation of antimicrobial agents

Antimicrobial agents such as magainin II, silver nitrate ($AgNO_3$), vancomycin and ciprofloxacin were utilized in this study.

Magainin II with 95 % purity and molecular mass of 2.467 kDa, was purchased from Cambridge Bioscience as a powder. The solutions for this peptide were prepared by dissolving powder in distilled water. Antimicrobial activities of magainin II (with different concentrations and time intervals) were tested against *Acanthamoeba* trophozoites, bacteria outside and inside *Acanthamoeba* and A549 cells. In parallel, cytotoxic effects on the A549 cell line were studied.

Silver nitrate ($AgNO_3$) is 0.170 kDa in mass and with ≥ 99 % purity, was dissolved in distilled water. Tested against *Acanthamoeba* trophozoites, A549 cell line and intracellular bacteria (alone and in combination with magainin II, vancomycin and ciprofloxacin).

Vancomycin molecular mass is 1.486 kDa. The solution was prepared by dissolving in sterile distilled water (100 μg/mL). It was used in drug combination experiments against Gram-positive bacteria inside *Acanthamoeba* trophozoites and A549 cells.

Ciprofloxacin antibiotic is 0.331 kDa compound. Stock solutions were prepared by dissolving antibiotic powder in distilled water (75 µg/mL). It was utilized in combinatorial drug experiments with magainin II and $AgNO_3$ against intracellular Gram-negative bacteria.

All solutions were filter sterilized using a 0.2 µm filter. Stocks were kept in -20 °C. The chemical structures of compounds are shown (Figure 3.1).

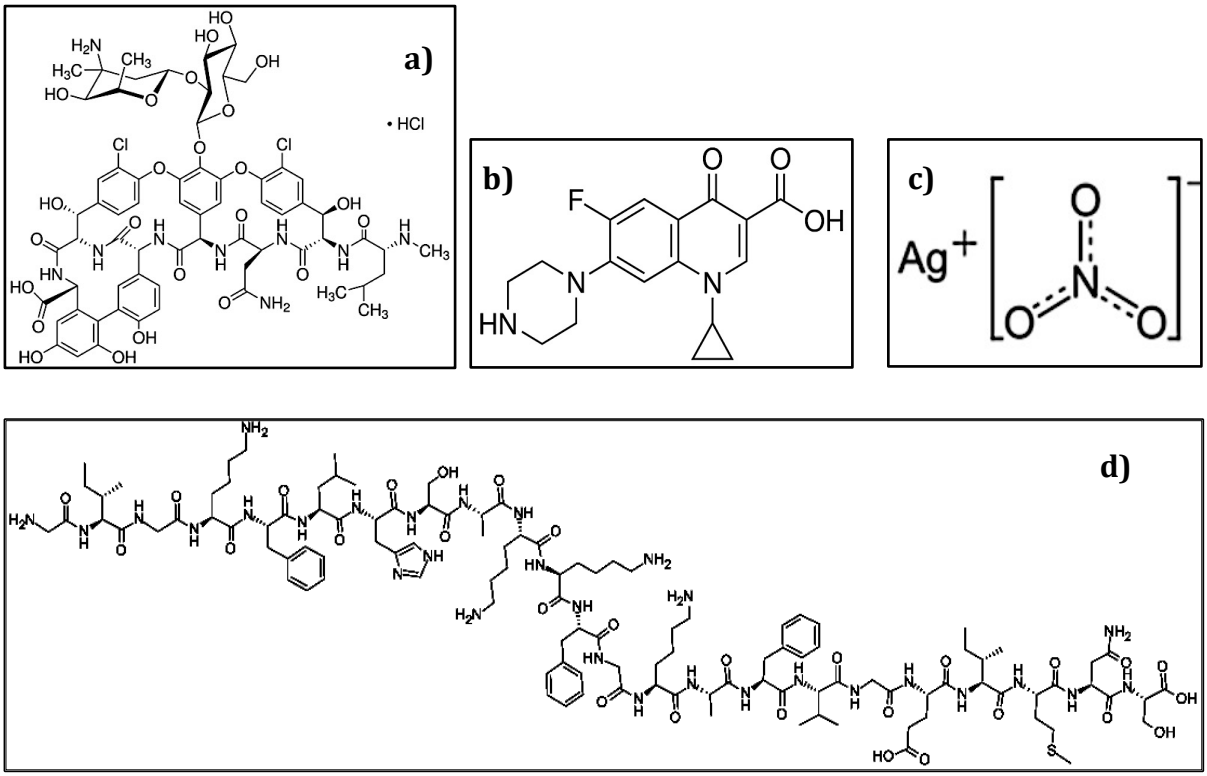


Figure 3.1 Chemical structure of vancomycin (a); ciprofloxacin (b); silver nitrate (c); and magainin II (d).

3.2.5 Antimicrobial assays

3.2.5.1 Antibacterial assays

To determine the antimicrobial activity of magainin II to inhibit bacterial growth, antimicrobial assays were performed. Bacterial strains were grown in their media (LB/BHI/BHI+FE) overnight at 37 °C. Then ODs were measured by spectrophotometer as described above. 96-well plates were used to perform these assays. Bacteria (~1x10^6 CFU/well) were incubated with different concentrations of magainin II for 2, 4, 6 and 24 h at 37 °C. Wells with untreated bacteria were used as controls. The number of viable bacteria in each well was determined by plating onto CLED/Blood/Chocolate agar using 20 µL volumes in duplicate. The percentage of bacterial killing was calculated as follows:

$$\left(R = \frac{[X_0 - X_t]}{X_0} \times 100\right)$$

where R is the bactericidal rate, X_0 the number of bacteria without magainin II (control), and X_t the number of bacteria after the treatment with magainin II (Qiu *et al.*, 2011).

3.2.5.2 Antiamoebic assays

To determine the antimicrobial activity of the magainin II on *Acanthamoeba*, antiamoebic assays were performed. *Acanthamoeba* were cultured and grown in 96-well plates in PYG (~1x10^5 cells per 0.150 ml in each well); next, plates were incubated until confluent. Then, the growth medium was discarded and wells washed with PBS once to remove non-adherent *Acanthamoeba*. Four different final concentrations of magainin II (3, 10, 20 and 30 µM) were added to different wells. All wells contained 150 µL of antimicrobial agent in RPMI, except wells of controls that contained only 150 µL of RPMI. Then *Acanthamoeba* with magainin II were incubated at 30 °C for 2, 4, 6 and 24 h. The number of viable and dead *Acanthamoeba* in each well was determined by adding 150 µL of 0.4% Trypan blue in each well on top of media and incubating for 15 min at room temperature. The numbers of viable and dead *Acanthamoeba*, as judged by dye exclusion or staining, were counted by a haemocytometer.

3.2.5.3 Cytotoxicity assays

To determine the cytotoxic activity of the magainin II on A549 cells, cytotoxic assays were performed. A549 cells were cultured and grown in 96-well plates in DMEM (~1 x10^5 cells per 0.150 milliliter in each well); and plates were incubated till confluent. Then, the growth medium was discarded and wells washed with PBS once to remove non-adherent cells. Four different concentrations of magainin II (3, 10, 20 and 30 μM) were added to different wells. All wells contained 150 μL of peptide in DMEM, except wells of controls that contained only 150 μL of DMEM. Then A549 cells with magainin II were incubated for 2, 4, 6 and 24 h in CO_2 incubator (37 °C in humidified 95 % air, 5 % CO_2). The number of viable and dead A549 cells in each well was determined by adding 150 μL of 0.4% Trypan blue in each well and incubating for 15 min at room temperature. The number of viable and dead A549 cells, as judged by dye exclusion or staining, were counted by a haemocytometer.

3.2.6 Intracellular antimicrobial assays

3.2.6.1 *Acanthamoeba* intracellular uptake

Drugs in combinations were used in this assay. Two combinations such as magainin II with silver nitrate and magainin II with antibiotic (vancomycin/ciprofloxacin) were applied against intracellular bacteria inside *Acanthamoeba*. Non-lethal concentration of magainin II (3 μM) to *Acanthamoeba* trophozoites was used along with silver nitrate (12.5 μM). Second combination is magainin II (3 μM) with vancomycin against Gram-positive intracellular bacteria (5 μM for *S. epidermidis* and 10 μM for MSSA). Or magainin II (3 μM) combined with ciprofloxacin against Gram-negative intracellular bacteria (7 μM for *E. coli* and 13 μM for *P. aeruginosa*).

Acanthamoeba were cultured in 96-well plates in PYG (~1x10^5 cells per 0.150 milliliter in each well); and plates were incubated until confluent. Then, the growth medium was discarded and wells washed with PBS once to remove non-adherent *Acanthamoeba*. Next, bacterial strains (~1.2x10^6 CFU/well) were added onto *Acanthamoeba* with RPMI medium and incubated for 1 h at room temperature. After incubation, supernatants were discarded and wells washed by PBS once to remove non-adherent bacteria. Next, the wells were washed once again with PBS, and then antibiotics were added to kill extracellular bacteria (vancomycin against MSSA and *S. epidermidis*;

ciprofloxacin against *P. aeruginosa and E. coli*; 100 µg/ml final concentration, for 45 min). Then the supernatants were discarded and wells washed once with PBS. Drugs alone and in two combinations were added. All wells contained 150 µL of antimicrobial agent combinations in RPMI, except wells of controls that contained only 150 µL of RPMI. Next, *Acanthamoeba* with bacteria inside and drugs were incubated at 30 °C for 2, 4, 6 and 24 h. After each incubation interval *Acanthamoeba* were lysed by adding Sodium Dodecyl Sulphate (0.5 % final concentration) (SDS) for 10 min. Finally, 20 µL of lysate was cultured onto CLED/Blood/Chocolate agar, and incubated overnight at 37 °C in CO_2 incubator. The bactericidal rates of drugs alone and in combinations on intracellular bacteria within *Acanthamoeba* were calculated as follows:

$$\left(R = \frac{[X_0 - X_t]}{X_0} \times 100\right)$$

where R is the bactericidal rate, X_0 the number of bacteria without drugs (control), and X_t the number of bacteria after the treatment with drugs (Qiu *et al.*, 2011).

3.2.6.2 A549 cell line intracellular uptake

To investigate the effects of drugs in combinations (peptide + silver nitrate; peptide + antibiotic) on the intracellular bacteria within A549 cell line, antimicrobial assays were performed. In brief, A549 cells were cultured and grown in 96-well plates in DMEM (~$1x10^5$ cells per 0.150 milliliter in each well); then plates were incubated till confluent. When confluent, the growth medium was discarded and wells washed with PBS once to remove non-adherent cell. Next, bacterial strains (~$1.2x10^6$ CFU/well) were added onto cells with DMEM medium and incubated for 1 h (37 °C in humidified 95% air, 5% CO2). Then, supernatants were discarded and cells were washed with PBS once to remove non-adherent bacteria. Next, antibiotics were added to kill extracellular bacteria (vancomycin against MSSA, *S. epidermidis*; and ciprofloxacin against *P. aeruginosa, E. coli*; 100 µg/ml final concentration, for 45 min). Then the supernatants were discarded and wells washed once with PBS. Following this, drugs alone and in combinations (as mentioned above in section 3.2.6.1.) were used in this assay. All wells contained 150 µL of antimicrobial agents in DMEM medium, except wells of controls that contained only 150 µL of DMEM medium. The A549 cell line with bacteria inside and drugs in combinations were incubated in CO_2 incubator (37 °C in humidified 95 % air, 5 % CO_2) for 2, 4, 6 and 24

h. Then, A549 cells were lysed by adding 150 µl of CelLytic M® for 10 min. In the end, 20 µL of lysed solution was cultured onto CLED/Blood/Chocolate agar, and incubated for overnight at 37 °C in O_2 incubator. The bactericidal rates of drugs alone and in combinations on intracellular bacteria within A549 cells were calculated as described in section 3.2.6.1.

3.2.6 Flow cytometry assays (Apoptosis)

To study the ability of magainin II to induce programmed cell death in A549 cell line and *Acanthamoeba* trophozoites, a fluorescence technique (flow cytometry assay to measure externalized plasma membranephosphatidyl serine) was performed. In brief, cells were seeded and grown in 24 well plates until confluent. Then, the growth medium was discarded, and cells were washed once with PBS. Various concentrations of magainin II (3, 10, 20 and 30 µM) were added to different wells to induce cell death. Control wells were devoid of peptides. Plates were incubated for 24 h at 37 °C in an incubator. After cell death induction, cells were harvested and collected with the supernatants in 1.5 mL eppendorf tubes for flow cytometry assays. Samples were centrifuged at 1500 x *g* for 5 min. The pellets of cells were washed twice with 200 µL of cold cell staining buffer (BioLegend). Then, cells (1 x 10^5) were resuspended in 100 µL of Annexin V binding buffer (BioLegend) containing 5 µL of Annexin V Fluorescein isothiocyanate (FITC) (BioLegend) and 1 µL of Zombie NIR (BioLegend). Samples were gently vortexed and incubated at room temperature for 20 min in the dark. Next, samples were washed once with cell staining buffer and fixed with 4 % paraformaldehyde (PFA) on ice for 20 min. Samples were centrifuged and supernatants were discarded. Pellets were washed once with cell staining buffer and then re-suspended in 400 µL of Annexin V binding buffer. Next, cells were analysed by flow cytometry [The BD AccuriTM C6] and each run was restricted to 10000 moments.

3.2.7 Transmission electron microscopy (TEM)

Images were obtained in collaboration with Dr. Matthew Hannah, Lead Electron Microscopist in the Virus Reference Department of National Infection Service, Public Health England in London. All samples were prepared in Medical Microbiology laboratory, School of Biological Sciences, Essex University.

The antimicrobial effects of magainin II on bacteria (MSSA, *Str. pneumoniae, P. aeruginosa and H. influenzae*) and *A. castellanii* were observed by TEM. Bacteria/*Acanthamoeba* were co-incubated with either peptide-treated or peptide-untreated media (RPMI) in 36 °C incubator. Next day, microorganisms were harvested, centrifuged and washed three times. The pellets obtained were fixed in 2.5 % glutaraldehyde (v/v) (in 0.1 M sodium cacodylate buffer, pH = 7.2) and incubated for 60 min at RT. Next, the samples were centrifuged (10 min at 300 x *g*) and re-suspended in 0.1 M sodium cacodylate buffer. This step was repeated twice to remove the fixative agent. Then, the pellets were washed again with 0.1 M sodium cacodylate buffer as mentioned in section 2.2.8 and embedded in 3 % Nobel agar prior to being cut into section of 1-2 mm cubes. *Acanthamoeba* trophozoites/bacteria were dehydrated using graded series of ethanol. Afterwards, the pellets were infiltrated with LR white resin and polymerized at 67 °C for 24 h. Sections of 80-100 nm were cut using a NOVA ultra-microtone, stained with lead citrate for 20 min, rinsed with distilled water and stained with uranyl acetate for 5 min. After drying, the grids were imaged in a JEOL JEM-1400 TEM operating at 120 kV. Images were acquired using an AMT XR60 digital camera.

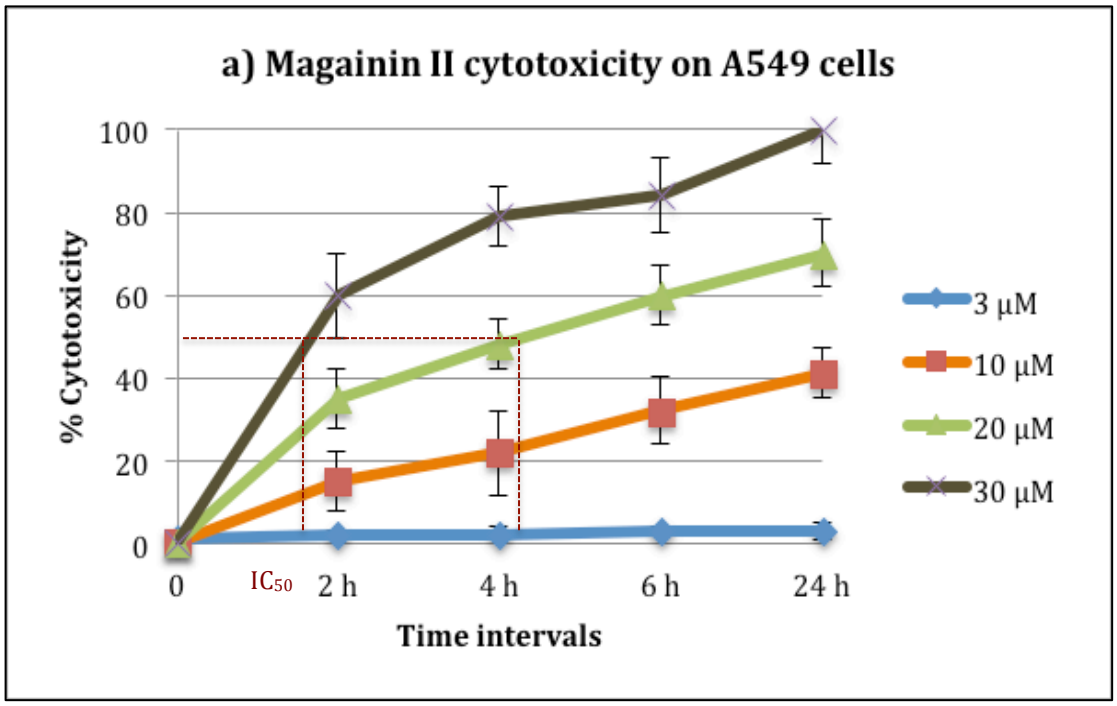
3.2.8 Presentation of results and statistical analysis.

The results shown in this work are representative of three independent experiments performed in triplicate by identical methods. The results are expressed as means ± 1 standard deviation. Differences were considered statistically significant if the *P* value was <0.05 by Student's *t* test.

3.3 Results

3.3.1 Peptides as biocidal agents

To determine the effects of magainin II, cytotoxicity assays on A549 cell line, and the antimicrobial assays on *Acanthamoeba* were carried out**.** Four different concentrations of magainin II (3, 10, 20 and 30 μM) at different time intervals (2, 4, 6 and 24 h) were applied. The antimicrobial effect of magainin II on the survival of both *Acanthamoeba* and A549 cell were dose and time dependent. The lowest concentration, 3 μM, did not demonstrate significant cytotoxicity on A549, resulting in 3 % dead cells when incubated for 24 h. The same concentration on *Acanthamoeba* trophozoite was more cytotoxic, resulting in 9 % killing. The greatest concentration, 30 μM, was 100 % fatal for both A549 cells and *Acanthamoeba* when incubated for 24 h. 20 μM of magainin II was able to decrease the number of A549 cells by 70 % and *Acanthamoeba* trophozoites by 51 % in 24 h of incubation. At 2 h incubation with 30 μM peptide, both *Acanthamoeba* and A549 cells viability decrease by 60 %. It was important to determine the effects of various concentrations at different time intervals since one of the aims of the study is to target intracellular pathogenic bacteria inside *Acanthamoeba* and A549 cells without harming host cells (Figure 3.2).



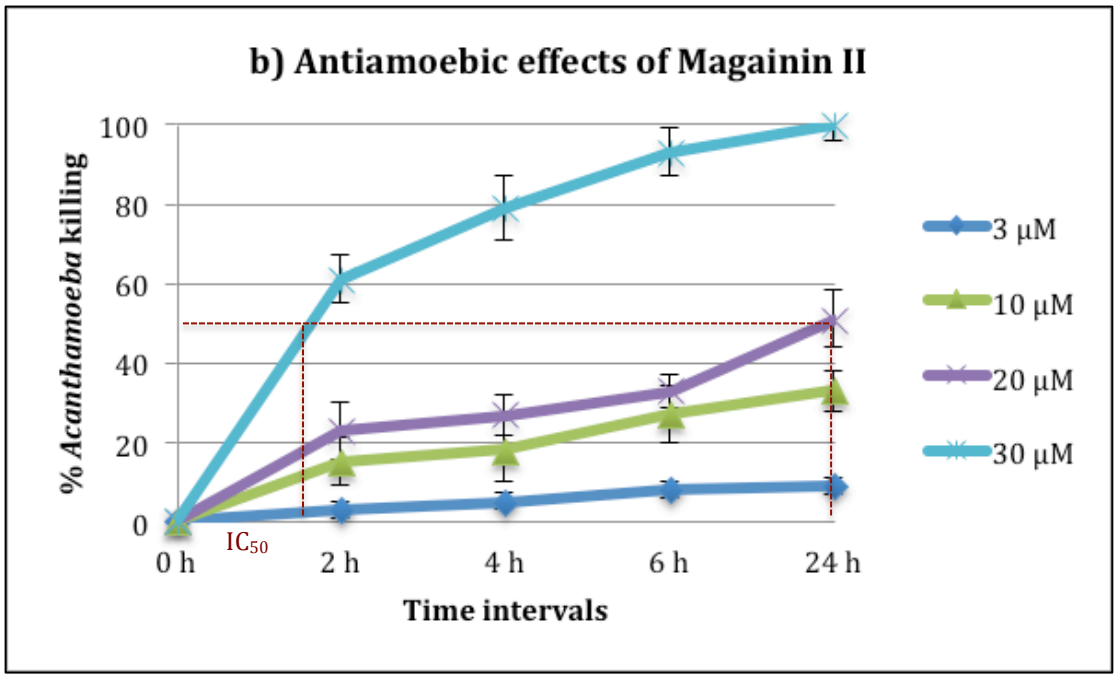


Figure 3.2 Cytotoxic effects of magainin II on a) A549 cells and b) *Acanthamoeba* trophozoites. *Acanthamoeba*/A549 cells were cultured and grown until confluent. Then, the growth medium was discarded and wells washed. Four different concentrations of magainin II were added to different wells and incubated. The number of viable and dead *Acanthamoeba*/A549 cells in each well was determined by adding 0.4% Trypan blue using haemocytometer. Results are the average of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

3.3.2 Induction of *Acanthamoeba* trophozoite and A549 cell death by magainin II at different concentrations

To study the antimicrobial/cytotoxic effects of cationic peptide magainin II, cell death induction was determined. Cell death induced by magainin II was determined at different concentrations (3, 10, 20 and 30 μM) to investigate the induction of different points of apoptosis and then necrosis. A549 cells and *Acanthamoeba* trophozoites were treated with various concentrations of cationic peptide for 24 h. Cells were stained with annexin V-FITC and Zombie NIR and analyzed by flow cytometry. Cells with double positive for both annexin V-FITC and Zombie NIR were dead cells, while cells staining positive for annexin V-FITC and negative for Zombie NIR were apoptotic cells. Live cells are those cells with low Zombie NIR and negative for annexin V-FITC.

Percentages of apoptotic and necrotic cells of both A549 cells and *Acanthamoeba* trophozoites were slightly and gradually increased after being treated with antimicrobial peptide, while they increased in A549 cell line (14 ±2 %) and *Acanthamoeba* trophozoites (7 ±2 %) for necrotic cells, and 54 ±11 % and 26 ±4 %, respectively, for apoptotic cells at 24 h post-treatment. Cell death induced by magainin II in A549 cell line was higher than death induced by peptide on *Acanthamoeba*. The percentage of apoptotic and necrotic A549 cells induced by magainin II was different when exposed to different concentrations. The percentage of apoptosis for 0 (control), 3, 10, 20 and 30 μM were 19 ±7 %, 23 ±6 %, 25 ±9 %, 37 ±7 % and 54 ±11 %, respectively. While, the percentage of necrosis were 3 ±1 %, 5 ±3 %, 6 ±1 %, 10 ±4 %and 14 ±2 %, respectively. Acquired results showed that the percentage of apoptosis and necrosis of *Acanthamoeba* was dose dependent. For apoptosis, results were 12 ±2%, 15 ±1%, 13 ±2%, 26 ±3% and 19 ±4%, respectively. And for necrosis, they were 1 ±0.5 %, 2 ±0.5 %, 3 ±1 %, 4 ±2 % and 7 ±1 %, respectively. (Figure 3.3 and 3.4).

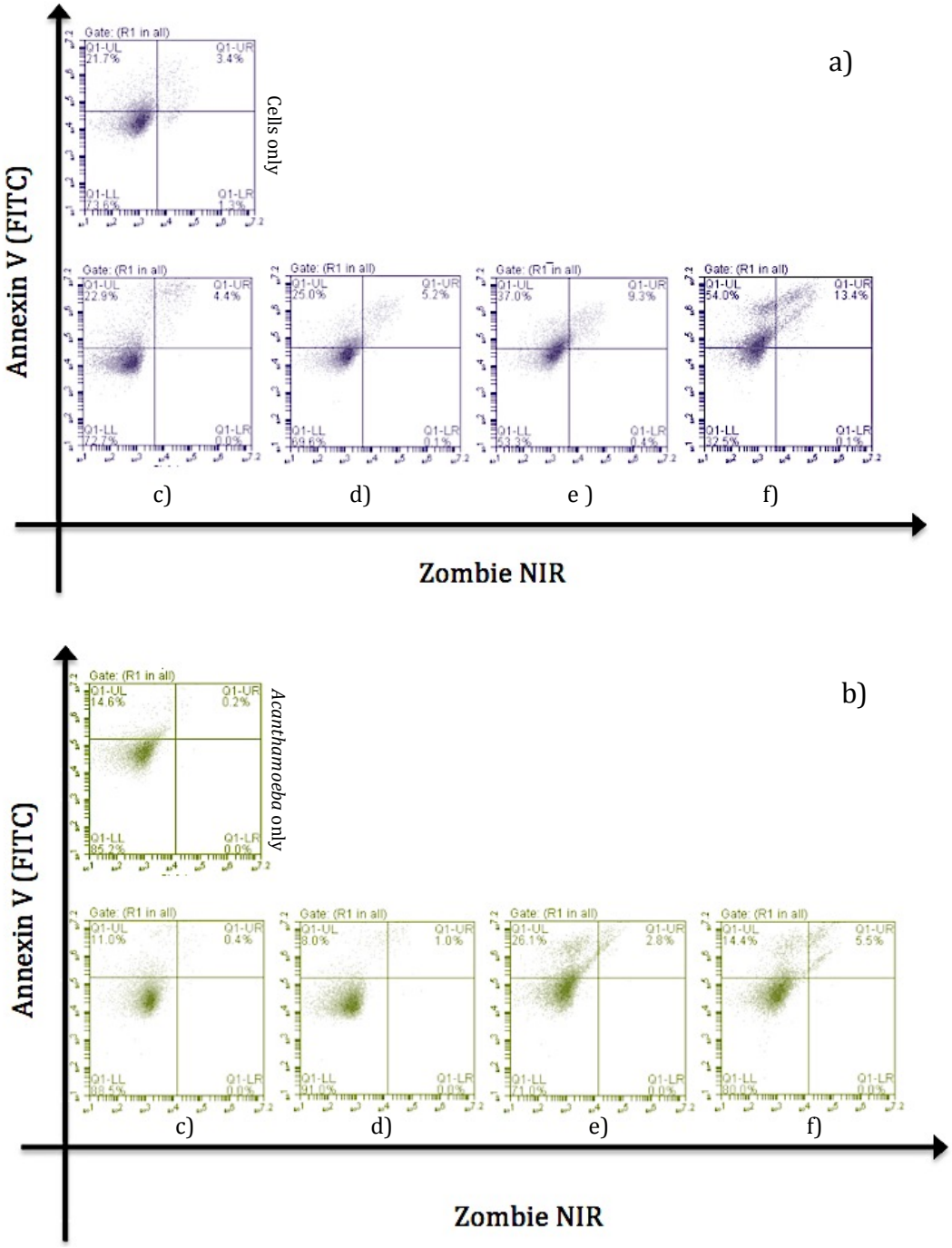
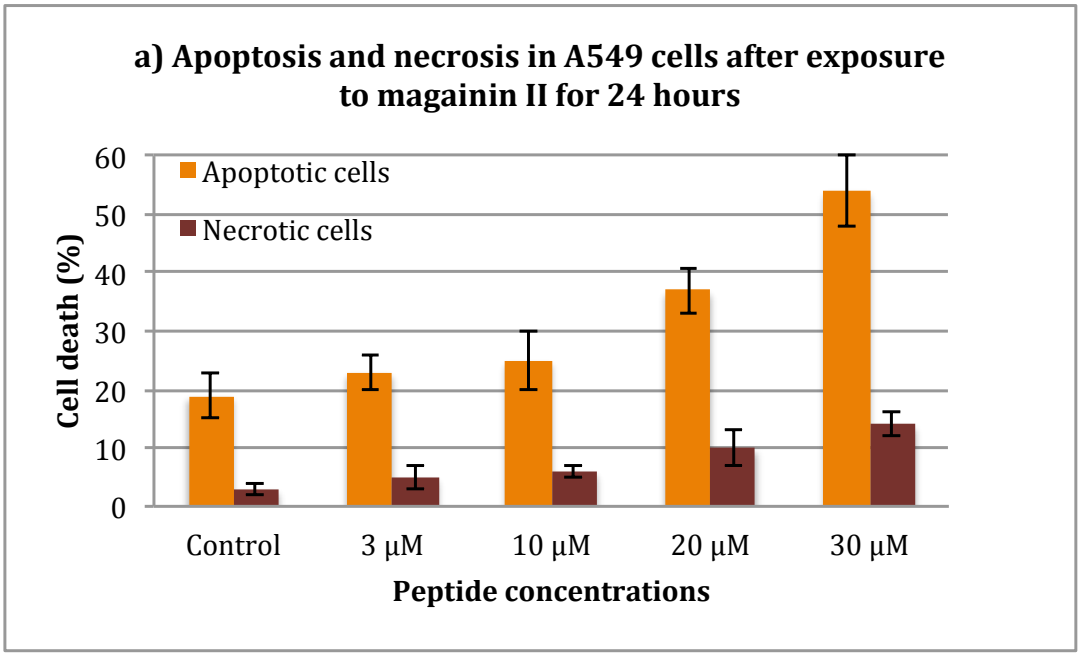


Figure 3.3 Evaluation of apoptosis in A549 (a) cells and *Acanthamoeba* (b) trophozoites after exposure to magainin II. Both *Acanthamoeba* and A549 cells were treated with 3 µM (c), 10 µM (d), 20 µM (e) and 30 µM (f) concentrations of peptide and incubated for 24 h. Cells were stained with annexin V-FITC, Zombie NIR and analyzed by flow cytometry. Cells double positive for both annexin V-FITC and Zombie NIR were dead cells, while cells positive for annexin V-FITC and negative for Zombie NIR were apoptotic cells. Live cells were those cells with low Zombie NIR and negative for annexin V-FITC. The data shown are representative of three experiments.



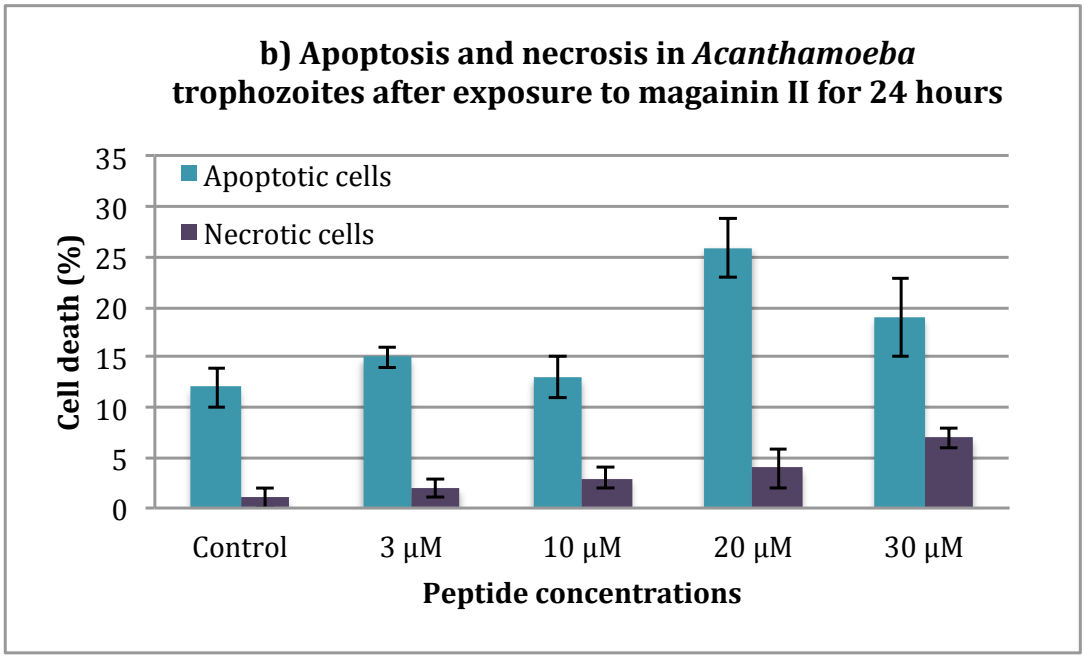


Figure 3.4 Evaluation of apoptosis and necrosis in A549 cells and *Acanthamoeba* trophozoites after exposure to magainin II. Both *Acanthamoeba* and A549 cells were treated with 3, 10, 20 and 30 µM concentrations of peptide and incubated for 24 h. Cells were stained with annexin V-FITC and Zombie NIR and analyzed by flow cytometry. Controls (cells only) are untreated cells. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error.

The effects of cationic peptide on the viability of bacterial strains were studied at similar concentrations and incubation timings to those used above. Three strains of Gram-positive and three strains of Gram-negative bacteria were studied. 100 % killing of bacteria in 24 h was achieved by different concentrations of the antimicrobial agent. For instance a 50 μM concentration eradicated *S. epidermidis* and MSSA by 100 %, whereas for *Str. pneumoniae* 40 μM was sufficient to kill all bacteria. The 50 μM peptide for 6 h was able to decrease the number of bacteria (*S. epidermidis* and MSSA) by 91 %. *Str. pneumoniae* was decreased by 93 % when exposed to 40 μM peptides for 6 h. 3 μM dosage was ineffective against all Gram-positive bacteria in all time intervals (2, 4, 6 and 24 h). 25 μM concentration of peptide was able to kill less than 50 % of *S. epidermidis* (44 %) but more than 50 % of MSSA (65 %) (Figure 3.5). Gram-negative bacteria were more susceptible to magainin II. For instance a 30 μM peptide concentrations was sufficient to kill 100 % of bacteria such as *E. coli* and *H. influenzae*, whereas for *P. aeruginosa* 40 μM was required. Similar concentrations demonstrated more than 68 % and 61 % reduction for *E. coli* and *P. aeruginosa*, respectively. Interestingly, a 30 μM concentration was not as effective against *H. influenzae* as against other strains in 2 h, decreasing the number of bacteria by 29 %. The ineffective dosage for Gram-negative bacteria was found to be 3 μM, where 98 % of all bacteria remained viable. So, magainin II had greater effect on Gram-negative bacteria when compared to Gram-positive ones (Figure 3.6).

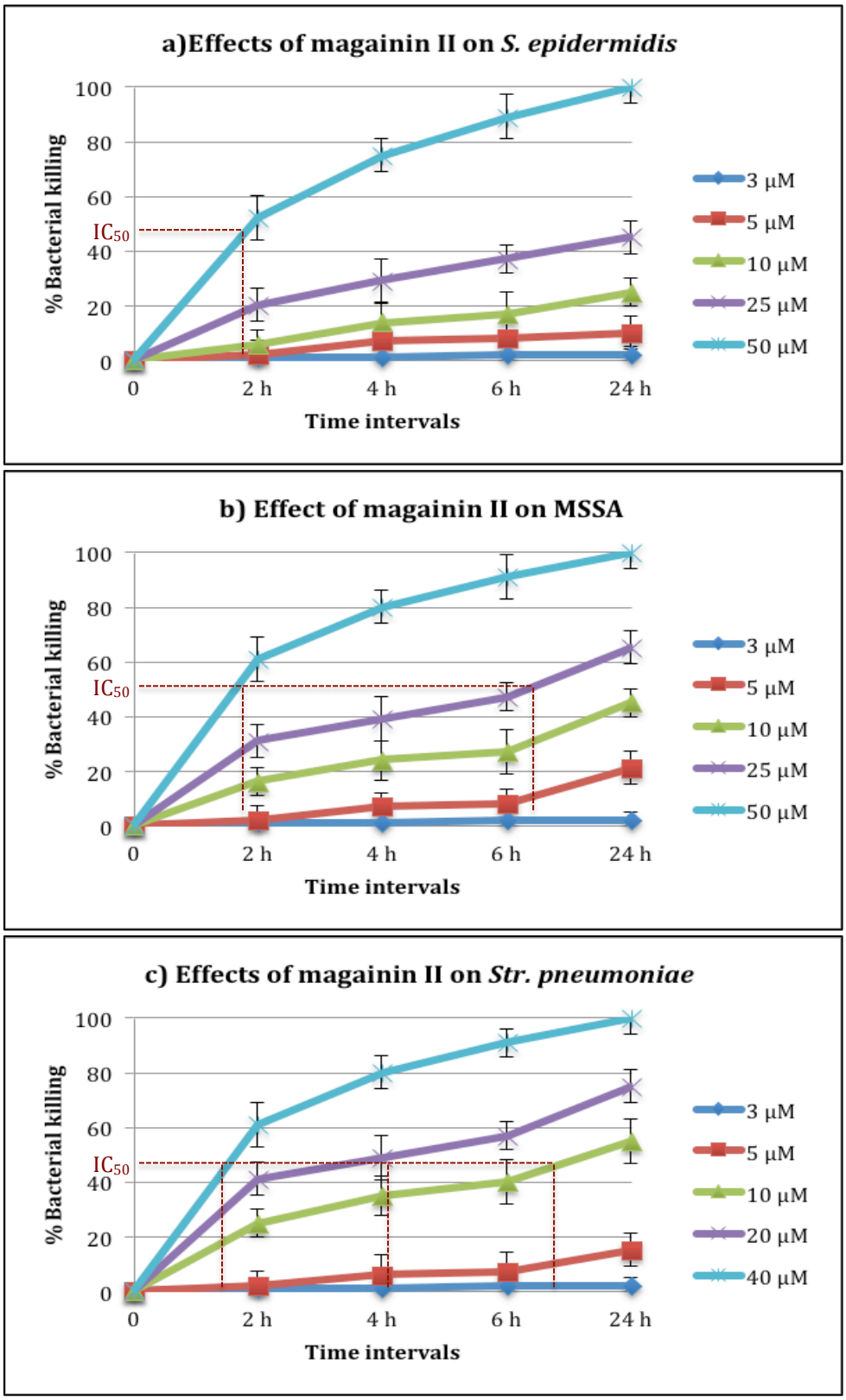


Figure 3.5 Antibacterial effects of magainin II on Gram-positive bacteria a) *S.epidermidis* b) MSSA c) *Str. pneumoniae,* for various incubation timings. Results are the average of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

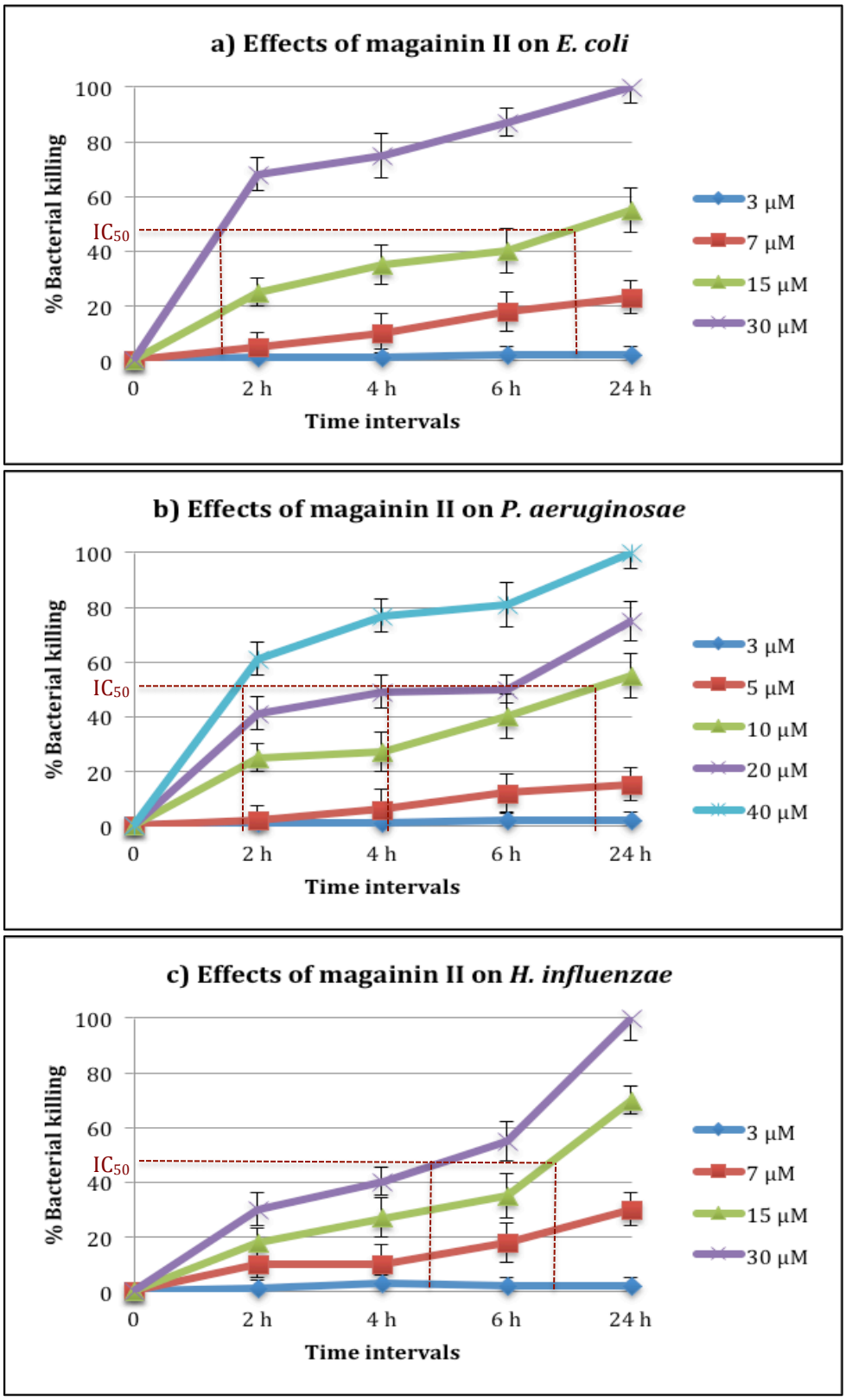


Figure 3.6 Antibacterial effects of magainin II on Gram-negative bacteria a) *E. coli* b) *P. aeruginosa* c) *H. influenzae,* for various incubation timings (2, 4, 6 and 24 h). Results are the average of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

To investigate the ultrastructural alterations caused by magainin II in the *Acanthamoeba* trophozoites and bacteria such as MSSA, *Str. pneumoniae, P. aeruginosa* and *H. influenzae*, TEM studies were performed. Microorganisms were grown as described previously and treated with antimicrobial peptide for 24 h. *Acanthamoeba* trophozoites were exposed to 20 μM, MSSA to 25 μM, *Str. pneumoniae* to 20 μM, *P. aeruginosa* to 20 μM and *H. influenzae* to 15 μM. Control strains for all microorganisms were free of antimicrobial peptides. Acquired micrographs revealed significant cytotoxic effects of peptide on microorganism cellular structures. It was observed that 24 h incubation with magainin II resulted in *Acanthamoeba*/bacterial cell envelopes disorganization with a loss of plasma membrane regions compared to control microorganisms exposed to medium alone (Figure 3.7 and 3.8).

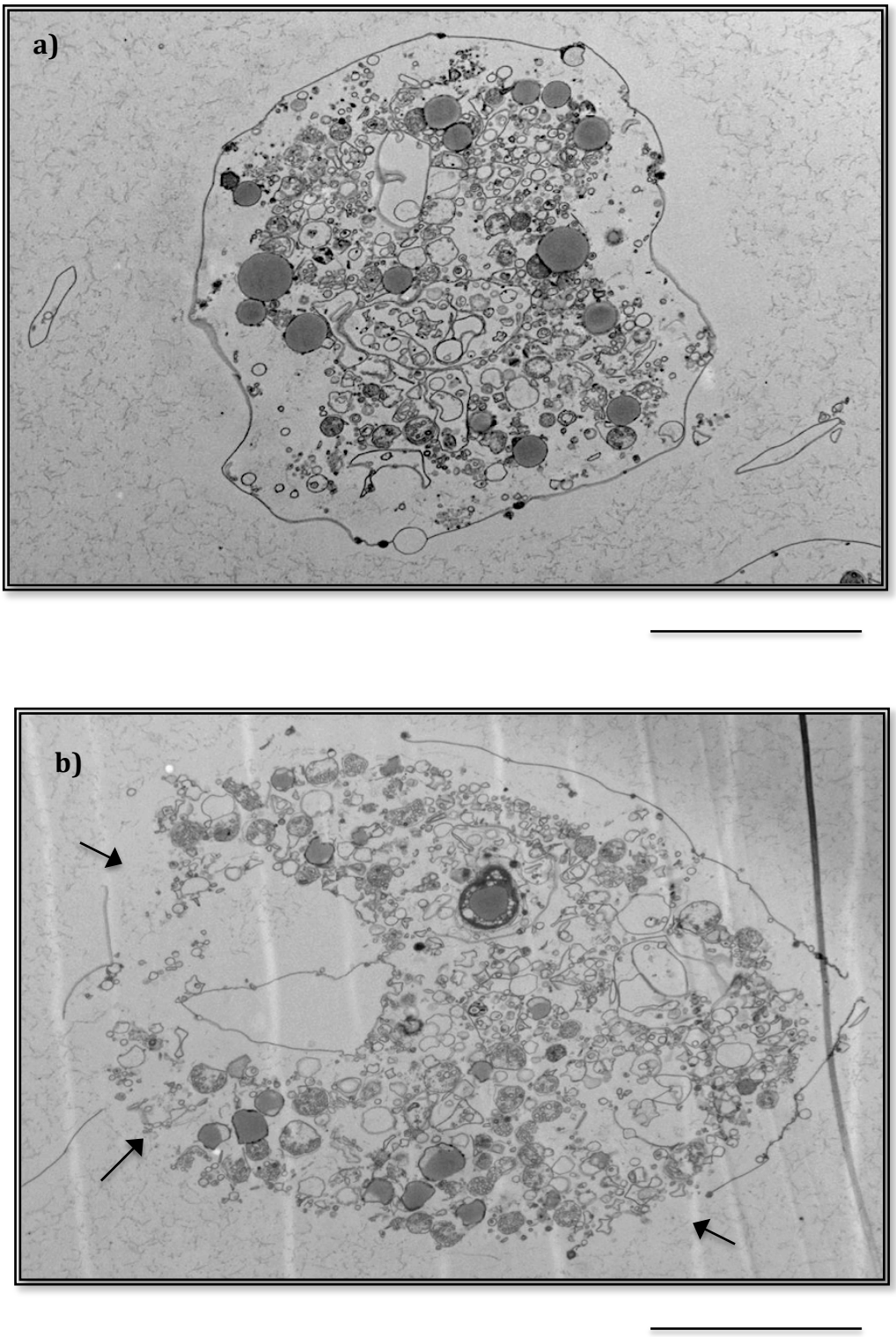


Figure 3.7 Transmission electron microscopy of *Acanthamoeba* trophozoites untreated and treated with magainin II. a) control, without peptide; b) with magainin II (20 μM). Trophozoites were co-incubated with either peptide-treated or peptide-untreated media for overnight. Next day, microorganisms were harvested, centrifuged, washed and fixed. Images were acquired using an AMT XR60 digital camera. bar = 2 μm. Electron micrographs revealed the disruption of cell membrane integrity of trophozoites. Swelling of the cell by becoming larger in size compared with control.

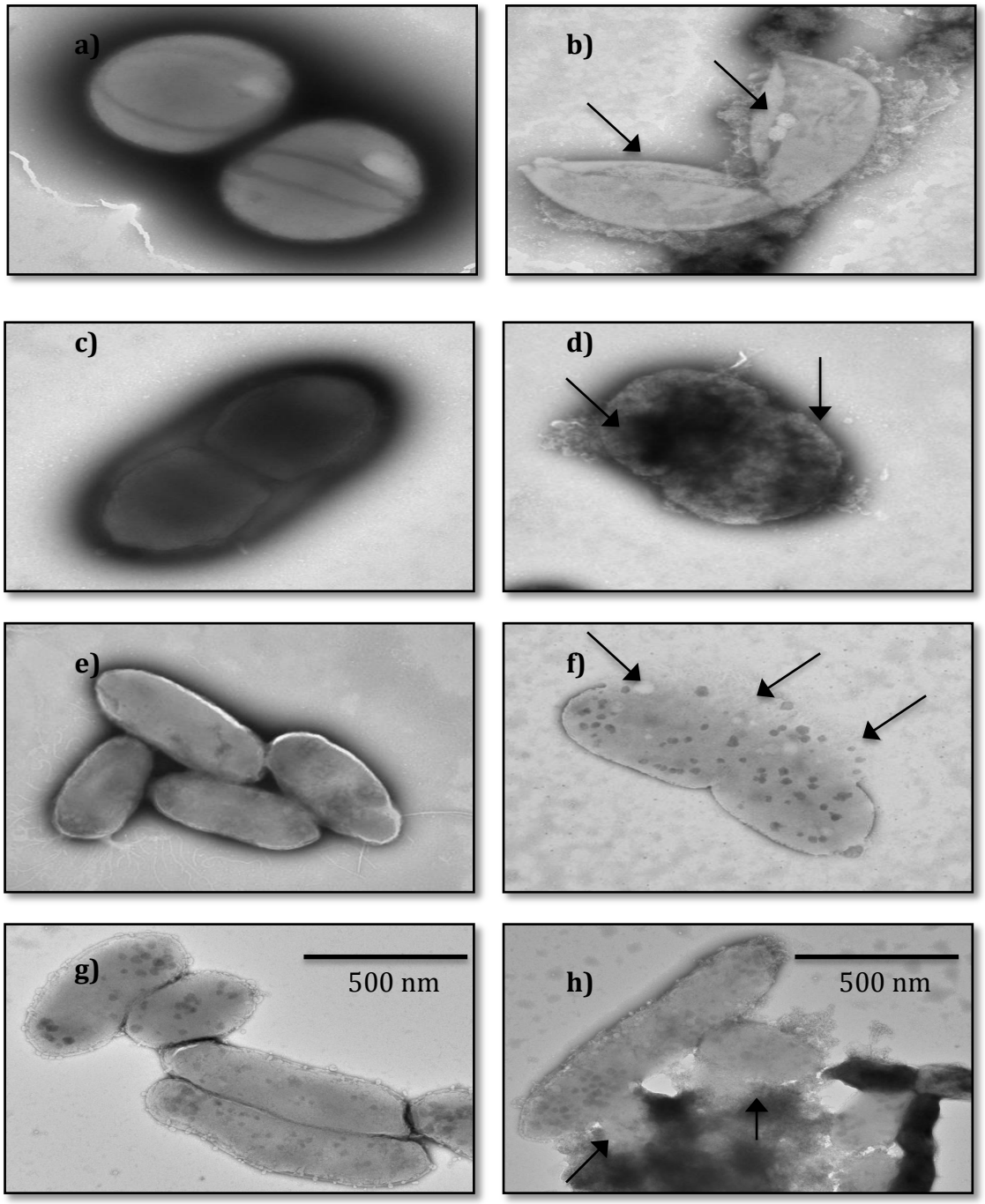
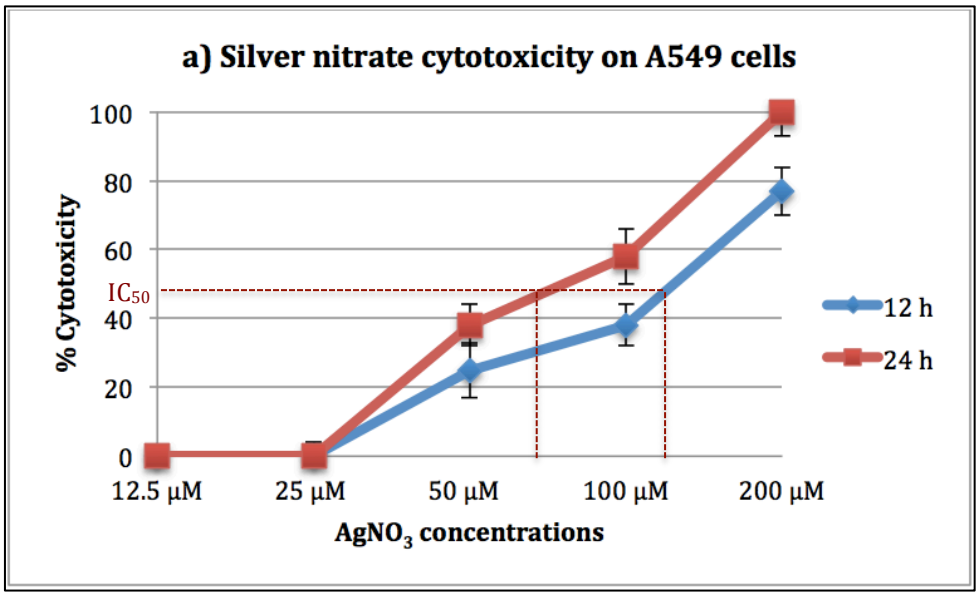


Figure 3.8 Transmission electron microscopy of bacteria untreated and treated with magainin II.

MSSA: a) control, without peptide; b) with peptide (25 μM); *Str. pneumoniae*: c) control; d) with peptide (20 μM); *P. aeruginosa* e) control; f) with peptide (20 μM); *H. influenzae*: g) control; h) with peptide (15 μM). Bacteria were co-incubated with either peptide-treated or peptide-untreated media for overnight. Next day, microorganisms were harvested, centrifuged, washed and fixed. Images were acquired using an AMT XR60 digital camera. As shown above, complete cell membrane and full intracellular contents were observed in the untreated bacteria. It can be seen that the treated bacteria cells by magainin II peptide exhibited obvious disrupted integrity of the bacterial membranes.

To identify a drug which could be used in combination with magainin II, silver nitrate ($AgNO_3$) was tested against A549 cells and *Acanthamoeba* trophozoites. A well-established antiseptic with antimicrobial properties silver nitrate was used in five different concentrations (200, 100, 50, 25 and 12.5 μM) with two incubation timings (24 and 12 h). All the concentrations used were not sufficient to eradicate all trophozoites or A549 cells in 12 h. 200 μM of silver nitrate in 12 h demonstrated high cytotoxicity on A549 cells, decreasing viability by 77 %. The similar concentration with incubation time exhibited 65 % antiamoebic activity. 25 and 12.5 μM dosages were found to be nontoxic to both A549 cells and *Acanthamoeba* trophozoites (Figure 3.9). Finding ineffective dosages were important. These dosages could be applied to target pathogenic intracellular bacteria inside host cells without harming host cells.



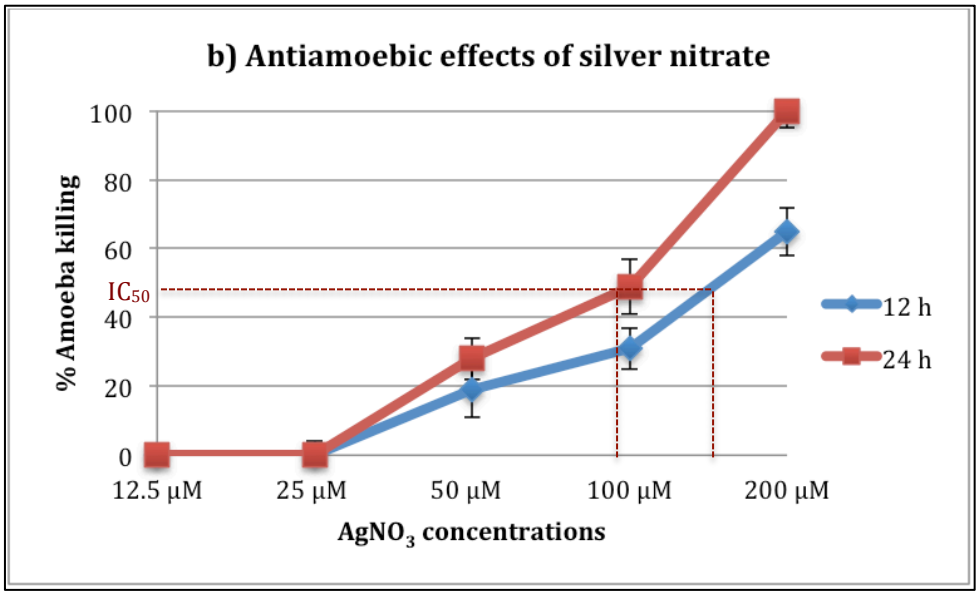


Figure 3.9 Cytotoxic effects of silver nitrate on a) A549 cells and antiacanthamoebic activity on b) *Acanthamoeba* trophozoites. *Acanthamoeba*/A549 cells were cultured and grown until confluent. Then, the growth medium was discarded and washed. Different concentrations of silver nitrate were added to different wells and were incubated for 12 and 24 h. The number of viable and dead *Acanthamoeba*/A549 cells was determined by adding 0.4% Trypan blue. Results are the average of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

Following the above experiments microbicidal effect of magainin II were examined against internalized bacteria could invade A549 cells and *Acanthamoeba*. As two bacteria (*Str. pneumoniae, H. influenzae*) were not able to survive and recover in interacting with A549 cells and in *Acanthamoeba*, they were excluded from antimicrobial drugs exposure.

Several other antimicrobial agents were tested as potential synergists for use with magainin II. These compounds were: silver nitrate, vancomycin and ciprofloxacin. Most promising of these was silver nitrate, which exhibited a higher antimicrobial effect on bacteria inside *Acanthamoeba* and A549 cells.

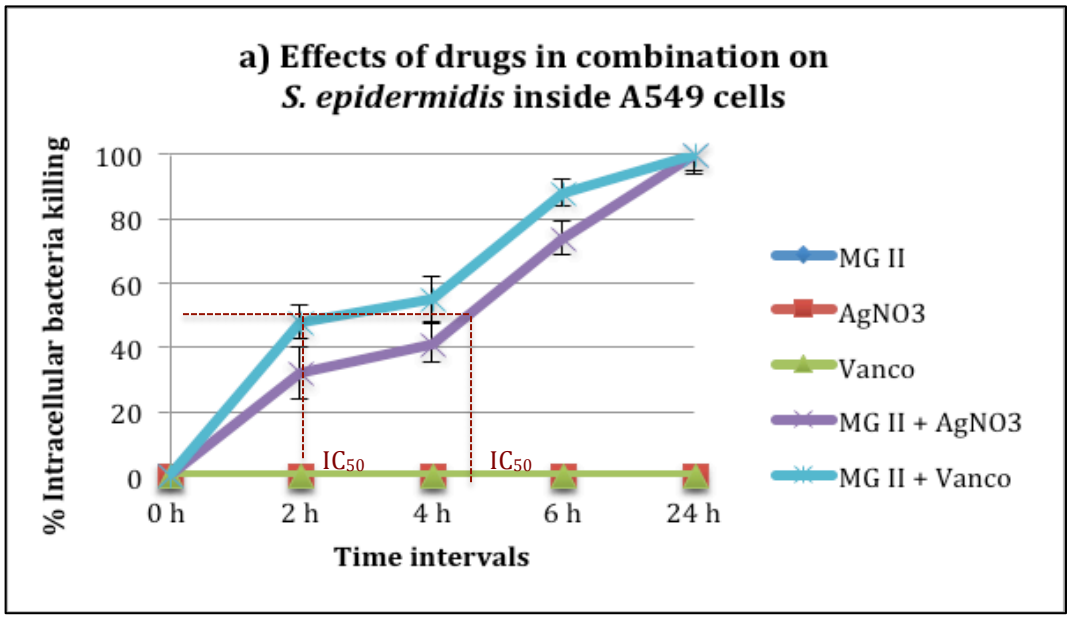
The 3 μM concentration of magainin II was used, since this dosage had been previously found least cytotoxic on A549 cells as well as *Acanthamoeba* trophozoites. Moreover, silver nitrate was used at 12.5 μM concentration, which had been found an ineffective dosage. Vancomycin antibiotic was used against Gram-positive intracellular bacteria, whereas ciprofloxacin was used for Gram-negative ones. The concentrations of antibiotics (5 and 10 μM for vancomycin and 7 and 13 μM for ciprofloxacin) applied in drug combination experiments were ineffective dosages on intracellular bacteria within A549 cells and *Acanthamoeba* trophozoites.

The drugs when used in combinations were designed so they do not disturb and not cytotoxic to host A549 cells as well as *Acanthamoeba* trophozoites.

S. epidermidis and MSSA in A549 cells exhibited normal growth when exposed to 5 and 10 μM concentrations of vancomycin, respectively. Furthermore, in *Acanthamoeba* trophozoites, similar bacteria could grow without any disturbance with antibiotic concentrations of 6 μM. The ineffective concentrations on Gram-negative bacteria such as *P. aeruginosa* and *E. coli* in A549 were 7 and 13 μM, and for the same bacteria inside *Acanthamoeba* trophozoites were 8 and 12 μM, respectively.

Four different time intervals (2, 4, 6 and 24 h) were applied to study the dynamics of drugs in combination. The initial numbers of intracellular bacteria per A549 cell, just before exposing to drugs, were as follows: *S. epidermidis*-2.6; MSSA-2.9; *E. coli*-2.5; and *P. aeruginosa*-1.4. Intracellular *S. epidermidis* inside A549 cells was susceptible to drugs in combination. When vancomycin was applied in combination with magainin II for 24 h, no viable intracellular bacteria were detected. This combination of drugs in just 4 h reduced the number of intracellular *S. epidermidis* by 55 %. The combination of peptide with

vancomycin displayed greater antimicrobial effect when compared to peptide with silver nitrate. Intracellular MSSA inside A549 cells exhibited susceptibility to drugs in combination. On this strain, the combination of peptide and silver nitrate exerted more cytotoxicity. In 2 h of incubation over 44 % of intracellular bacteria were calculated to be dead. Moreover, in 6 h this combination was successful in reducing the number of intracellular MSSA by 78 %. Inside A549 cells, intracellular Gram-negative bacteria such as *E. coli* and *P. aeruginosa* largely survived up to 6 h of incubation with peptide-ciprofloxacin combination, exhibiting more than 78 % viability. The peptide-silver nitrate combination reduced the number of *E. coli* and *P. aeruginosa* by 43 % and 22 % in 2 h, respectively. The same combination was capable of eradicating 57 % of *E. coli* and 55 % of *P. aeruginosa* in 6 h (Figure 3.10). It was essential to design the concentrations of drugs used against intracellular bacteria as low as possible with minimal effects to *Acanthamoeba* and A549 cell.



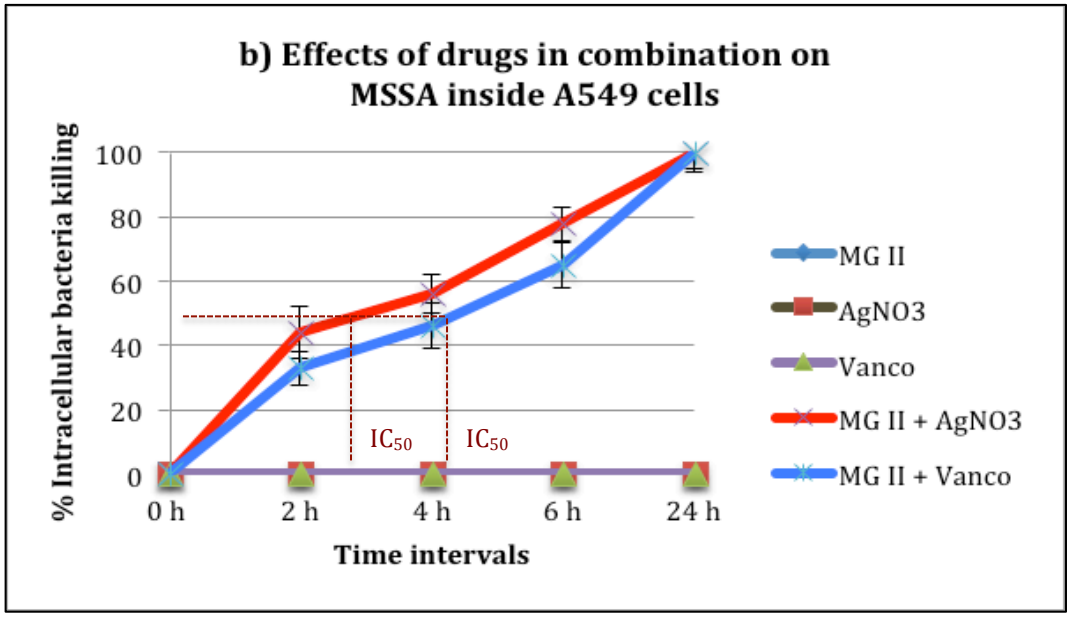
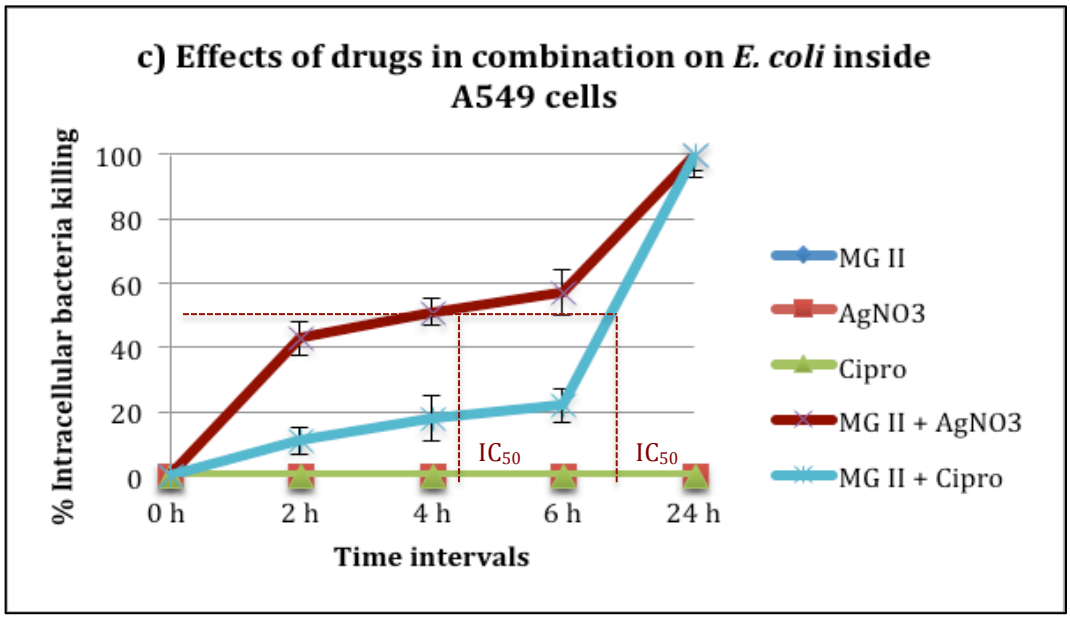


Figure 3.10 Drugs in combinations tested against intracellular bacteria inside A549 cells: a) *S. epidermidis*; b) MSSA. Magainin II (3 μM); silver nitrate (12.5 μM) and various concentrations of vancomycin (5 and 10 μM) were applied. Two sorts of combinations were used experimentally: magainin II + silver nitrate; and magainin II + antibiotics. Abbreviations: MG II- magainin II; Vanc-vancomycin. Results are the average of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.



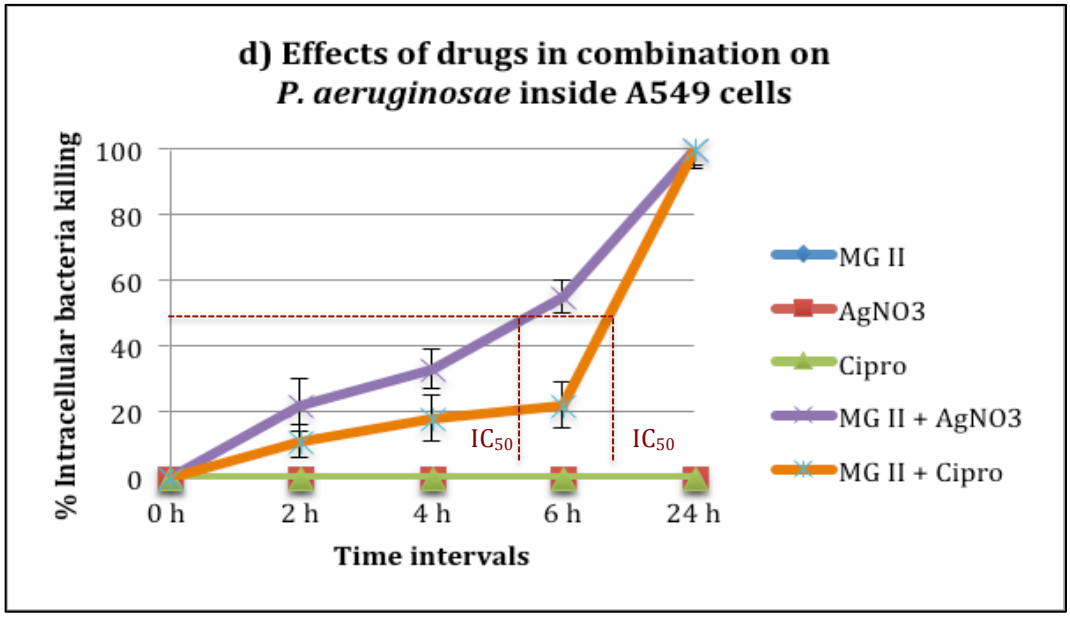


Figure 3.10 Drugs in combinations tested against intracellular bacteria inside A549 cells: c) *E. coli*; d) *P. aeruginosa*. Magainin II (3 μM); silver nitrate (12.5 μM) and various concentrations of ciprofloxacin (7 and 13 μM) were applied. Two sorts of combinations were used experimentally: magainin II + silver nitrate; and magainin II + antibiotics. Abbreviations: MG II- magainin II; Cipro- ciprofloxacin. Results are the average of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

The effects of drugs in combination were tested against intracellular bacteria inside *Acanthamoeba* trophozoites. Magainin II (3 μM), silver nitrate (12.5 μM), vancomycin (6 μM for *S. epidermidis* and 6 μM for MSSA), and ciprofloxacin (12 μM for *E. coli* and 8 μM for *P. aeruginosa*) were antimicrobial agents used in this series of experiments. The initial numbers of intracellular bacteria per *Acanthamoeba*, just before exposing to drugs, were as follows: *S. epidermidis*-1350; MSSA-820; *E. coli*-880; and *P. aeruginosa*-1480.

At 24 h of incubation with either of the combination drugs (peptide-silver nitrate; peptide-antibiotic), no bacteria under investigation were able to survive inside host *Acanthamoeba* trophozoites. When chemical agents were used alone, 100 % viability with abilities to multiply and survive intracellularly was documented. The peptide-silver nitrate combination in 2 h was more bactericidal on Gram-positive bacteria compared with Gram-negative bacteria. MSSA displayed the highest susceptibility to this combination of drugs, with numbers reduced by 30 %. On the other hand, Gram-negative *P. aeruginosa* exhibited the least susceptibility displaying 67 % viability rate at 6 h co-incubation. 24 h of co-incubation with both combination drugs were fatal for intracellular bacteria inside *Acanthamoeba* trophozoites.

Pathogenic strains (MSSA and *P. aeruginosa*) demonstrated lower viability when exposed to peptide-antibiotic (vancomycin, ciprofloxacin) combination. 6 h of co-incubation with the same combination drugs was sufficient to eradicate more than 50 % of pathogenic strains (MSSA, *P. aeruginosa* to 69 and 57 %, respectively) (Figure 3.11).

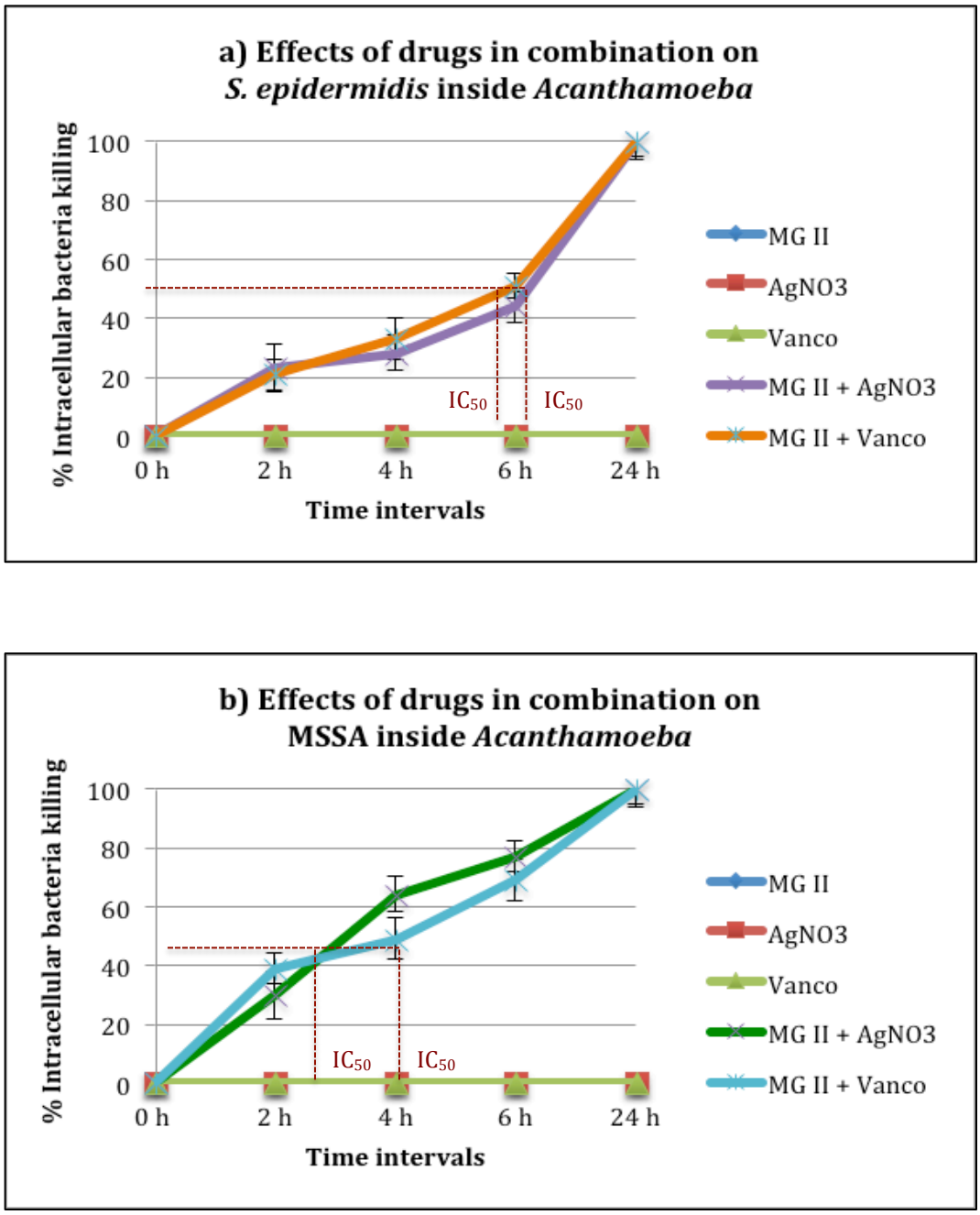
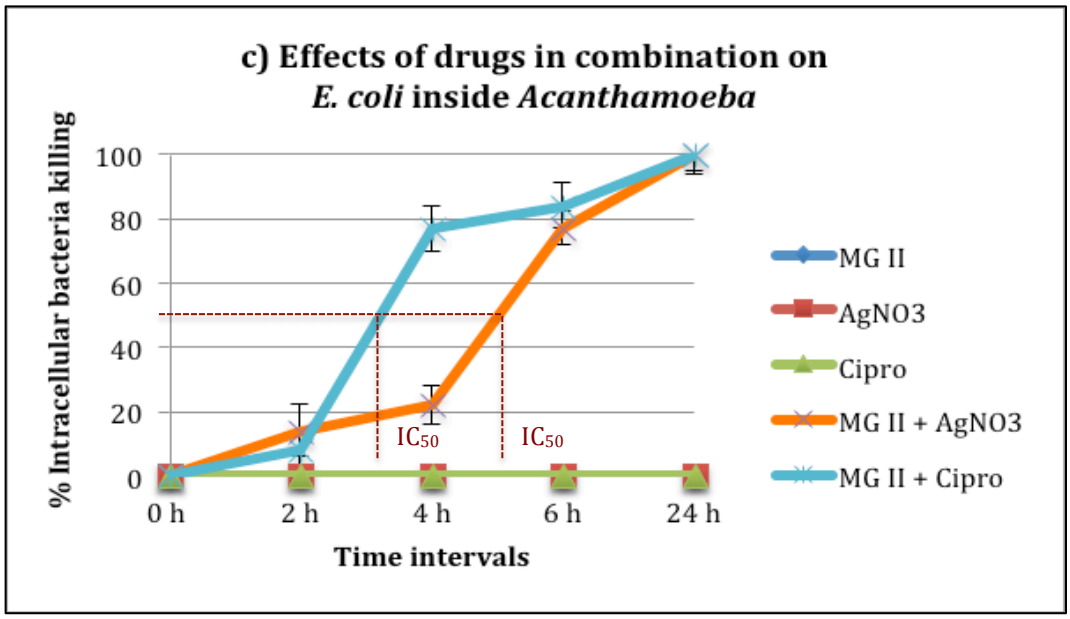


Figure 3.11 Drugs in combinations tested against intracellular bacteria inside *Acanthamoeba*: a) *S. epidermidis*; b) MSSA. Magainin II (3 µM); silver nitrate (12.5 µM) and various concentrations of vancomycin (6 µM) were applied. Two combinations were used experimentally: magainin II + silver nitrate; and magainin II + antibiotics. Abbreviations: MG II- magainin II; Vanco-vancomycin. Results are the average of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.



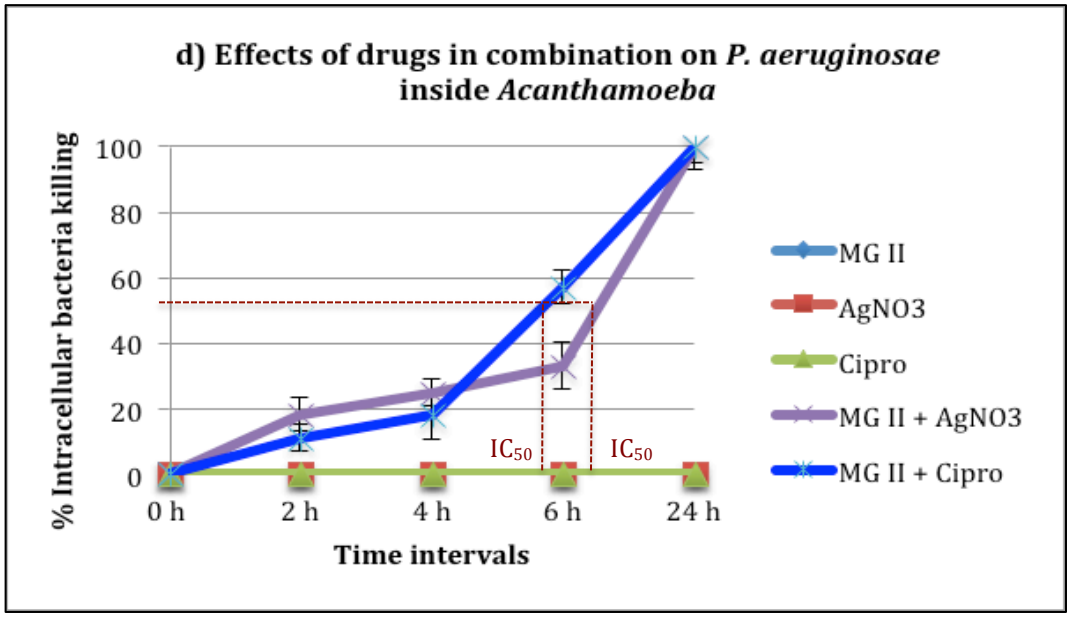


Figure 3.11 Drugs in combinations tested against intracellular bacteria inside *Acanthamoeba*: c) *E.coli*; d) *P. aeruginosa.* Magainin II (3 μM); silver nitrate (12.5 μM) and various concentrations of ciprofloxacin (8 and 12 μM) were applied. Two combinations were used experimentally: magainin II + silver nitrate; and magainin II + antibiotics. Abbreviations: MG II- magainin II; Cipro- ciprofloxacin. Results are the average of three autonomous experiments carried out in duplicate. Error bars symbolize standard error.

3.4 Discussion

It is well known that several bacterial pathogens are associated with CF and that they are the major cause of morbidity and mortality among patients. However, the understanding of pathogenesis and pathophysiology of these infections as well as the number of effective treatments remain insufficient. A fundamental question is how bacteria interact with epithelial cells of the respiratory tract, causing infections. In our previous studies we have revealed that all six bacteria studied in this project demonstrated an ability to associate with A549 cells and *Acanthamoeba* trophozoites. But, just four of them invade and survive internally inside host cells. In this study, various concentrations of magainin II exhibited an antimicrobial effect on *Acanthamoeba* trophozoites, extracellular and intracellular bacteria.

In recent years, the rapid increase in drug-resistant bacteria has prompted a growing interest in antimicrobial peptides as an alternative to conventional antibiotics. Comparing with conventional antibiotics, which act by interfering with a specific biochemical reaction inside the cell, antimicrobial peptides are believed to disrupt the bacterial membrane in a non-receptor-mediated fashion. The exact mechanism of action has not yet been fully described.

Information on antimicrobial effects of magainin II peptide against *Acanthamoeba castellanii* and internalized bacteria remains insufficient. Here, various time intervals along with different concentrations of magainin II were found to demonstrate antimicrobial efficacy *in vitro*. Similar studies revealed that magainins had been shown to be effective against *Acanthamoeba polyphaga* which had been isolated from clinics (Schuster & Jacob, 1992). The incubation of *Paramecium caudatum* with magainin II resulted in the contractile vacuoles immediate swelling and then rupture (Zasloff *et al*., 1988). In other studies, magainins displayed effectiveness against *Leishmania (Guerrero et al., 2004), Blastocystis hominis, Tryponosoma cruzi, and Entamoeba histolytica* in laboratory research (Huang *et al*., 1990). Flow cytometry assays demonstrated that negative control wells had 10-19 % early apoptotic cells. This could happen because of various factors, such as time of incubation with annexin buffer, higher speeds of centrifugations, harsh pipetting when re-suspending etc. Annexin is an indicator of very early apoptosis. At this early stage of apoptosis, AnnexinV conjugates have a direct access to the outer PS, even before the loss of membrane integrity. Overall the dynamics of

magainin II by increasing concentration can be correlated with that of trypan blue assay. Further studies to understand the mechanisms of this action on *Acanthamoeba* are essential.

On the other hand, as cationic antimicrobial peptides show broad-range activity against Gram-positive and Gram-negative bacteria (Hancock & Sahl, 2006), magainin II was tested against bacteria employed in this study and it displayed significant antibacterial potency. Interestingly, all Gram-positive bacteria employed in this study exhibited higher MIC when compared with *Acanthamoeba* trophozoites. Moreover, Magainin II was found to be more cytotoxic to *Acanthamoeba* when compared with *P. aeruginosa*. Recently, a microbial flow cytometry method was described which quantified magainin II activity within 3 h, called Minimum Membrane Disruptive Concentration (MDC). Increasing peptide concentration positively correlated with the extent of MRSA and *E. coli* membrane disruption and the calculated MDC was equivalent to its Minimum Bactericidal Concentration (MBC) (O'Brien-Simpson *et al.*, 2016). Magainin II inhibited the growth of *P. aeruginosa* (Dosler & Karaaslan, 2014), *S. epidermidis* (Imura *et al.*, 2007), *Str. pneumoniae* (Kovács *et al.*, 2006), and *H. influenzae* (Bishop-Hurley *et al.*, 2005). It has been shown with coarse-grained computer simulations using MARTINI force field, which is suited for molecular dynamics simulations of biomolecules that magainin produces water-filled toroidal holes in biological membranes (Santo & Berkowitz, 2012).

In preliminary experiments magainin II showed synergistic antimicrobial effect against bacteria in extracellular conditions when used in combination with silver nitrate and antibiotics such as vancomycin and ciprofloxacin.

Bacteria able to survive could contribute to re-infections. It is crucial to be able to eradicate intracellular bacteria without harming host cells. In this case, it was A549 cells. Magainin II in sub-cytotoxic concentrations was not able to eradicate intracellular bacteria, but could do so when used in combination with other antimicrobials such as silver nitrate, vancomycin and ciprofloxacin. The mechanism of positive synergistic activity between peptides and antibiotics appears to be complicated. Magainin II might play role in increasing of permeability of host cell membrane (He *et al.*, 2015).

Silver nitrate has a reputable place in the pharmacopoeia as an effective and well-tolerated antimicrobial reagent (Unger & Luck, 2012). By combining magainin II with silver nitrate, greater activity was seen in the experiments against intracellular bacteria.

This may allow for use of magainin II and silver nitrate at concentrations sub-cytotoxic concentrations, with continuous activity. The reasons for the greater efficacy of silver when used in combination with magainin II may be associated with influx into the *Acanthamoeba* or A549 cell line cytoplasm as a result of increased membrane permeability. This hypothesis can be supported by results, where we combined sub-lethal doses of antibiotics such as vancomycin and ciprofloxacin with magainin II. It was stated that the magainin II interacted synergistically with all tested β-lactam antibiotics (Giacometti *et al.*, 2000c).

The magainin II was found to be cytotoxic to the A549 cell line. This is consistent with another study (Pascariu *et al*., 2012). The greater cytotoxicity could be related to the outer negative charge of cancerous cells. The outer leaflet of eukaryotic plasma membranes normally comprises neutral phospholipids, like phosphatidylcholine (PC) and sphingomyelin (SM), whereas the negatively charged phospholipid phosphatidylserine (PS) as well as phosphatidylethanolamine (PE) are situated in the inner leaflet (Bevers *et al.*, 1996). It has been stated previously that this asymmetry could be lost due to decreased activity of the ATP dependent phospholipid translocase, which specifically transports PS and PE between bilayer leaflets and is susceptible to oxidative damage, or due to the activation of a scramblase stimulated by amplified intracellular Ca^{2+} levels which leads to non-specific movement of phospholipids (Seigneuret & Devaux, 1984). In turn, this loss of asymmetry ends up in exposure of the negatively charged PS on the surface of cancerous and other pathological cells (Zwaal *et al.*, 2005), as well as apoptotic cells (Martin *et al.*, 1995) and thus PS represents a promising target for cationic magainin II peptide.

In future studies, a similar combination drug experiment could be conducted applying antimicrobial agents against the cyst stage of *Acanthamoeba*. Antimicrobial reagents active against trophic *Acanthamoeba* cannot equally be active against cysts. In the majority of cases, cysticidal concentrations of reagents are substantially greater than amoebicidal concentration amounts (Osato *et al*., 1991). Moreover, microdilution checkerboard data can be used to assess pharmacodynamic interactions based on Fractionally Inhibitory Concentration (FIC) index analysis.

The outcomes of these investigations encourage further studying of magainin II and its derivatives for their ability to kill *Acanthamoeba* trophozoites and cysts as well as pathogenic bacteria. Although these outcomes are preliminary, they suggest that magainin II in combination with silver nitrate or antibiotics have *in vitro* efficacy against intracellular bacteria, and that they could be suitable in handling various types of infections caused by *Acanthamoeba* and bacteria.

Chapter 4

"Bacteria-host cell interactions and antimicrobial efficacy of magainin II against bacteria in conditions more relevant to Cystic Fibrosis"

4.1 Background

Bacterial respiratory infections are the leading causes of morbidity and mortality in CF patients (Ciofu *et al.*, 2013). *P. aeruginosa* and *S. aureus* strains are identified as opportunistic pathogens which trigger some of the most widespread infections of eyes, ears, wounds and lung (Park *et al.*, 2011). These pathogens are notorious with an extensive range of drug resistance abilities. Nonetheless, AMPs constitute a novel group of antimicrobials, since they are totally different from the antibiotics that eradicate pathogens. Although antibiotics require definite intracellular targets for their activity, AMPs usually do not require a specific target in the microorganism cell (Perron *et al.*, 2006). Instead, AMPs bind to the bacterial cell membrane and disassemble the membrane construction. Moreover, certain AMPs display selective inhibition of intracellular targets within the microorganism cells (Giuliani *et al.*, 2007). This action makes AMPs invulnerable to bacterial resistance, for which the microorganisms require to change the whole membrane lipid arrangement. Consequently, AMPs are appealing for their prospective therapeutic effect against drug-resistant microorganisms. Moreover, rational design of peptides could produce potentially therapeutic membrane active drugs with characteristics tailored to their function (Mason *et al.*, 2009).

AMPs have been shown to possess antiviral, antimicrobial, anticoagulant and antifreeze specifities by previous investigators, and the number of novel AMPs isolated is growing (Zasloff, 2002b). Since primary studies proposed that numerous AMPs target membranes (Matsuzaki *et al.*, 1991), the molecular mechanism of membrane permeabilization has been investigated comprehensively, exploiting predominantly model membranes. For instance, magainin II, an α-helical peptide isolated from the African clawed frog *Xenopus laevis* (Zasloff, 1987b) was proposed to form a toroidal pore with a diameter of 2–3 nm, stimulating lipid flip-flop and the translocation of peptides into the inner leaflet of the bilayer coupled to membrane permeabilization (Huang, 2006).

Pleurocidin, a cationic 25-residue AMP, was discovered in the skin-secreted mucous fluid of winter flounder (*Pleuronectes americanus*), which was examined against bacterial and fungal clinical isolates, exhibited broad-spectrum antimicrobial activity (Cole *et al.*, 2000). The sequence of the pleurocidin complete molecule, (GWGSFFKKAAHVGKHVGKAALTHYL), indicates sequence homology with the dermaseptin and ceratotoxin classes of antimicrobial peptides (Cole *et al.*, 2000). In a

study, where a variety of spectroscopic techniques were applied to study mechanisms of pleurocidin antibiotic activity with model membranes it was shown that pleurocidin interacts with both the zwitterionic phosphatidylethanolamine and anionic phosphatidylglycerol (PG) but disrupts the lipid acyl chain order of the anionic PG lipids more effectively (Mason *et al.*, 2006c).

The elaboration of antimicrobial peptides has been hindered by numerous complications. One of these difficulties is salt sensitivity (Park *et al.*, 2004). The antimicrobial ability of human β-defensin-1 is prominently dropped in the presence of high salt concentrations in broncho-pulmonary fluids in CF patients (Goldman *et al.*, 1997a). Analogous complications were spotted in the clinically active histidine-rich peptide P-113, indolicidins, gramicidins, bactenecins, and magainins (Wang *et al.*, 2009b). Nonetheless, not all AMPs are salt sensitive, and some peptides demonstrate potent salt-insensitive antimicrobial efficacy such as clavanins and tachyplesins (Tam *et al.*, 2002). NRC-13's action is similarly consistent with other pleurocidin, that it is not sensitive to salt conditions such as NaCl, $MgCl_2$ and $CaCl_2$ (Cole *et al.*, 2000). Concerning interactions of AMPs with mammalian cell membranes, there have been merely a few researches (Lau *et al.*, 2005). It is not surprising that maximum research on AMPs has concentrated on their antibacterial efficacies. Nevertheless, the cytotoxic effects of AMPs also need to be examined, because they hinder the systemic exploitation of AMPs (Hancock & Sahl, 2006).

The bactericidal abilities of silver ion has been identified since ancient eras, and its range is rather extensive (Clement & Jarrett, 1994). Silver ion reacts with the thiol group in vital enzymes and inactivates them (Liau *et al.*, 1997) or interacts with DNA (Feng *et al.*, 2000), resulting in noticeable development of pyrimidine dimerization by a photodynamic process and probable inhibition of DNA replication (Russell & Hugo, 1994). Silver nitrate reveals various functions against bacteria dependent upon its quantity (Kumar Pandian *et al.*, 2010). Silver-based antimicrobials can be efficient in the treatment of infections based on non-toxicity of active Ag+ to human cells (Ewald *et al.*, 2006).

CF patients experience chronic lung infections which need long-term antibiotic therapy (Jansen *et al.*, 2016). One of the reasons for the long-term presence of pathogens could be the presence of *Acanthamoeba* in lungs of the CF patient. *Acanthamoeba* is a ubiquitous, opportunistic protozoan pathogen, the causative agent of granulomatous encephalitis, keratitis and feeds on bacteria (Marciano-Cabral & Cabral, 2003). Being in the CF lung it could phagocytize pathogenic bacteria protecting them and giving them

conditions to grow (Cardas *et al.*, 2012b). This in turn could result in reinfections complicating the treatment of patients. Under unfavorable conditions or chemotherapeutic drugs, *Acanthamoeba* changes into a resistant double-walled cyst form, resulting in prolong presence of pathogens, and causing problems in the treatment of bacterial infections (Coulon *et al.*, 2010).

Based on this hypothesis, the aim of this part of the project was to study the growth of bacteria, *Acanthamoeba* and A549 cells in artificial sputum media (ASM) to mimic the natural physiological environment in the CF lung. Bacterial intracellular survival was examined within *Acanthamoeba* and A549 cells in ASM. Moreover, the antimicrobial and cytotoxic effects of magainin II were tested.

4.2 Materials and Methods

All chemicals were purchased from Sigma-Aldrich (UK), unless otherwise stated.

4.2.1 Preparation of Artificial Sputum Medium (ASM)

To prepare artificial sputum medium 4 g of DNA from fish sperm were added to 250 mL distilled water very slowly over a period of several h. The DNA takes a long time to completely dissolve and can be stirred overnight at RT. Next, 5 g of mucin from porcine stomach (type II) were added slowly to 250 mL sterile water until the mucin had dissolved completely. The solution can be stirred overnight at 4 °C and 0.25 g of each essential and non-essential L-amino acid, with the exception of L-tyrosine and L-cysteine, were dissolved in 100 mL sterile water. 0.25 g of L-cysteine in 25 mL of 0.5 M potassium hydroxide (KOH) (56.11 g/mol) and 0.25 g of L-tyrosine were dissolved in 25 mL sterile water. On top of that 5.9 mg diethylenetriaminepentaacetic acid (DTPA), 5 g NaCl and 2.2 g of KCl were added in 100 mL of distilled water. The DNA, Mucin, L-amino acids, DTPA, NaCl and KCl were combined in a 1-litre volume bottle. Into this mixture 5 mL of egg yolk emulsion were added and filled to approximately 850 mL with distilled water. pH was adjusted to 6.9 with 1 M Tris (pH 8.5; Mr 121.14) and the volume was brought to 1 litre with distilled water. The ASM was sterilized by filtration using a 0.22 µm syringe filter. Unfiltered and filtered ASM were stored at 4 °C in the dark.

4.2.2 Cell line and cell culture

A549 cell line was grown and cultured as described in section 2.2.3.

Normal human urothelial cell line (TERT-NHUC) was kindly provided by Prof. Knowles, from St. James's University Hospital in Leeds. The TERT-NHUC cell line was provided as a frozen sample. The frozen sample is a mixture of growth medium with 10% DMSO and 10% fetal calf serum (FSC). Cells were passaged into T25 tissue culture flasks and incubated at 37 °C in a CO_2 incubator (95 % humidity, 5 % CO_2) using Keratinocyte Growth Medium 2 (KGM) as a medium. Cells became confluent within 2 days (~$4x10^6$ cells). All passages used in this study were between passage numbers 4 to 7.

4.2.3 *Acanthamoeba* culture

Acanthamoeba strains (T4 genotype) were grown and cultured as described in section 2.2.1.

4.2.4 Bacterial cultures

Six clinical bacterial strains were utilized (MSSA, *Str. pneumoniae*, *S. epidermidis*, *P. aeruginosa*, *H. influenzae* and *E. coli*). All strains were cultured, inoculated and optical densities (OD) were measured by spectrophotometer as described in section 2.2.2.

4.2.5 Intracellular survival assays

To determine the long-term fate of bacteria inside A549 cells or *Acanthamoeba*, intracellular survival assays were performed. Briefly, A549 cells or *Acanthamoeba* were incubated with bacteria, followed by the addition of antibiotic for 45 min. After incubation, wells were washed with PBS. A549 cells were incubated in 0.2 mL of DMEM/ASM, while *Acanthamoeba* were incubated in 0.2 mL RPMI 1640/ASM for 24 h at 30 °C. Finally, cells were lysed and cultured onto agar and the number of bacteria survived inside cells counted.

4.2.6 Preparation of antimicrobial agents

Antimicrobial agents such as magainin II, silver nitrate ($AgNO_3$), vancomycin and ciprofloxacin were utilized in this study.

Magainin II was purchased and prepared as described in section 3.2.4. Antimicrobial activities of magainin II (with various concentrations for 24 h) were tested against *Acanthamoeba* trophozoites, bacteria in extracellular and intracellular conditions. In parallel, cytotoxic effects on the A549 cell line were studied.

Pleurocidin (NRC-13) with 95 % purity and molecular mass 2.652 kDa, was purchased from Cambridge Bioscience as a powder. The solutions for these peptides were prepared by dissolving powder in sterile water. Antimicrobial activities of pleurocidin were tested against *Acanthamoeba* trophozoites (25, 50, 100, 150 and 200 μM), bacteria (5 and 10 μM) in their own media and ASM for 24 h. Moreover, cytotoxic effects on the A549 cell line and TERT-NHUC (25, 50, 100, 150 and 200 μM) cell line were studied.

Silver nitrate ($AgNO_3$) is 0.170 kDa in mass and with ≥ 99 % purity, was dissolved in sterile water. It was tested against *Acanthamoeba* cysts in combination with magainin II.

All solutions were filter sterilized using a 0.2 µm filter. Stocks were kept at -20 °C.

4.2.7 Antimicrobial assays

4.2.7.1 Antibacterial assays

To determine the antimicrobial activity of magainin II to inhibit bacterial growth in ASM, antimicrobial assays were performed. Bacterial strains were grown in ASM and media (Luria broth (LB)/Brain heart infusion (BHI)/BHI+Fildes extract (FE)) overnight at 37 °C. Then ODs were measured by spectrophotometer and the number of viable bacteria was calculated as described in 3.2.5.1.

4.2.7.2 Antiamoebic assays

To determine the antimicrobial activity of the magainin II on *Acanthamoeba* in ASM, antiamoebic assays were performed. *Acanthamoeba* were cultured and grown as described in 3.2.5.2. Seven different concentrations of magainin II (2.5, 5, 10, 20, 30, 40 and 50 µM) were added to different wells. All wells contained 150 µl of antimicrobial agent in PYG/ASM, except wells of controls that contained only 150 µl of PYG/ASM. Then *Acanthamoeba* with magainin II were incubated at 30 °C for 24 h. The number of viable and dead *Acanthamoeba* was determined as described in section 3.2.5.2.

4.2.7.3 Cytotoxicity assays

To determine the cytotoxic activity of the magainin II on A549/TERT-NHUC cells, cytotoxic assays were performed. A549/TERT-NHUC cells were cultured and grown in 96-well plates in DMEM/KGM/ASM (~1 $x10^5$ cells per 0.150 milliliter in each well); and plates were incubated till confluent. Then, the growth medium was discarded and wells washed with PBS once to remove non- adherent cell. Five different concentrations of magainin II (2.5, 5, 10, 20, 30, 40 and 50 µM) or pleurocidin (NRC-13) were added to different wells. All wells contained 150 µl of peptide in DMEM/KGM/ASM, except wells of controls that contained only 150 µl of DMEM/KGM/ASM. Then A549/TERT-NHUC cells

with magainin II/pleurocidin (NRC-13) were incubated for 24 h at 37 °C (humidified 95 % air, 5 % CO_2). The number of viable and dead A549/TERT-NHUC cells in each well, was determined as described in section 3.2.5.2.

4.2.7.3 Cysticidal activity of magainin II in combination with AgNO3

To determine the cytotoxic effect of drugs in combination (magainin II-$AgNO_3$) on *Acanthamoeba* cysts, *Acanthamoeba* trophozoites were grown and incubated for up to 7 days onto non-nutrient agar to trigger their encystment. Next, cysts were collected, counted and 2 x 10^5 cysts / mL were incubated for 24 h with different concentrations of drugs in combination magainin II-silver nitrate (50, 100, 200, 300 and 400 μM). Wells with cysts, but without drugs were used as controls. The viability of cysts was calculated by exclusion method using trypan blue stain (0.2 % final concentration).

4.2.7.4 Transmission electron microscopy

The cysticidal activities of magainin II on *A. castellanii* cysts were observed by TEM. *Acanthamoeba* cysts were co-incubated with either magainin II-treated (400 μM) or magainin II-untreated media (ASM) overnight. Next day, cysts were harvested, centrifuged, washed and fixed. Images were obtained as described in section 2.2.8.

4.2.8 Presentation of results and statistical analysis.

The results shown in this work are representative of three independent experiments performed in triplicate by identical methods. The results are expressed as means ± 1 standard deviation. Differences were considered statistically significant if the *P* value was <0.05 by Student's *t* test.

4.3. Results

4.3.1 Bacterial survival in ASM

To determine whether the pathogenic bacteria under investigation survive in conditions mimicking the CF environment, ASM medium was used. RPMI, BHI, BHI+FE and LB were used as controls (for *S. epidermidis*, MSSA, *P. aeruginosa* and *E. coli*)/BHI (for *Str. pneumoniae*)/BHI+FE (for *H. influenzae*). All bacteria are isolates from CF lower respiratory tracts. ASM has all the components, which are found in sputum of a CF patient. *S. epidermidis* (4×10^7), MSSA (1×10^9), *Str. pneumoniae* (6×10^6), *E. coli* (4×10^9), *P. aeruginosa* (4×10^9) and *H. influenzae* (2×10^8) were found to have the ability to survive in artificial sputum medium. *S. epidermidis* (6×10^7) and MSSA (8×10^8) displayed growth similar to those when they are incubated in ASM. However for *Str. pneumoniae*, *E. coli*, *P. aeruginosa* and *H. influenzae* the growth rate in ASM was shown to be better compared to RPMI (1×10^5, 1×10^9, 8×10^9 and 3×10^7 respectively) (Figure 4.1).

All bacteria employed in this project exhibited growth potency in ASM. This leads us to conclude that sputum environment in CF lung is optimal for functioning of bacteria.

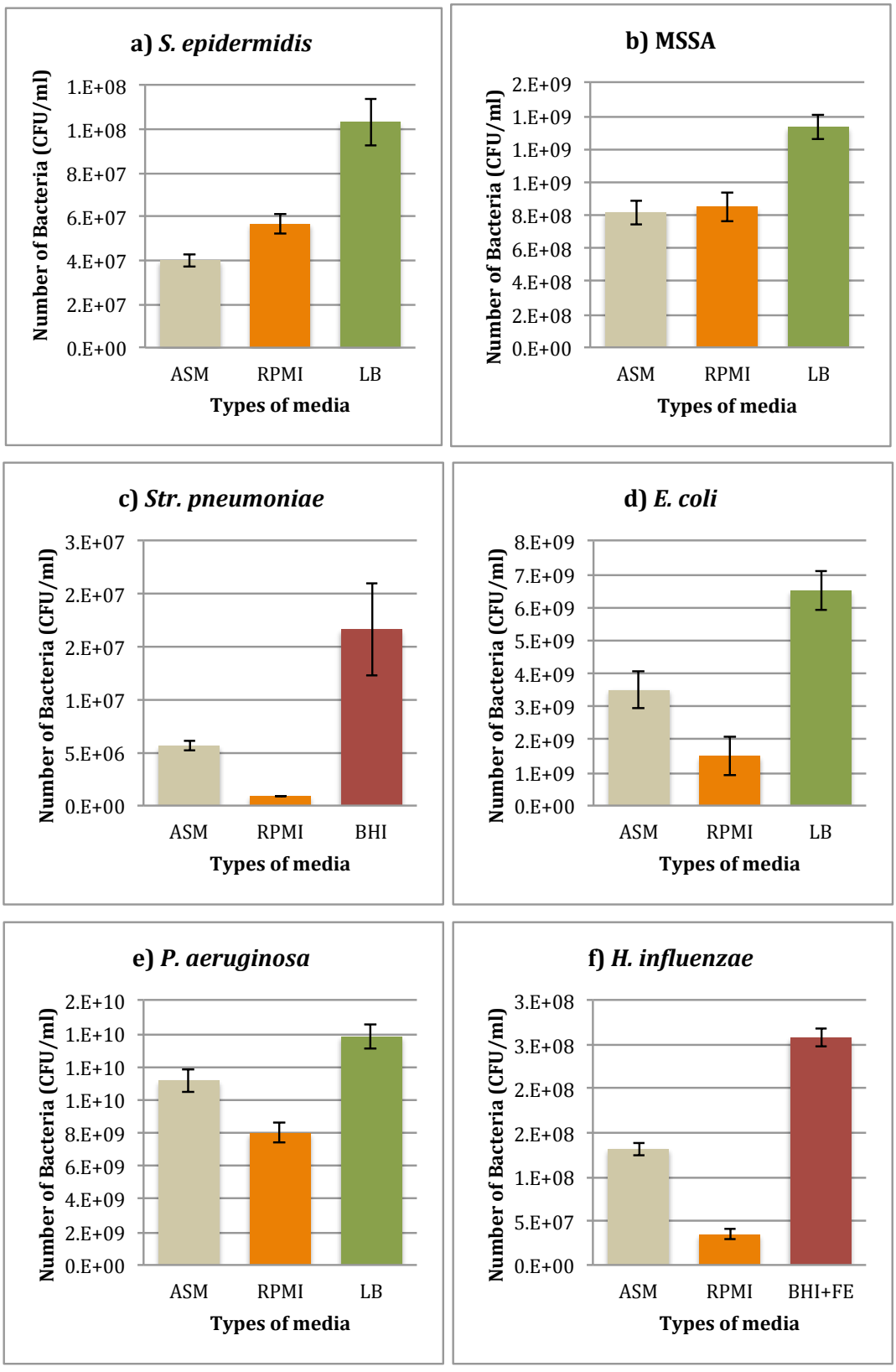


Figure 4.1 Bacterial growth in different media such as Artificial Sputum Medium (ASM); Roswell Park Memorial Institute medium (RPMI); Luria Berthani (LB); Brain heart infusion (BHI); BHI+Fildes extract (BHI+FE).

4.3.2 *Acanthamoeba* and A549 cells survival in ASM

The hypothesis leading to this work was that *Acanthamoeba* could have all necessary conditions for growth and development in the lower respiratory tract. A549 cells were used for comparison in this assay. The aim was to maintain a similar environment to the physiological state of the CF lung. To analyze whether *Acanthamoeba* could survive in the lungs of CF patients, ASM was used to mimic the environment. PYG and RPMI media were used as controls for *Acanthamoeba* and DMEM for A549 cells. Trypan blue exclusion assays were performed to analyze survival of both *Acanthamoeba* and A549 cells. *Acanthamoeba* exhibited growth ability in ASM (65 x 10^5/mL), RPMI (40 x 10^5/mL) and PYG (77 x 10^5/mL). A549 cells also showed ability to grow in ASM (25 x 10^5) whilst in DMEM (41 x 10^5). ASM is evidenced to be sufficient media for *Acanthamoeba* growth (Figure 4.2).

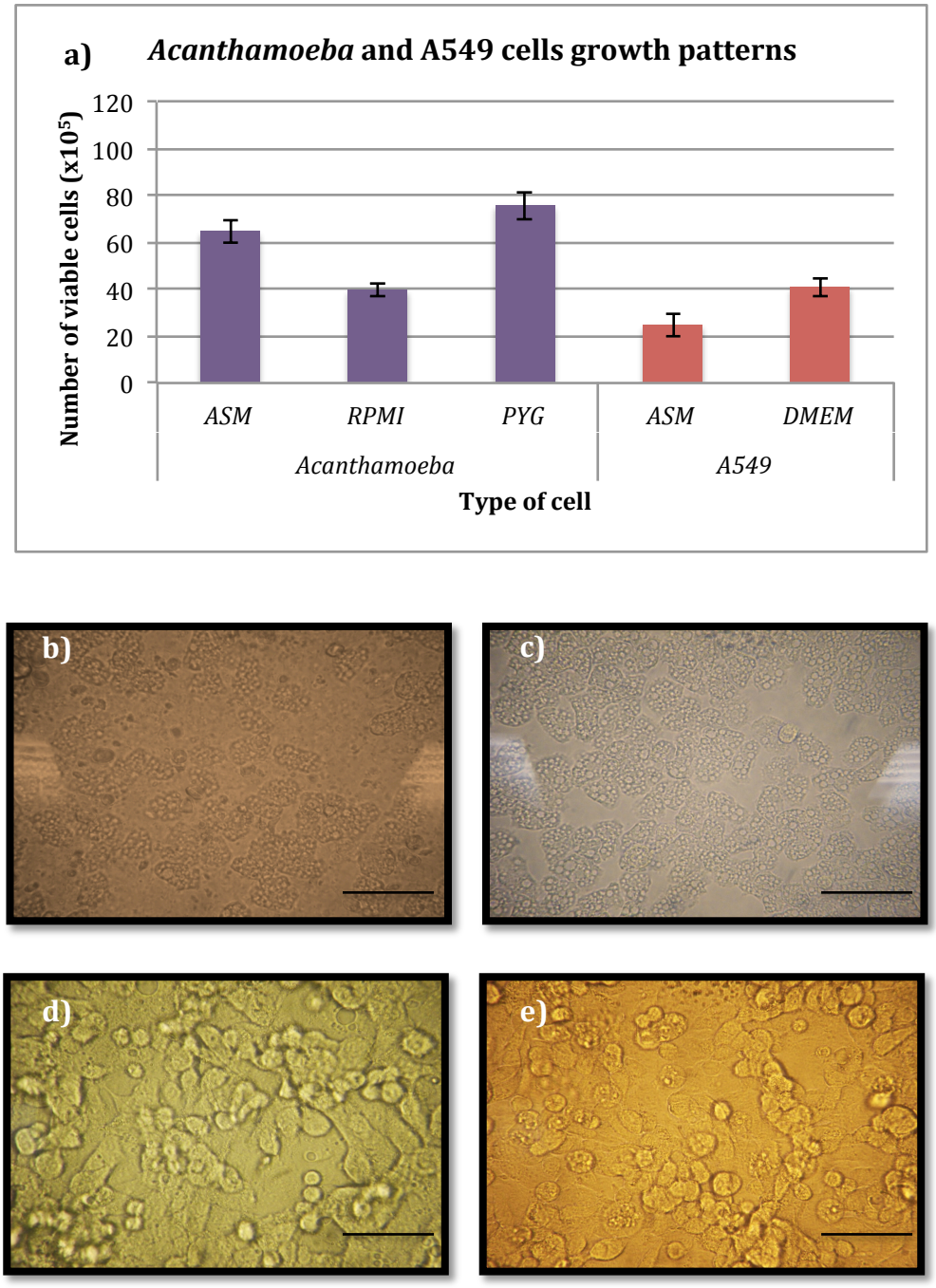
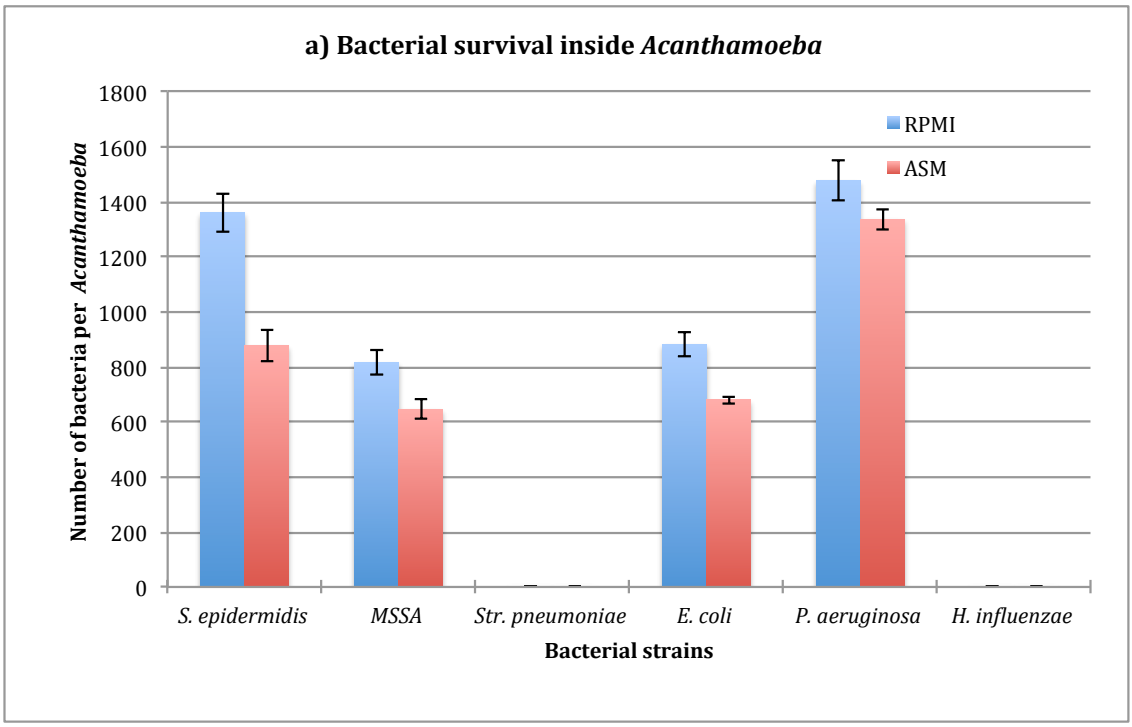


Figure 4.2 *Acanthamoeba* and A549 cell line growth patterns in different media. Comparison of *Acanthamoeba* trophozoites and A549 cells growth patterns in different media (a). *A. castellanii* trophozoites were incubated with b) ASM and c) PYG and A549 cells with d) ASM and e) DMEM. Data are represented as the means and standard errors of at least three independent experiments performed in duplicate. Scale bars, 30 µm.

4.3.3 Intracellular survival assays

Acanthamoeba present in the lower respiratory tract could interact with bacteria by feeding on them. It has been demonstrated that pathogenic bacteria after engulfment develop strategies to stay undigested in *Acanthamoeba* and even keep the ability to multiply. Pathogenic bacteria could use *Acanthamoeba* as a "Trojan horse" in lower respiratory tracts of CF patients. Examining interaction patterns between *Acanthamoeba* and bacteria associated with CF in ASM is therefore essential. To determine if bacteria survive in *Acanthamoeba* in the artificial sputum environment, survival assays were performed. At the beginning of assays 10 bacteria per *Acanthamoeba* were added. More than 840 *S. epidermidis*, 620 MSSA, 630 *E. coli* and 1370 *P. aeruginosa* per *Acanthamoeba* survived in ASM (Figure 4.3a). It is obvious that bacteria- *Acanthamoeba* interaction in ASM is occurring. However, survival was lower in A549 cell: per A549 cell *S. epidermidis*, MSSA, *E. coli* and *P. aeruginosa* were 2.49, 2.58, 2.51 and 1.43 respectively (Figure 4.3b). *Str. pneumoniae* and *H. influenzae* were unable to survive neither in *Acanthamoeba* nor in A549 cells in either type of medium.



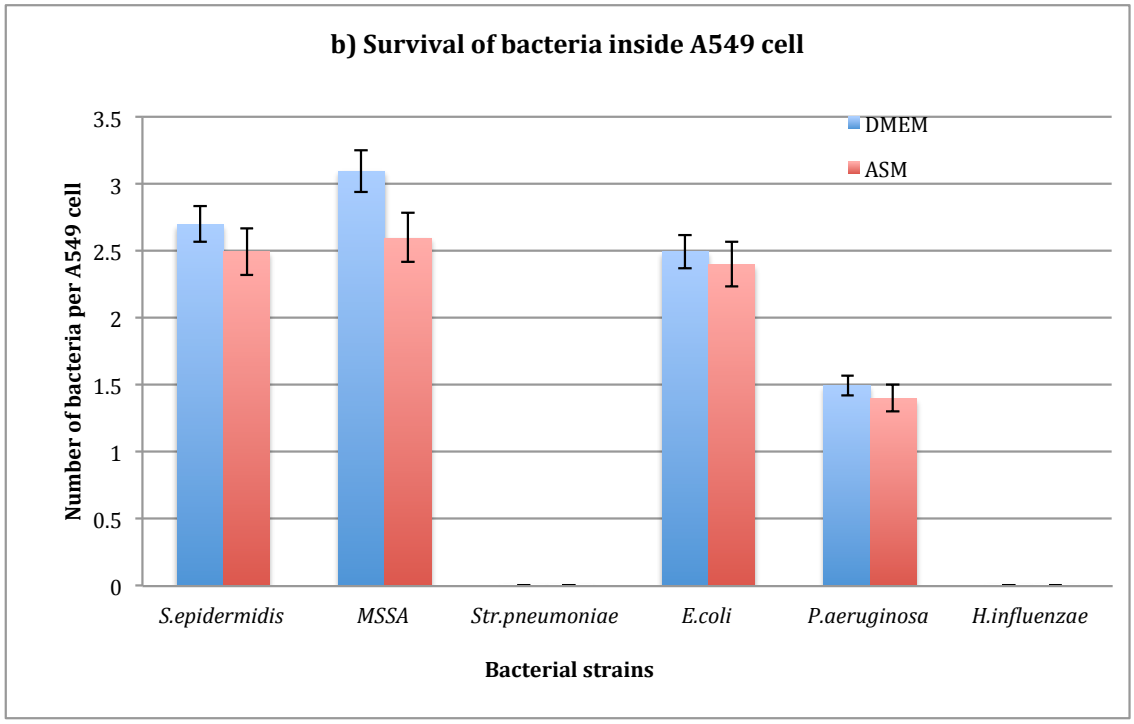


Figure 4.3 Intracellular survival rates of bacteria in different media. Above it is shown that all bacteria exhibit intracellular survival in ASM inside *Acanthamoeba* and A549 cells except *Str. pneumoniae* and *H. influenzae*. These two bacteria were found to be encapsulated which can be the reason of no invasion and survival. Data are represented as the means and standard errors of at least three independent experiments performed in duplicate.

4.3.4 Biocidal assays

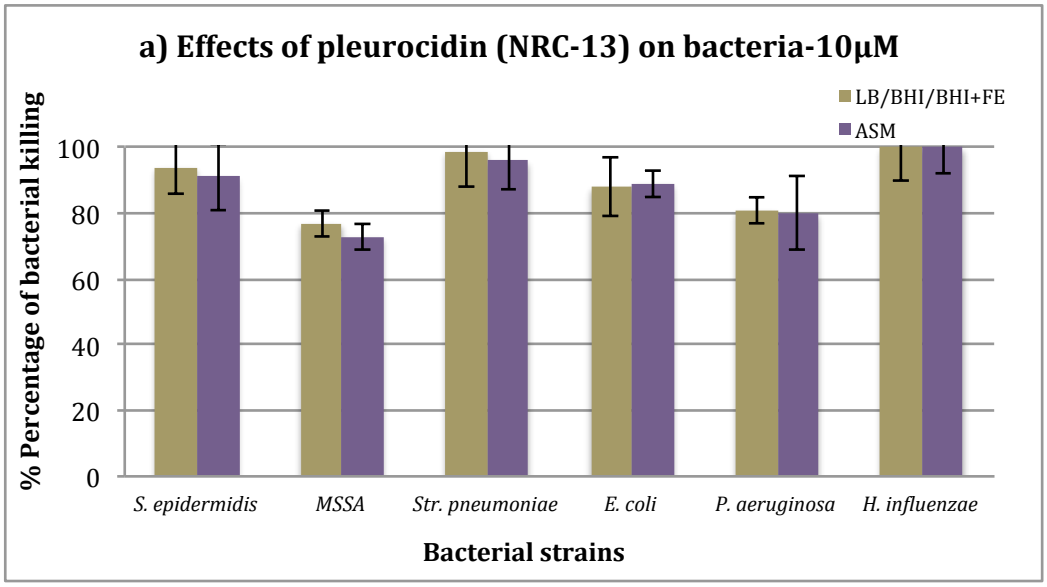
4.3.4.1 Antimicrobial activity of magainin II on bacteria in ASM

To determine antimicrobial efficacy of magainin II, antimicrobial assays were carried out (Table 4.1). After 24 h of incubation magainin II showed bactericidal effects on *S. epidermidis* (50 μM in LB; 90 μM in ASM), MSSA (50 μM in LB; 90 μM in ASM), *Str. pneumoniae* (40 μM in BHI; 80 μM in ASM), *E. coli* (30 μM in LB; 70 μM in ASM), *P. aeruginosa* (40 μM in LB; 60 μM in ASM), *H. influenzae* (30 μM in BHI+FE; 60 μM in ASM).

Table 4.1 Minimum inhibitory concentrations of magainin II on bacteria in different media: in conventional (LB/BHI/BHI+FE), and in ASM. Data are represented as the means and standard errors of at least three independent experiments performed in duplicate.

	LB/BHI/BHI+FE	**ASM**
S. epidermidis	50 μM	90 μM
MSSA	50 μM	90 μM
Str. pneumoniae	40 μM	80 μM
E. coli	30 μM	70 μM
P. aeruginosa	40 μM	60 μM
H. influenzae	30 μM	60 μM

Pleurocidin, another antimicrobial peptide less sensitive to salt was examined in the salty environment of ASM. Two different concentrations (10 μM and 5 μM) of pleurocidin were tested against all bacteria employed in this project (Figure 4.4). 10 μM of pleurocidin eradicated *S. epidermidis* (91 % in ASM, 94 % in LB), MSSA (73 % in ASM, 77 % in LB), *Str. pneumoniae* (96 % in ASM, 99 % in BHI), *E. coli* (89 % in ASM, 88 % in LB), *P. aeruginosa* (80 % in ASM, 81 % in LB) and *H. influenzae* (100 % in ASM, 100 % in BHI+FE). 5 μM of pleurocidin eradicated *S. epidermidis* (40 % in ASM, 41 % in LB), MSSA (33 % in ASM, 34 % in LB), *Str. pneumoniae* (44 % in ASM, 46 % in BHI), *E. coli* (41 % in ASM, 38 % in LB), *P. aeruginosa* (37 % in ASM, 39 % in LB) and *H. influenzae* (61 % in ASM, 57 % in BHI+FE).



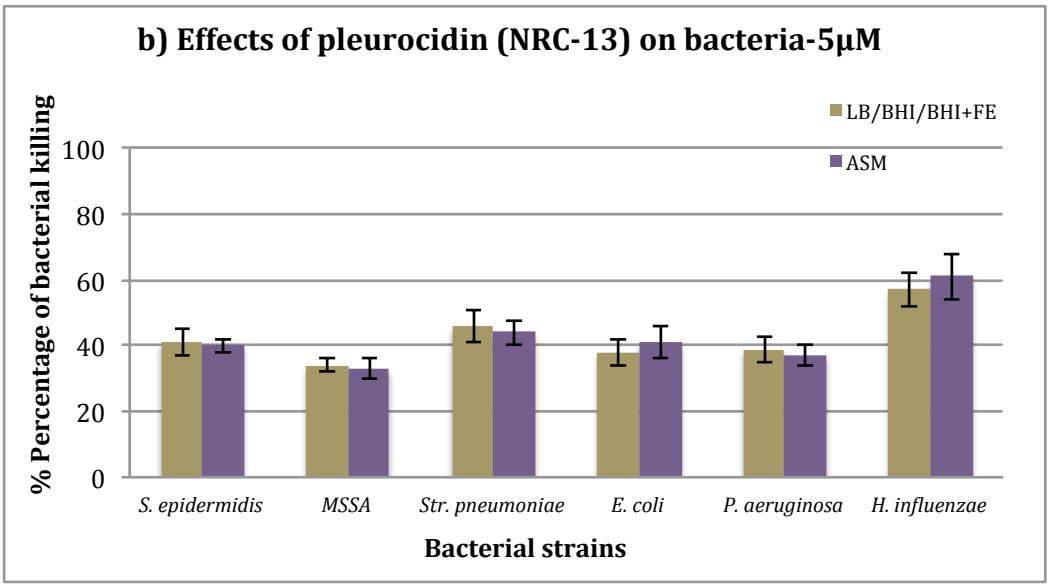
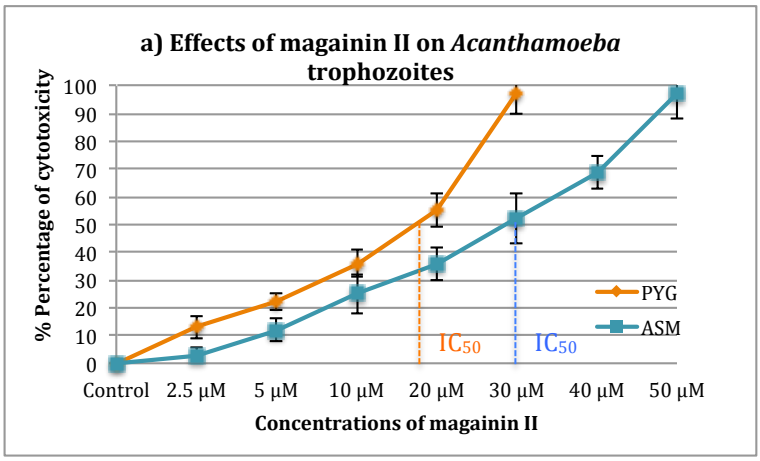
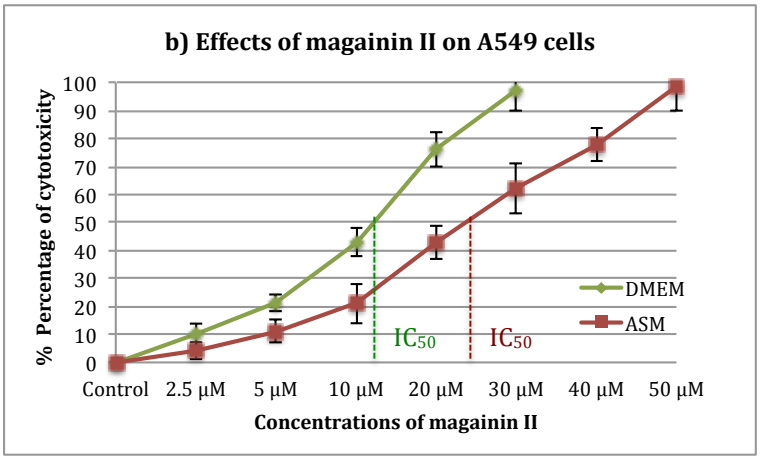


Figure 4.4 The antibacterial activity of pleurocidin (NRC-13) against employed bacteria in ASM with control media (LB, BHI and BHI+FE). Two various final concentrations of pleurocidin (10 and 5 µM) were tested. Pleurocidin exhibited similar effects on bacteria regardless in which media bacteria present. Pleurocidin shows salt tolerance against ASM salinity. Data are represented as the means and standard errors of at least three independent experiments performed in duplicate.

4.3.4.2 Amoebacidal and cytotoxic effects of magainin II on A549 in ASM medium

To determine the amoebacidal and cytotoxic effects of magainin II on cells, cytotoxicity assays were performed. 30 μM of magainin II were completely amoebicidal in PYG and 50 μM in ASM. This effect was significant using t-test $P<0.5$. Similar concentrations (30 μM in DMEM and 50 μM in ASM) were 100 % cytotoxic to A549 cells. Magainin II against TRET-NHUC cells did not show similar cytotoxic effect as on A549 cells. 50 μM of antimicrobial peptide were cytotoxic to 40 % in KGM and 30 % in ASM to TERT-NHUC cells (Figure 4.5).





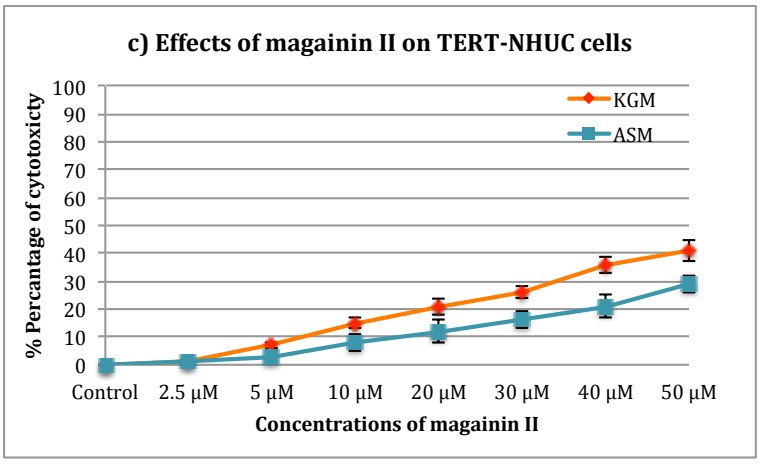


Figure 4.5 Biocidal effects of magainin II on a) *A. castellanii* trophozoite; b) A549 cells; c) TERT-NHUC cells. Magainin II is less amoebicidal in ASM when compared with PYG; Magainin with 50 µM final concentration, was 100 % cytotoxic against A549 cells and 30 % cytotoxic against TRET-NHUC cells. Data are represented as the means and standard errors of at least three independent experiments performed in duplicate.

Salt tolerant pleurocidin (NRC-13), was examined against *Acanthamoeba* (in ASM and PYG), A549 cells (in ASM and DMEM), and TRET-NHUC (in ASM and KGM). Five various concentrations of cationic peptide (25, 50, 100, 150 and 200 μM) were tested. Results show 150 μM of pleurocidin exhibited more than 90 % cytotoxic effect on A549 cells. Amoebicidal effect was reached when concentration of pleurocidin was increased to 200 μM. The least cytotoxic effect was found on TRET-NHUC cells, where 200 μM killed 70 % of cells (Figure 4.6).

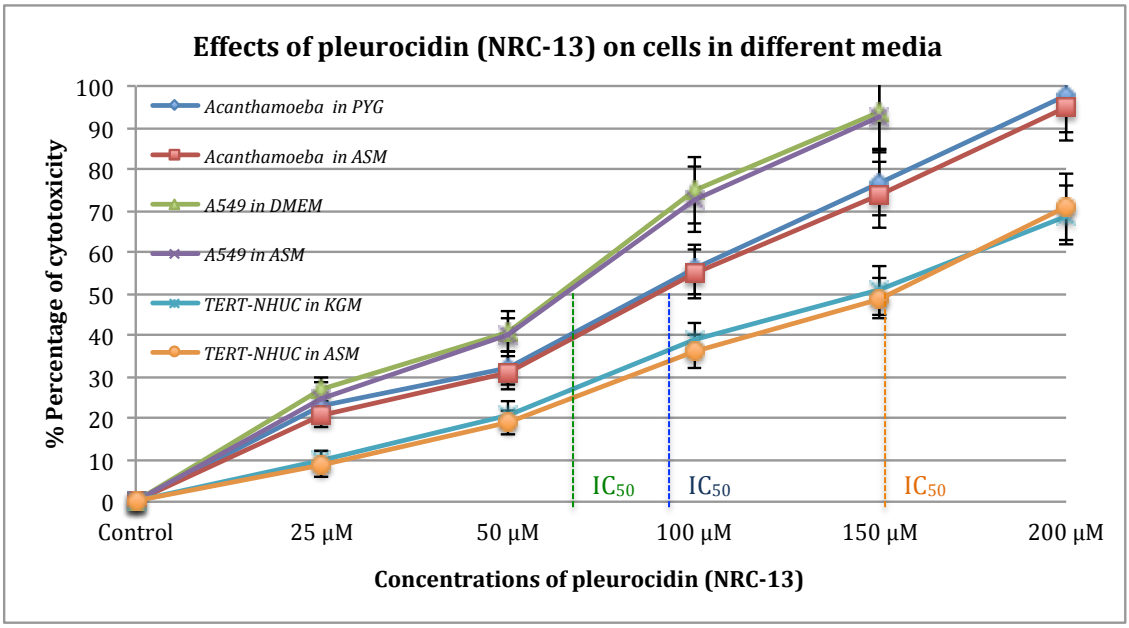
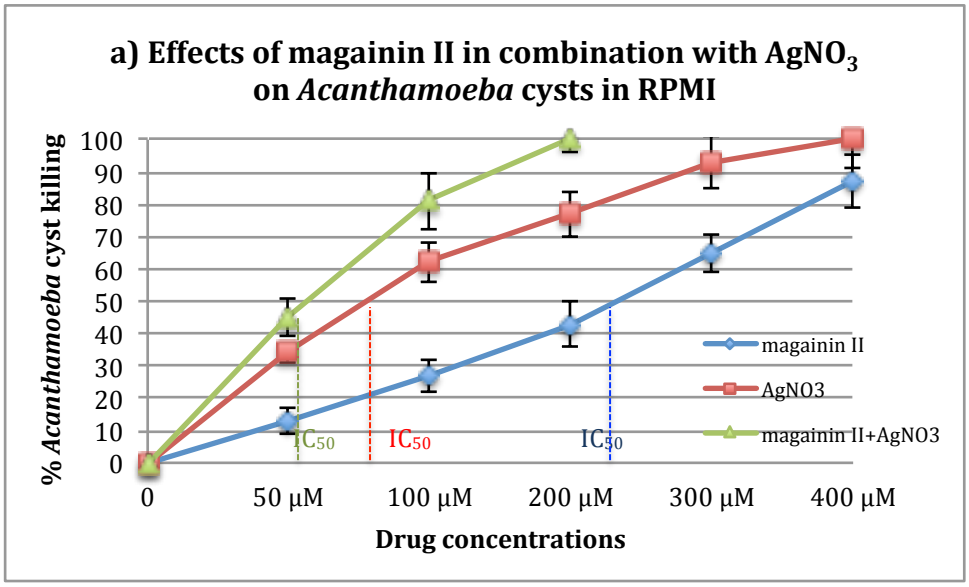


Figure 4.6 Biocidal effects of pleurocidin (NRC-13) on *A. castellanii* trophozoites, A549 cells and TERT-NHUC cells. As shown above pleurocidin has similar antiamobic effects in ASM and PYG. With the final concentration of 150 µM, pleurocidin is 100 % cytotoxic to A549 cells. The 200 µM of pleurocidin was 70 % cytotoxic to TERT-NHUC in ASM and KGM. Data are represented as the means and standard errors of at least three independent experiments performed in duplicate.

4.3.4.3 Cysticidal activities of magainin II alone and in combination with $AgNO_3$

As cysts are more resistant due to their cell wall, it was aimed primarily to test the cysticidal effect of magainin II in RPMI. The concentration of peptide to reach cysticidal effect was 400 μM. It was decided to use silver nitrate in combination with peptide to reduce the concentration of peptide used. 200 μM of magainin II in combination with 200 μM of silver nitrate exhibited cysticidal effect after 24 h of co-incubation. Magainin II cysticidal effects in ASM were less than in RPMI. At 400 μM concentration magainin II in ASM was able to kill 40 % of cysts. Drugs in combination were able to reach complete cysticidal effect with higher doses (magainin II 400 μM and silver nitrate 400 μM) when compared to those in RPMI (Figure 4.7).



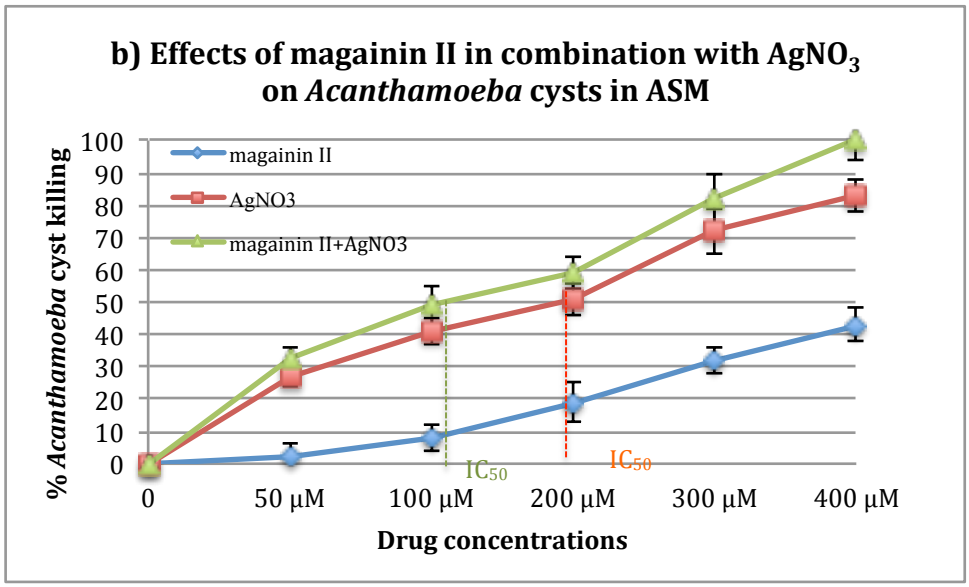


Figure 4.7 Cysticidal efficacy of magainin II, silver nitrate and both in combination in a) RPMI and b) ASM. To determine the cysticidal effect of AMP on *Acanthamoeba* cysts, *Acanthamoeba* trophozoites were incubated for up to 7 days onto non-nutrient agar to facilitate encystment. Next, cysts were collected, counted and 2 x 10^5 cysts / mL were incubated for 24 h with different concentrations of AMP alone or in combination wit silver nitrate. Cysts without antimicrobial agents were used as controls. The viability of cysts was performed by trypan blue exclusion assay (0.2 % final concentration).

The MIC indices of magainin II were different for all bacteria, *Acanthamoeba* and A549 cells in different media. MIC indices were higher in ASM media (*S. epidermidis*-50, 90; MSSA-50, 90; *Str. pneumoniae*-40, 80; *E. coli*-30, 70; *P. aeruginosa*-40, 60; *H. influenzae*-30, 60; *Acanthamoeba*-30, 50; A549 cells-30, 50). To investigate the ultrastructural alterations caused by magainin II in the *Acanthamoeba* cysts TEM studies were performed. *Acanthamoeba* cysts were treated with antimicrobial peptide (400 μM) for 24 h. *Acanthamoeba* cyst controls were free of antimicrobial peptides. Encystment of the *Acanthamoeba* is evident. Cell wall is present. Exposure of cysts to magainin II produced many of the effects on cytoplasmic integrity, alterations and swelling of the cyst wall. Inside the cyst, the trophozoite presents signs of damage. Electron-dense granules also appear. In cyst, loss of the inner wall and cell wall disruption are observed (Figure 4.8).

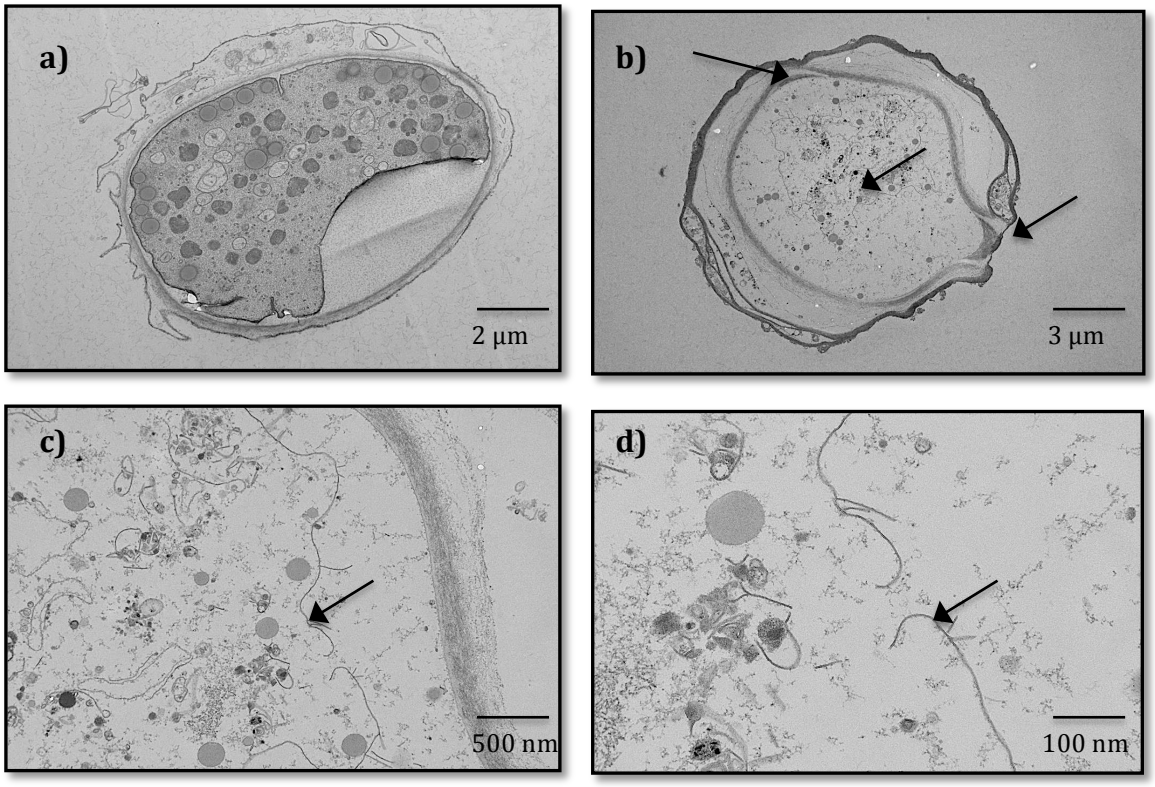


Figure 4.8 Transmission electron microscopy of *Acanthamoeba* cysts untreated and treated with magainin II. a) control, without peptide; b) with peptide (400 µM); c & d) magnification of *Acanthamoeba* cyst incubated with magainin II. Exposure of cysts to magainin II produced many of the effects on cytoplasmic integrity, alterations and swelling of the cyst wall (arrows in b). Inside the cyst, the trophozoite presents signs of damage. In cyst, loss of the inner wall and cell wall disruption are observed (arrows in c and d). Images were acquired using an AMT XR60 digital camera.

4.4. Discussion

In order to better comprehend bacterial infections associated with CF, it is essential to generate model conditions to imitate those in the CF lung, for example media designed to mimic the nutritional environment of CF sputum (Behrends *et al.*, 2013). Furthermore, numerous studies utilize a more-conventional rich medium, such as LB, BHI and RPMI. All of these media support development of bacteria to high densities, but the issue remains how the variations in composition affect the growth, physiology and metabolism of the experimented bacteria and, consequently, the importance to CF infections. Hence, in this study, growth patterns of bacteria, *Acanthamoeba* and A549; survival rate of bacteria within *Acanthamoeba* and A549 cells; Antimicrobial and cytotoxic effects of magainin II assays were performed in artificial sputum medium to check if it is cytotoxic to cells under treatment conditions.

Some of the CF patients are suffering from prolong treatment due to the bacterial infections and re-infection is not uncommon. We hypothesise that the presence of *Acanthamoeba* could play a role as they feed on bacteria and protect them from unfavourable conditions. Bacteria multiply inside and burse *Acanthamoeba* to re-infect the surroundings. In the presence of any medication, *Acanthamoeba* turn to very rigid double walled cysts which is very hard to treat. Without treating *Acanthamoeba*, the bacterial infection will continue in the CF lungs.

Wide-ranging heterogeneity was observed in natural populations of *P. aeruginosa* recovered from CF patient sputum (Mowat *et al.*, 2011). Previous studies showed that both phenotypic and genotypic divergence can be detected during growth in ASM, making it an appealing *in vitro* model of the CF lung conditions (Sriramulu *et al.*, 2005). ASM was exploited to examine biofilm formation by *Stenotrophomonas maltophillia* (Fouhy *et al.*, 2007). Bacteria (MSSA, *Str. pneumoniae, P. aeruginosa and H. influenzae*) employed in this study were isolated from CF patients, which previously mentioned in various researches (Lipuma, 2010). All bacteria were able to exhibit growth potential in ASM. The components of artificial sputum such as sodium, chloride, potassium, calcium, ammonium, and magnesium (Sanders *et al.*, 2006) had no inhibiting effect on growth of bacteria.

Acanthamoeba is an opportunistic protist that is universally dispersed in the environment. The routes of access into human organism for *Acanthamoeba* include the

respiratory tract (Siddiqui & Khan, 2012). *Acanthamoeba* feeds on bacteria (Martinez & Visvesvara, 1997). It is expected that each human inhales one cyst of *Acanthamoeba* a day (Warhurst, 1968). All these facts lead to the importance of studying bacteria-*Acanthamoeba* interactions in ASM. It would appear that the presence of *Acanthamoeba* in the lung of CF patients could play a role in the persistence of bacterial infections and prolong treatment process. In this project it was demonstrated that *S. epidermidis,* MSSA*, E. coli and P.aeruginosa* were capable of surviving intracellularly. These bacteria were able not only to survive but even to multiply within *Acanthamoeba* and A549 cells. Recent studies have demonstrated that *Vibrio cholerae* O1 and O139 are capable of surviving and replicating inside *Acanthamoeba castellanii* (Abd *et al.*, 2009). Several microbes have developed strategies to become resistant to protists, since they are able to survive, grow, and exit free-living amoebae after internalization (Greub & Raoult, 2004). It was shown that MRSA and MSSA were able to survive and multiply within *Acanthamoeba* (Cardas *et al.*, 2012b) which is consistent with the results in this study.

It was found that magainin II was the only peptide examined in one study, from the group of antimicrobial peptides analyzed, that exerted antimicrobial efficacy against particular strains of the *Burkholderia cepacia* complex, which is associated with chronic opportunistic lung infections of CF patients (Thwaite *et al.*, 2009).

In this project an investigation of the antimicrobial potential of magainin II against bacterial strains associated with CF, with the ultimate goal of elaborating an additional effective treatment for infections with these pathogens is reported. Antimicrobial activity of magainin II is shown against bacteria such as *S. epidermidis,* MSSA, *Str. pneumoniae, E. coli, P. aeruginosa, H. influenzae* and *Acanthamoeba*. Magainins are broad-spectrum, positively charged, antimicrobial peptides (Zasloff, 1987b). Magainin II has earlier been shown to have antimicrobial potency against multidrug-resistant bacteria such as meticillin-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Giacometti *et al.*, 2005).

The present work has demonstrated that magainin II activity was inhibited (2-fold) when used in ASM compared to conventional laboratory media. This could be, because of the increased salt concentration in ASM. Magainins have been reported to be salt sensitive (Lee *et al.*, 1997). It was determined that healthy sputum fluid had Cl^- concentrations of 84 ± 9 mM, whereas CF fluid had higher Cl^- concentrations of 129 ± 5 mM (Gilljam *et al.*,

1989). Therefore, the salt insensitive antimicrobial peptide, pleurocidin, was tested in ASM including conventional media. Pleurocidins exert broad-spectrum antimicrobial potency at micro molar concentrations and also exhibit synergistic activity with several antimicrobial agents (Cole *et al.*, 2000). Additionally, in contrast to many cationic peptides, pleurocidin is insensitive to NaCl concentrations up to 150 mM, and could therefore have employment in relatively high-salt body fluids and in treatment of lung infections in CF patients, who possess higher NaCl content (Goldman *et al.*, 1997b). Consistent with previous studies in this project pleurocidin, unlike magainin II, was equally effective in all media without significant difference, and showed salt-insensitivity properties. Although here we hypothesize that the main cause of magainin II activity decrease is saltiness it is essential to note that the potency of magainin II could be effected by the ingredients of PYG and ASM.

Magainin II demonstrated antimicrobial efficacy, but at the same time it was cytotoxic on A549 cells. Researches have revealed a substantial cytotoxic effect of magainin II against a broad spectrum of cancer cell lines comprising melanoma, bladder, breast and lung cancers as well as lymphomas and leukaemia (Lehmann *et al.*, 2006). It has been proposed that the moderately greater quantities of anionic constituents, such as phosphatidyl serine in tumour cells, may predominantly attract cationic magainins (Iwasaki *et al.*, 2009). Magainin II was less cytotoxic against TERT-NHUC in comparison with A549 cells. Higher pleurocidin concentrations did not exert cytotoxic effects on TERT-NHUC and exhibited no significant difference in either media including ASM.

A transmission electron microscope study has revealed both structural and cytoplasmic membrane damage to *A. castellanii* cysts when treated with higher concentrations of magainin II alone or in combination with silver nitrate. The antiamoebic effect of magainins has been previously established (Feldman *et al.*, 1991). Schuster *et al* showed that magainin peptide in combination with silver nitrate or other antimicrobial agents have *in vitro* activity against *Acanthamoeba trophozoites and cysts* (Schuster & Jacob, 1992b).

Acanthamoeba infections are threatening and commonly noncompliant to antimicrobial treatment. An additional aspect of drug response to take into account is the differential sensitivity of trophozoites and cysts, the former being more sensitive than the latter (Osato *et al.*, 1991). Cysticidal dosages for various antimicrobial agents range from 10-1000 times the doses effective against trophozoites of *Acanthamoeba* (Nagington &

Richards, 1976). Magainin II alone and silver nitrate alone were used against *Acanthamoeba* cysts. At high concentrations they were able to eliminate cysts. Several studies have suggested that magainin II acts synergistically with numerous antimicrobial agents including piperacillin, ceftazidime, imipenem, meropenem, clarithromycin polymyxin E (Giacometti *et al.*, 2000d) and silver nitrate (Schuster *et al*. 1992). A substantial advantage of the magainins is their apparent role in increasing cell membrane permeability (Zasloff *et al.*, 1988). This has been exploited in the current study by combining magainin II with silver nitrate to observe increased antimicrobial potency *in vitro* against *A. castellanii* cysts. Silver nitrate has an reputable place in the pharmacopoeia as an efficient and well-tolerated antimicrobial agent, utilized in the treatment of neonatal ophthalmia (Moore *et al.*, 2015). By combining silver nitrate with magainin II, greater activity was established in the reactions against cysts. The nature of boosted antimicrobial potency of magainin II when used in combination with silver nitrate may relate to influx into the *Acanthamoeba* cytoplasm as a result of increased membrane permeability.

Here, it has been concluded that these findings are preliminary, but they indicate that magainin II has potential to be used against bacterial pathogens and *Acanthamoeba* in CF patients.

Chapter 5

"Magainin II inhibits the biofilm formation of bacteria associated with Cystic Fibrosis"

5.1 Background

The lung of CF patients is in a prolonged inflammatory condition and reacts inadequately to antimicrobial treatment. CF patients normally suffer from tenacious and recurrent lung infections instigated by pathogens in the CF air ways (Heijerman, 2005). Chronic bacterial infections are related to a deteriorating clinical status of the CF patients and a worsened prognosis, and once established, existing antibiotic procedures are impotent to eliminate these infections in airways (Römling *et al.*, 1994). The difficulties in the treatment efficacy is considered to be because of the formation of antibiotic resistant biofilms in the CF lungs (Costerton *et al.*, 1999). The capability of bacteria to develop and produce drug resistant biofilms in the airways of the CF patients is assumed to be eased by the hyper-secretion of a gluey mucus layer, which leads to a low oxygen environment, and the release of DNA and actin resulting from the necrosis of neutrophils that are employed as part of the innate immune response (Boucher, 2004). Bacterial biofilms develop when microorganisms permanently adhere to a surface and manufacture extracellular polymers which enable the adhesion and supply a structural matrix which stabilizes the biofilm (Sasirekha *et al.*, 2015). One possible aspect of biofilm-mediated drug resistance can be credited to the differential gene expression of biofilm cells compared to their planktonic counterpart (Whiteley *et al.*, 2001).

Biofilms can exist on inner, non-living surfaces like medical equipment or living surfaces like wounded tissue (Gayvallet-Montredon *et al.*, 1998). Furthermore, these biofilm cells can avoid the host immune response and remain unrecognized by antimicrobial agents in the host (Crossley *et al.*, 2009). The worrying medical situation of medical sphere is that the biofilm population contributes to nearly 80% of the overall microbial infection (Yakandawala *et al.*, 2007). Biofilm likewise facilitates swift horizontal gene transfer between bacteria which can lead to escalation in the quantity of virulent strains (Lewis, 2001).

AMPs are considered to have the prospective for use as anti-biofilm agents for their diverse mechanisms, which comprise membrane-disrupting ability, functional inhibition of proteins, adhere to DNA, and detoxification of polysaccharides (lipopolysaccharide and lipoteichoic acid) (Waters & Bassler, 2005b).

5.1.1 *P. aeruginosa*

This is an opportunistic, leading pathogen in the CF air way, and by late adolescence (Lyczak *et al.*, 2002), 80 % of the CF patients are chronically infected contributing to the extreme level of illness and death (Goossens, 2003). In the majority of cases it has been shown that the pathogenesis triggered by them is conditional on biofilm development (Wagner & Iglewski, 2008b).

5.1.2 *S. aureus*

It is widely abundant bacterium and is a commensal of the human skin, particularly front nares and skin wrinkles in CF and non-CF. An estimated 30–50% of healthy individuals are occasionally or chronically colonised by *S. aureus*, with chronic nasal carriage being a risk factor for *S. aureus* bacteraemia. *S. aureus* grows normally aerobically but additionally as facultative anaerobe and is capable of biofilm formation (Wertheim *et al.*, 2004).

5.1.3 *H. influenzae*

It is a critical human respiratory tract pathogen (Murphy, 2000). It regularly infects the CF patients lungs, particularly early in childhood and grows adherent biofilms on the apical surface airway epithelia with reduced sensitivity to antimicrobial agents (Starner *et al.*, 2006).

5.1.4 *Str. pneumoniae*

Persistence of indistinguishable clones with growing frequencies of antibiotic resistance throughout prolonged periods suggests the opportunity of biofilm based growth among the CF *Str. pneumoniae* isolates (del Campo *et al.*, 2005). Besides, biofilm formation is accelerated by the anaerobic or microaerophilic environment that is typical in the CF airways (García-Castillo *et al.*, 2007).

5.1.5 Antimicrobial cationic peptides

AMPs have considerable appeal attention as substitute antibiotics for their prospective potency, fast action, and broad spectrum of activities against Gram-negative and -positive bacteria, viruses, fungi and parasites (Dosler *et al.*, 2015). They can be used against biofilms or in the prevention of bacterial biofilm formation. They demonstrate

numerous mechanisms of action and, subsequently, a low possibility to induce *de novo* resistance, which restricts the use of other antibiotics (Yeung *et al.*, 2011).

5.1.5.1 Magainin II

Magainin II was reported to have anti-biofilm activity against *Streptococcus* species (Wei *et al.*, 2006). Human cationic host immune peptides revealed potent inhibitory activity against biofilm formation of bacterial infections associated with CF and reduced pre-grown biofilms (Moreau-Marquis *et al.*, 2008b). Melittin and colistin, were capable of reducing attachment of *P. aeruginosa*, *E. coli* and *Klebsiella pneumoniae* (Dosler *et al.*, 2015).

5.1.6 Artificial sputum medium

Acquiring an adequate quantity of human sputum for research is problematic and time consuming. Various studies use ASM to culture respiratory bacteria with an environment that sufficiently imitates CF lung sputum (Sriramulu, 2005). This medium assists clinical and research scientists to pay attention at the expression of specific genes and proteins in an "nearly *in-vivo*" setting (Palmer *et al.*, 2007).

Meanwhile, it has been confirmed that medically essential antibiotics, comprising aminoglycosides, fluoroquinolones, and tetracycline, among others, function inadequately in chronic infections and, on the contrary, even perform as inter-microbial signalling mediators which contribute to bacterial biofilm formation at sub-inhibitory doses (Linares *et al.*, 2006), so novel antimicrobial drugs are required to fight chronic infections. Lately, screening techniques for anti-biofilm drugs have been established (Pitts *et al.*, 2003), and numerous high-throughput screenings of environmental natural compounds were carried out to detect substances with anti-biofilm characteristics that could be employed as potential agents in biofilm-associated infections (Junker & Clardy, 2007). Nevertheless, only partial achievement has been succeeded to date. The anti-biofilm properties of magainin II, with its antimicrobial potency, could provide the foundation for the development of novel drugs that could assist in combating chronic infections.

In the present report, all efforts were directed to inhibit the biofilms of bacteria associated with CF so that the level of pathogenesis can be monitored.

5.2 Materials and Methods

All chemicals were purchased from Sigma-Aldrich (UK), unless otherwise stated.

5.2.1 Preparation of Artificial Sputum Medium

ASM was prepared as indicated in section 4.2.1

5.2.2 Bacterial growth analyses

S. epidermidis, MSSA, *Str. pneumoniae*, *E. coli*, *P. aeruginosa* and *H. influenzae* (~1 x 10^6 CFU ml^{-1}) were inoculated in ASM and LB/BHI/BHI+FE media in a 24 well plate. The plate was incubated at 37 °C for 48 h. After the incubation period, the OD at 595 nm was measured for approximate growth. Each well was washed three times with 450 µL PBS and air-dried. The cells were then fixed by adding 250 µL of 99 % methanol for 15 min, aspirated and then allowed to air dry. The wells were then stained with 1 % CV solution for 5 min. Excess stain was removed by washing three times with 450 µL PBS. When the wells had dried light microscope images were recorded. The stain was then solubilised with 160 µL of 33 % glacial acetic acid and absorbance was read at an OD of 570 nm using a plate reader (Verhoeven *et al*, 2010). The background (values of the negative control) was subtracted from experimental and control values.

5.2.3 Biofilm formation assessment by crystal violet (CV)

Biofilm formation of six types of bacteria was monitored by performing CV assay. Overnight saturated cultures of bacteria were separately inoculated in equal numbers (~1 x 10^6 CFU mL^{-1}) in different wells of 24 well plate containing LB/BHI/BHI+FE/ASM media supplemented with varied concentrations of sub-MIC doses of magainin II (1, 2, 3 µM). In the control set, the microorganisms in same number were grown in the absence of antimicrobial peptide. Plates were incubated at 37 °C for 48 h. After the incubation, culture broths from each well were discarded. Wells were washed with distilled water twice and dried adequately. After that, to each well, 1 % CV was added and incubated for 15 min at RT. CV solution was then discarded from each well, and then the wells were again washed with distilled water twice to remove any unabsorbed CV from the wells. Thereafter, 160 µL of 33 % glacial acetic acid were added to each well to dissolve the CV

absorbed into bacterial biofilm and the intensity of colour was then measured by recording the absorbance at 570 nm.

5.2.4 Bacterial growth patterns analyses

To examine the effect of the sub-MIC doses of magainin II on bacteria, the growth pattern of both the magainin II treated and untreated samples of bacteria were compared. To do this experiment, equal numbers of bacteria (~1 x 10^6 CFU mL^{-1}) were inoculated separately in different 24 well plates containing media (*S. epidermidis*, MSSA, *E. coli* and *P.* were inoculated in LB broth, *Str. pneumoniae* in BHI and *H. influenzae* in BHI enriched with Fildes Extract). After that, various sub-MIC doses (1, 2, 3 μM) of antimicrobial peptide were added to each growth medium. In the control set, only the organism was allowed to grow devoid of peptide. All the experimental growth media were then incubated at 37 °C for 48 h. At regular time intervals (2, 4, 6 and 48 h), the culture broths were separately taken from each growth medium and absorbance was recorded on 595 nm in a spectrophotometer.

5.2.5 Bacterial viability measurements

To examine the viability of bacteria in different media, identical numbers of microorganisms treated with various sub-MIC doses of magainin II peptide were inoculated. In the control set, peptide was not added. All plates were incubated at 37 °C for 24 h. For CFU calculation between peptide treated and untreated conditions, 1/10 dilutions were performed. 20 μL of these diluted supernatants were plated onto solid CLED/Blood agar/Chocolate agar plates. Thereafter, plates were incubated at 37 °C for 2 days and CFU were counted.

5.2.6 Microscopic Observation of bacterial attachment

To identify bacterial adherence on the surface of wells, overnight, cultures of bacteria were inoculated in 24 well plates containing sterile media supplemented with various concentrations of magainin II (1, 2, 3 μM). In the control wells, no peptide was added. Each well was washed three times and air-dried. The cells were then fixed by adding 250 μL of 99 % methanol for 15 min, aspirated and then allowed to air dry. The wells were then stained with 1 % CV. Excess stain was removed by washing three times distilled water. When the wells had dried light microscope images were recorded.

5.2.7 Statistical analysis

All experiments were performed in biological duplicates and repeated at least thrice in independent experiments. All data were expressed as arithmetic means ± standard deviations. Comparisons between groups were carried out using the unpaired Student *t-test*. P-values were determined by the unpaired Student *t-test*, using Excel software. Statistical significance was set at $P<0.05$.

5.3 Results

5.3.1 Biofilm formation assessment by CV

5.3.1.1 Comparison of biofilm formation in ASM

To determine the effect of ASM on biofilm formation, CV assays were carried out. The difference in biofilm formation in ASM and conventional laboratory media is highlighted (Figure 5.1). ASM was hypothesized to be unfavorable environment to growth as well as for biofilm formation. In this experiment conventional media were used as controls. All clinical isolates confirmed that ASM is less favorable condition to form biofilms. According to results acquired, *P. aeruginosa* demonstrated the highest rate of biofilm formation in ASM, whereas the lowest was displayed by MSSA.

If to compare the ability of biofilm formation between Gram-negative and Gram-positive bacteria, it is obvious that the Gram-positive bacteria are less able of biofilm formation. It is interesting that *S. epidermidis* exhibited highest biofilm formation among Gram-positive bacteria, as it is associated with medical complexities by forming biofilms in medical devices such as catheters. However, the biofilm formation in ASM was not significantly lower when compared with biofilm formation in conventional media that is known to be favorable condition.

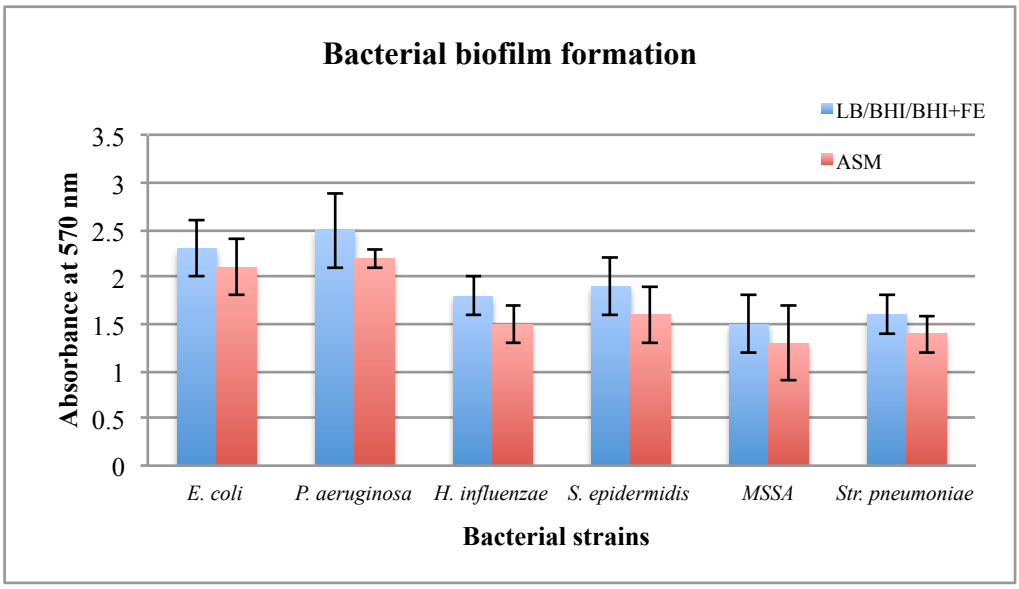


Figure 5.1 Formation of Bacterial biofilm in ASM. Biofilm formation was observed by carrying out the CV assay. Overnight cultures of bacteria were inoculated in media. Plates were incubated for 48 h. CV was added, incubated and were washed. Glacial acetic acid was added to dissolve the bound CV and the intensity of colour was measured by recording the absorbance. Results are obtained from at least three independent experiments. Error bars represent standard error.

5.3.1.2 Antibiofilm activity of magainin II

In order to test anti-biofilm activity of magainin II, biofilm formation assays were carried out by using the CV technique. The results revealed that all the tested sub-MIC concentrations of antimicrobial peptide exhibited significant biofilm attenuation compared to controls where the microorganisms were grown and allowed to form biofilm in absence of peptide. It was noticed that biofilm formation was sub-MIC concentration dependant (Figure 5.2). The anti-biofilm activity of magainin II against Gram-positive bacteria was lower in ASM media when compared with LB or BH. The greatest sub-MIC dose, 3 μM, in ASM medium, decreased biofilm formation of *S. epidermidis*, MSSA and *Str. pneumoniae* to 72 %, 57% and 77% respectively. However, in LB/BHI the same bacteria potential dropped to 81 %, 66% and 85% respectively. Among Gram-positive bacteria, *Str. pneumoniae* was the most sensitive to magainin II in both types of media. The Gram-negative bacteria, *E. coli*, *P. aeruginosa* and *H. influenzae* were less susceptible to magainin in ASM media (65 %, 81% and 79 respectively) than in LB/BHI (69 %, 86% and 87% respectively). Magainin II demonstrated efficacy in preventing biofilm formation by pathogenic Gram-negative bacteria in ASM and conventional media.

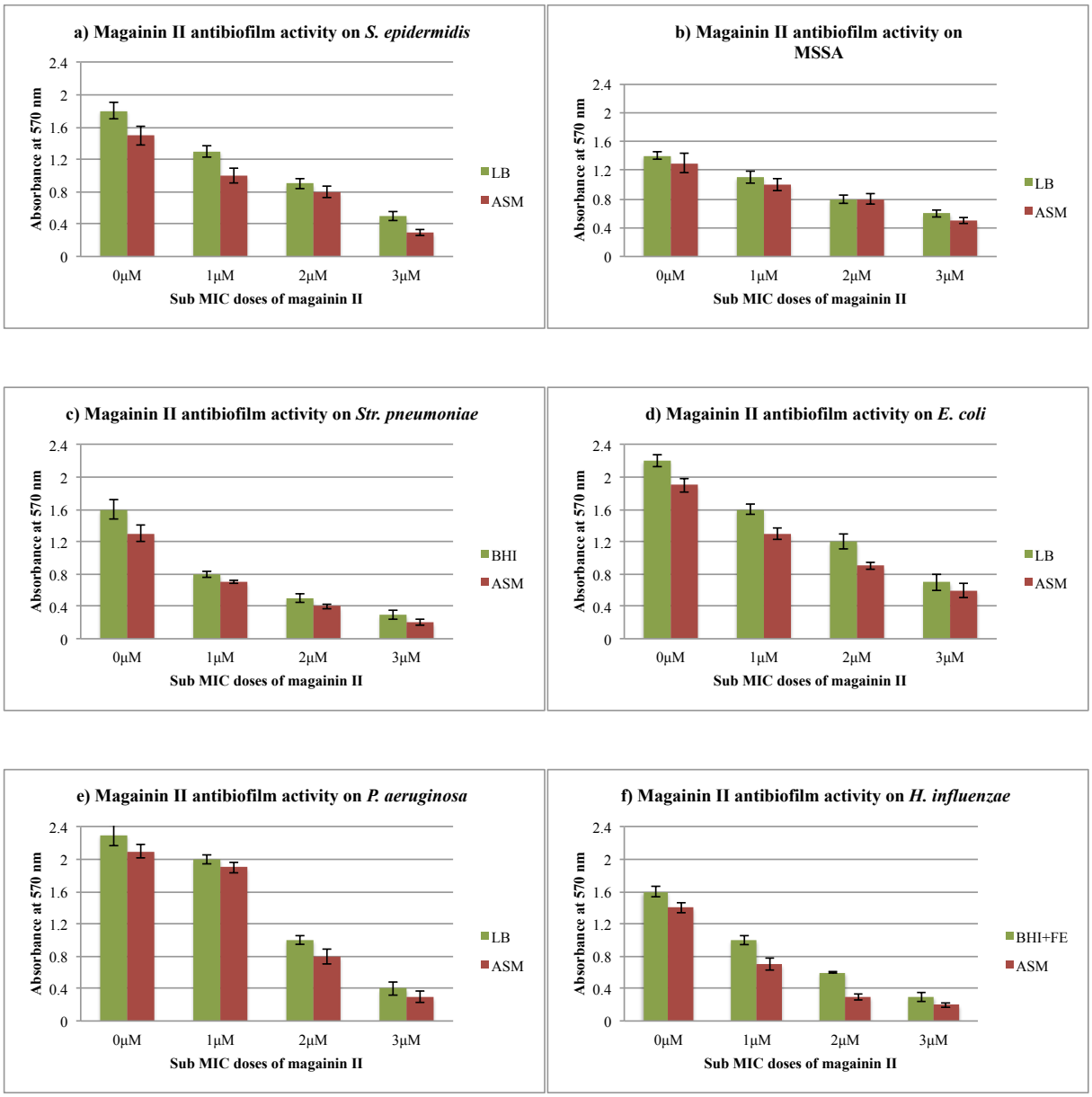
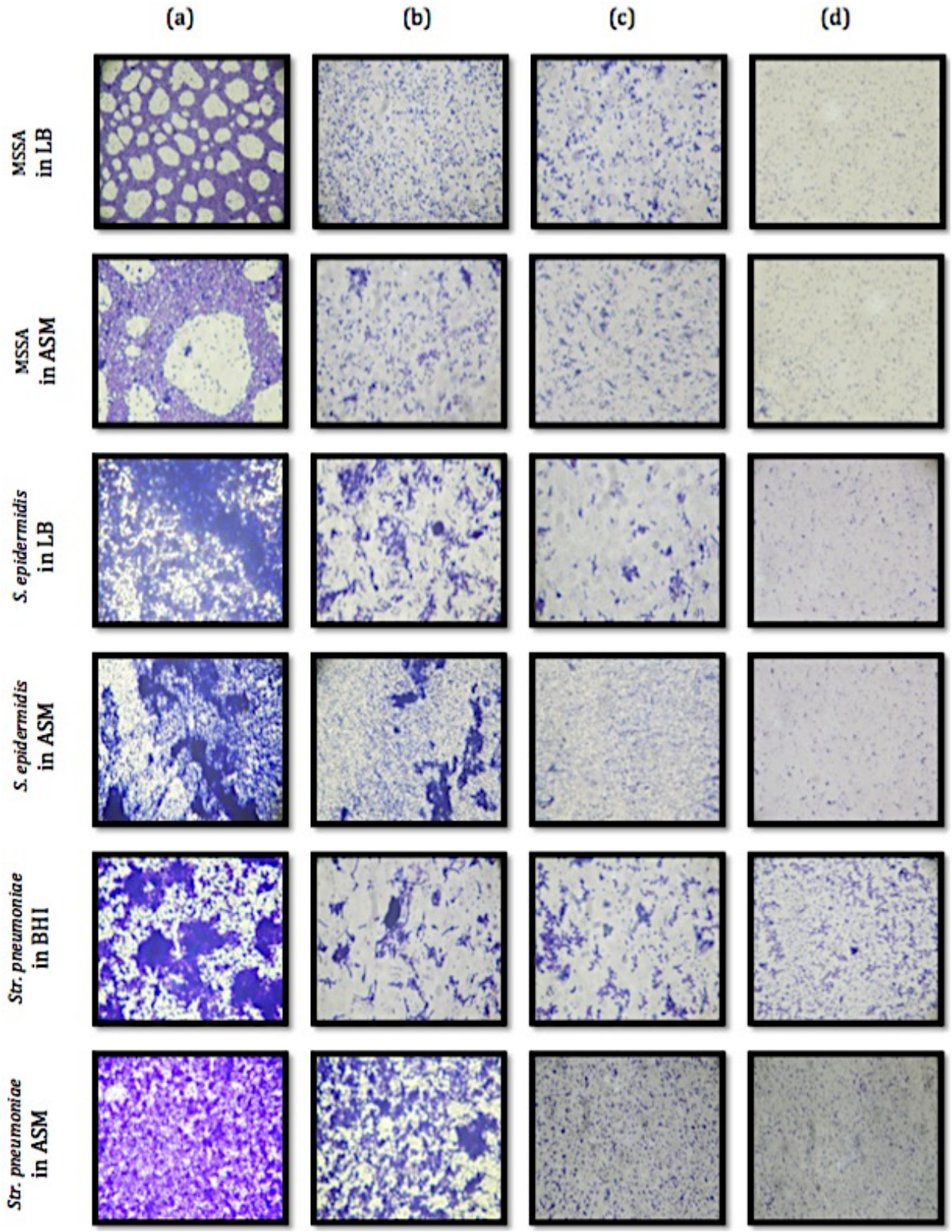


Figure 5.2 Antibiofilm activities of magainin II in ASM of bacteria such as *S. epidermidis* (a), MSSA (b), *Str. pneumoniae* (c), *E. coli* (d), *P. aeruginosa* (e) and *H. influenzae* (f). Conventional media were used as controls. Overnight cultures of bacteria were inoculated with various concentrations of sub-MIC doses of magainin II. Plates were incubated for 48 h. Next, CV was added, incubated and washed. Glacial acetic acid was added to dissolve the bound CV and the intensity of colour was measured by recording the absorbance. Results are obtained from at least three independent experiments. Error bars represent standard error.

Identification of bacterial adherence on the surface of a well, supplemented with various concentrations of magainin II (1, 2, 3 μM) was carried out. MSSA, *Str. pneumoniae, S. epidermidis, E. coli, P. aeruginosa and H. influenzae* were examined in ASM. Conventional media such as LB, BHI and BHI+FE were used as controls for ASM medium. In the control wells, no peptide was added. Images that obtained from bright field microscope indicated that the sub-lethal concentrations of magainin II were able to decrease bacterial surface attachment. Interestingly, bacterial attachment was less where the highest sub-MIC doses were used showing dose dependant efficacy (Figure 5.3).


(a)
(b)
(c)
(d)
MSSA in LB
MSSA in ASM
S. epidermidis in LB
S. epidermidis in ASM
Str. pneumoniae in BHI
Str. pneumoniae in ASM

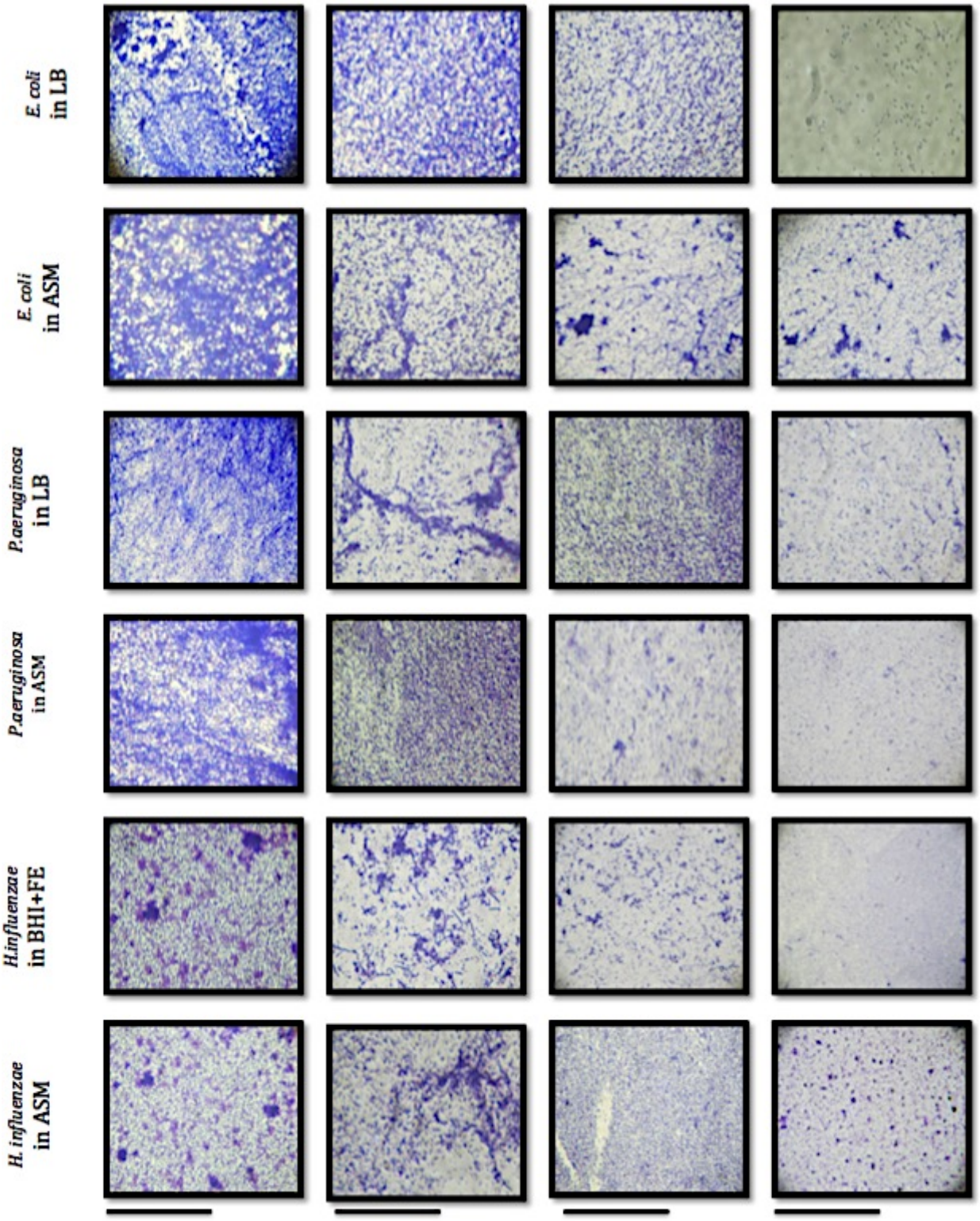
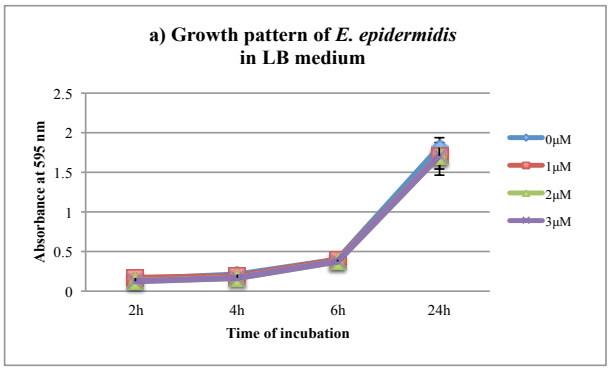
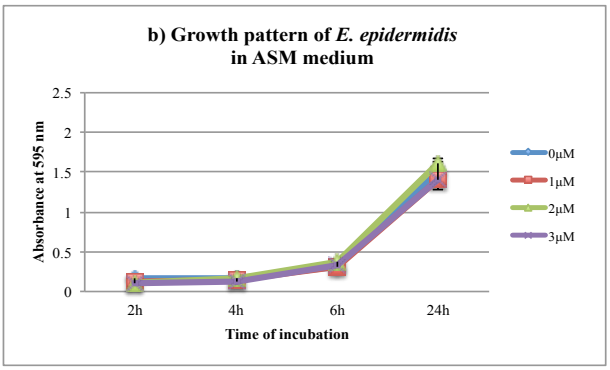


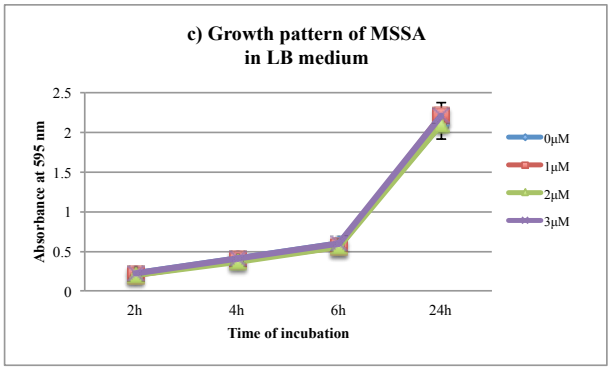
Figure 5.3 Microscopic observation of bacterial attachment. Micrographs of CV stained bacterial population on the surface of wells under different treatment: a) 0 μM; b) 1 μM; c) 2 μM and d) 3 μM. The figure is representative of images obtained from bright field microscope and from three independent experiments. The scale bar, 80 μm.

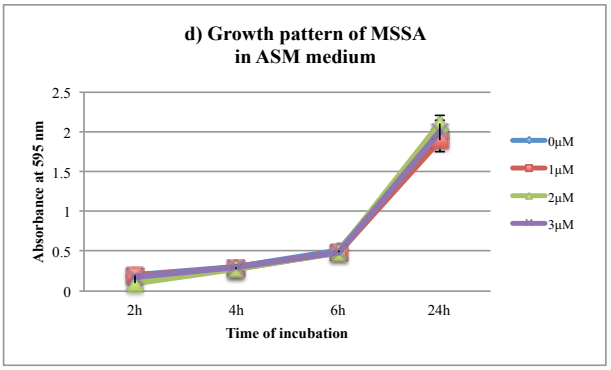
5.3.2 Growth Pattern Analyses

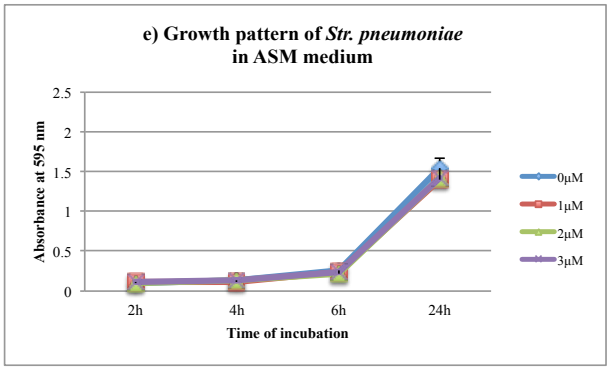
To examine the effect of sub-MIC doses of magainin II on the growth cycle of bacteria associated with CF infections, bacteria were co-incubated with different sub-MIC concentrations of antimicrobial peptide (1, 2, 3 μM). *S. epidermidis*, MSSA, *E. coli* and *P. aeruginosa* were inoculated in LB broth, *Str. pneumoniae* in BHI and *H. influenzae* in BHI enriched with Fildes Extract. After the identical period of incubations (2, 4, 6 and 48 h) it was observed that the magainin II treated and untreated (control) microbes exactly follow the same trend in their growth cycle (Figure 5.4). The trends of growth of all bacteria were compared with that of in ASM. The growth of bacteria was less in ASM but sub-MIC doses of magainin II did not impact on growth patterns of Gram-positive as much as Gram-negative bacteria. These results suggest that sub-MIC doses of magainin II tested in the experiments do not alter the normal growth behaviour of the microorganisms seen in either their own bacterial growth media (LB, BHI and BHI+FE) or ASM.

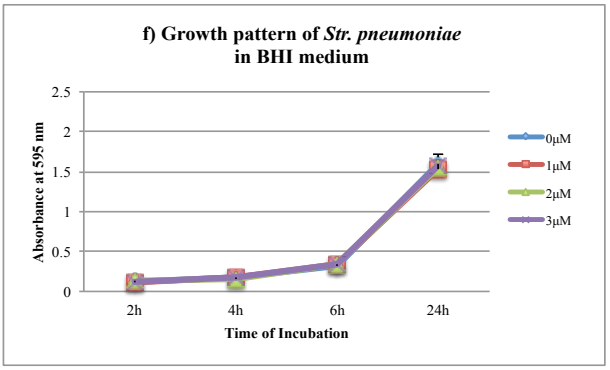

a) Growth pattern of E. epidermidis in LB medium
Absorbance at 595 nm
2.5
2
1.5
1
0.5
0
2h
4h
6h
24h
Time of incubation
0µM
1µM
2µM
3µM


b) Growth pattern of E. epidermidis in ASM medium
Absorbance at 595 nm
2.5
2
1.5
1
0.5
0
2h
4h
6h
24h
Time of incubation
0µM
1µM
2µM
3µM


c) Growth pattern of MSSA in LB medium
Absorbance at 595 nm
2.5
2
1.5
1
0.5
0
2h
4h
6h
24h
Time of incubation
0µM
1µM
2µM
3µM


d) Growth pattern of MSSA in ASM medium
Absorbance at 595 nm
2.5
2
1.5
1
0.5
0
2h
4h
6h
24h
Time of incubation
0µM
1µM
2µM
3µM


e) Growth pattern of Str. pneumoniae in ASM medium
Absorbance at 595 nm
2.5
2
1.5
1
0.5
0
2h
4h
6h
24h
Time of incubation
0µM
1µM
2µM
3µM


f) Growth pattern of Str. pneumoniae in BHI medium
Absorbance at 595 nm
2.5
2
1.5
1
0.5
0
2h
4h
6h
24h
Time of Incubation
0µM
1µM
2µM
3µM

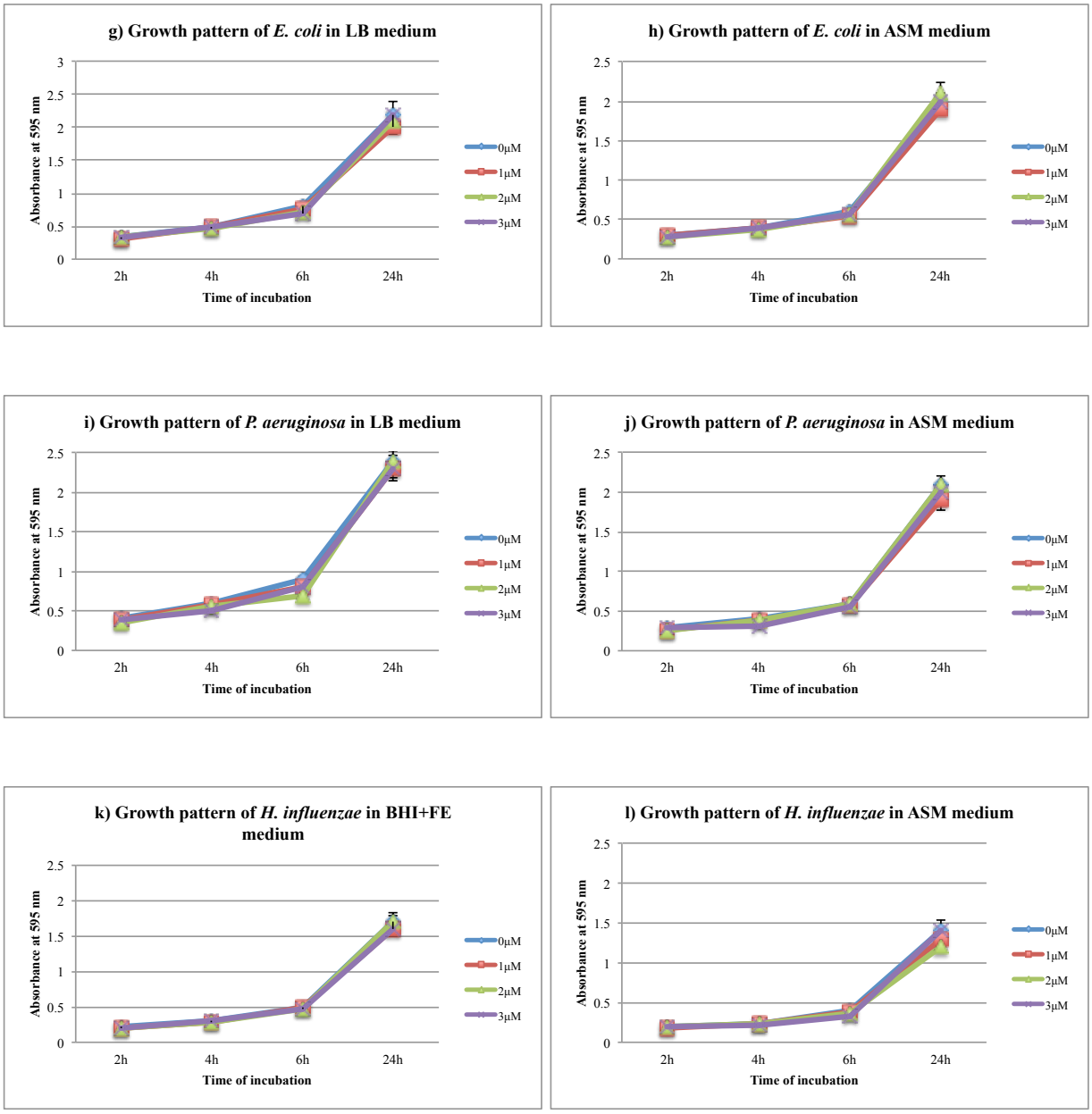
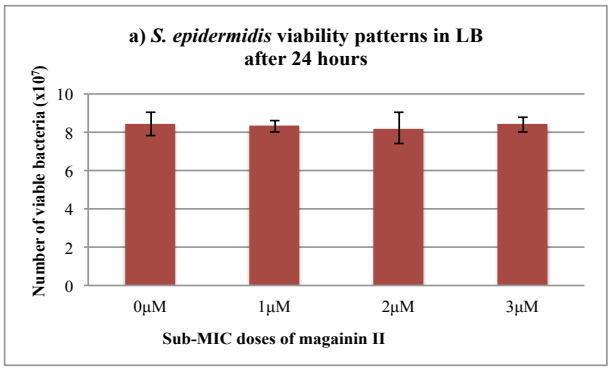
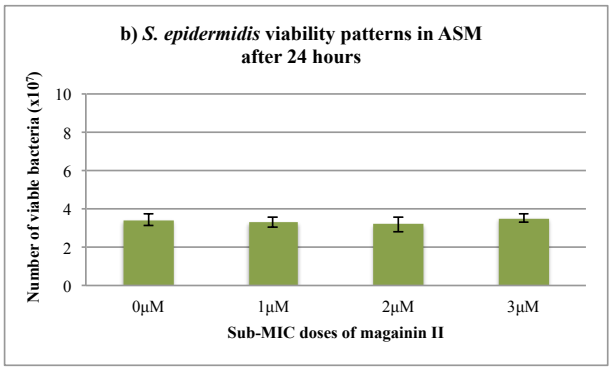


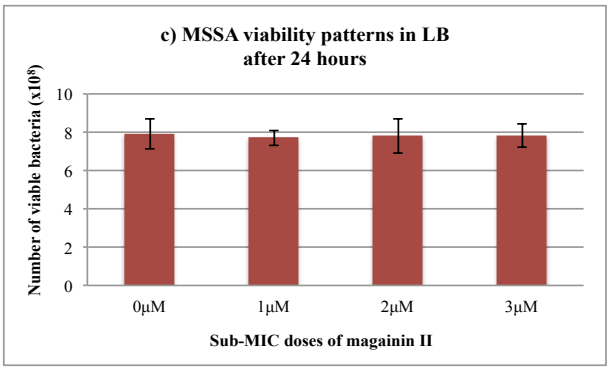
Figure 5.4 Growth patterns of bacteria in ASM with sub-MIC doses of magainin II. To examine the effect of the sub-MIC doses of magainin II on bacterial growth patterns treated and untreated samples of bacteria were studied. Bacteria were incubated with various sub-MIC doses of AMP and incubated for 48 h. At regular time intervals absorbance was recorded. Results are obtained from at least three independent experiments. Error bars represent standard error.

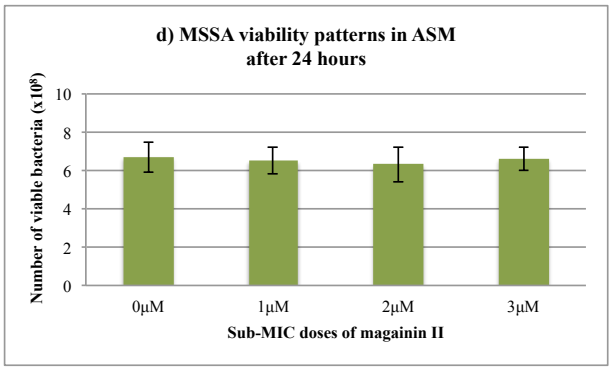
5.3.3 Bacteria viability measurements

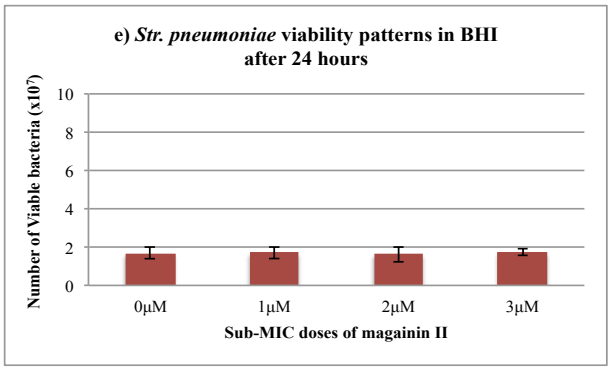
To gain further information about effects of sub-MIC doses of antimicrobial peptide on the growth of bacteria, bacterial viability was measured. All bacteria inoculated for 24 h with sub-MIC doses of magainin II (1, 2, 3 μM) either in bacterial own media or ASM. Control wells were inoculated without peptides. After incubation time, media were diluted and inoculated onto CLED agar (*S. epidermidis*, MSSA, *E. coli* and *P. aeruginosa*), Blood agar (*Str. pneumoniae*) and Chocolate agar (*H. influenzae*). Following the incubation of plates the CFU was counted. No significant differences were observed in the number of CFU between peptide treated and untreated cultures (Figure 5.5). This assay showed that none of the tested sub-MIC doses of magainin II exhibited cytotoxicity or growth arresting properties.

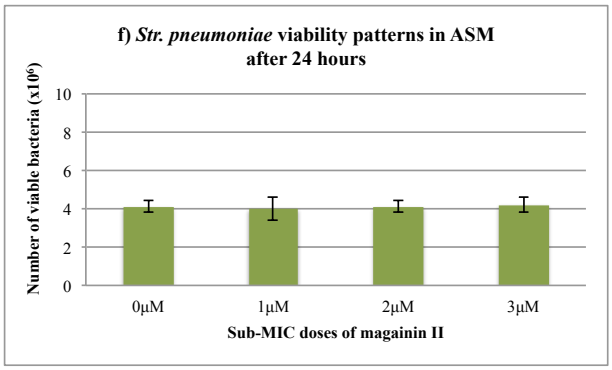

a) S. epidermidis viability patterns in LB after 24 hours
Number of viable bacteria (x10⁷)
0
2
4
6
8
10
0μM
1μM
2μM
3μM
Sub-MIC doses of magainin II


b) S. epidermidis viability patterns in ASM after 24 hours
Number of viable bacteria (x10⁷)
0
2
4
6
8
10
0μM
1μM
2μM
3μM
Sub-MIC doses of magainin II


c) MSSA viability patterns in LB after 24 hours
Number of viable bacteria (x10⁸)
0
2
4
6
8
10
0μM
1μM
2μM
3μM
Sub-MIC doses of magainin II


d) MSSA viability patterns in ASM after 24 hours
Number of viable bacteria (x10⁸)
0
2
4
6
8
10
0μM
1μM
2μM
3μM
Sub-MIC doses of magainin II


e) Str. pneumoniae viability patterns in BHI after 24 hours
Number of Viable bacteria (x10⁷)
0
2
4
6
8
10
0μM
1μM
2μM
3μM
Sub-MIC doses of magainin II


f) Str. pneumoniae viability patterns in ASM after 24 hours
Number of viable bacteria (x10⁶)
0
2
4
6
8
10
0μM
1μM
2μM
3μM
Sub-MIC doses of magainin II

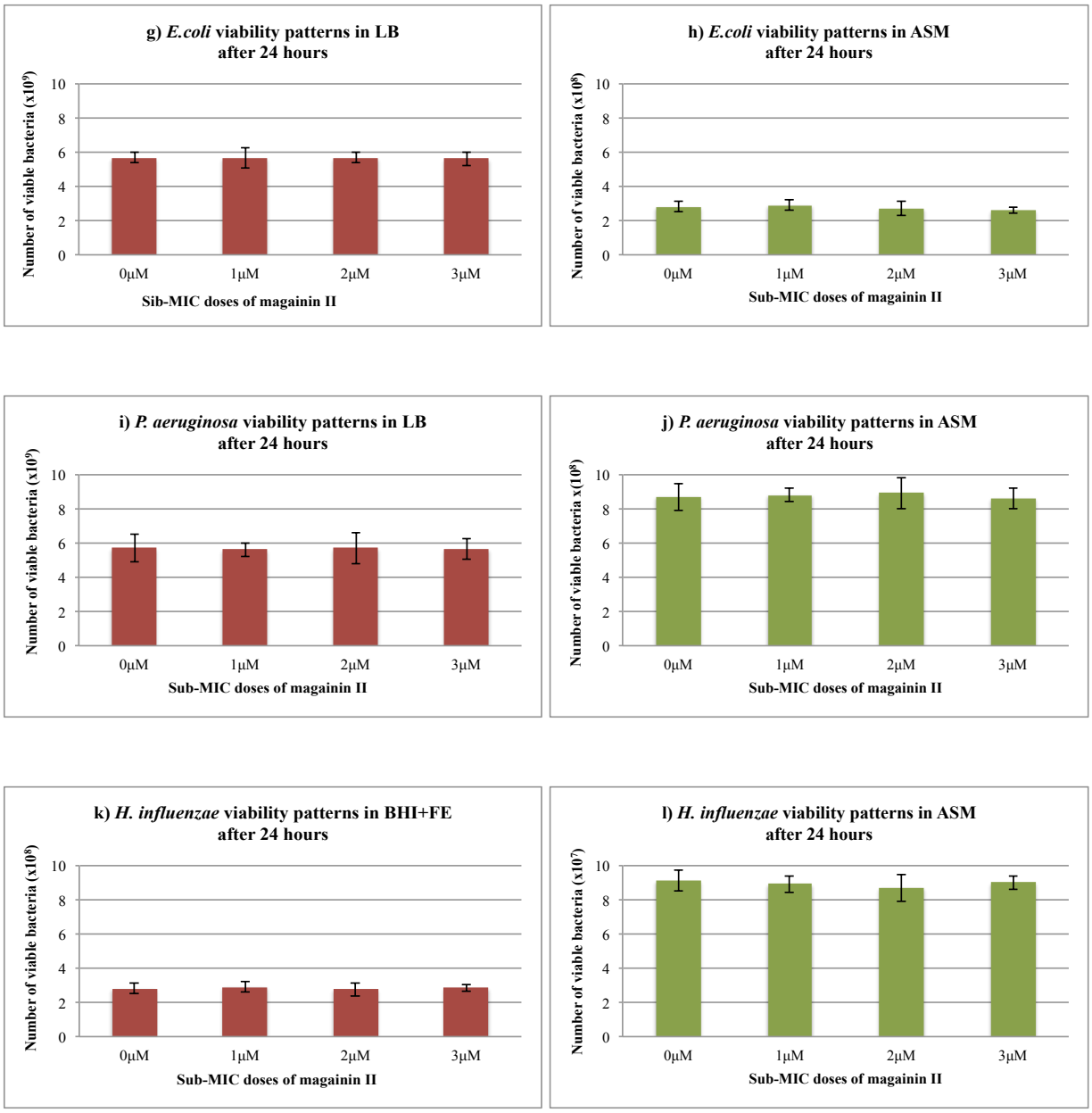


Figure 5.5 Bacterial viability measurements. To examine the viability of bacteria in different media, identical numbers of microorganisms were inoculated and treated with various sub-MIC doses of magainin II peptide. In the control set, peptide was not added. All plates were incubated at 37 °C for 24 h and plated onto solid CLED/Blood agar/Chocolate agar plates. Results are obtained from at least three independent experiments. Error bars represent standard error.

5.4 Discussion

The infected lung of CF patients is in a chronic inflammatory state and responds inadequately to antimicrobial treatments. This deficiency in treatment efficiency is thought to be due in part to the formation of antibiotic resistant biofilms forming in the CF lung (Moreau-Marquis *et al.*, 2008c). The occurrence of quorum-sensing molecules in CF sputum, utilized by *P. aeruginosa*, *S. aureus* and *H. influenzae* for cell to cell communication *in vitro* (Fuqua *et al.*, 2001) has been reported as indication that *P. aeruginosa* in the CF lung produce biofilms (Singh *et al.*, 2000).

Biofilm formation is an essential strategy for bacterial sustainability and the expansion of disease (Donlan, 2002). A reduction in biofilm growth could be seen as a prospective approach to make the bacterial population more susceptible to antibiotics (Bjarnsholt *et al.*, 2013).

In this study the anti-biofilm activity of magainin II against *P. aeruginosa*, *H. influenzae*, *E. coli*, MSSA, *Str. pneumoniae* and *S. epidermidis* was examined. Artificial sputum medium was used to mimic physiological circumstances in the CF lung. The results showed that all the tested sub-MIC doses (1, 2 and 3 μM) of magainin II for all six types of strains demonstrated significant biofilm attenuation for all six types of strains compared with controls where bacteria were grown and allowed to form biofilm in the absence of magainin II. Previously, magainin II showed anti-biofilm activity against *Streptococcus mutans* (Wei *et al.*, 2006). It has been reported that a human cationic host immunity peptide, LL-37, exhibited a potent inhibitory activity in biofilm formation at a concentration of 2 μM against *P. aeruginosa* biofilm (Moreau-Marquis *et al.*, 2008c). Moreover, Dashper *et al*. stated that kappacin, non-glycosylated κ-casein, displayed a significant reduction of *Streptococcus* biofilm (Dashper *et al.*, 2013).

All the observations under the microscope with sub-MIC doses of magainin II showed ability to attenuate microbial attachment to the well surface considerably compared to the controls. It was observed that in the control sets, where the bacteria were untreated, a dense microbial biofilm developed over the well surface. But in peptide treated conditions, it was seen that with the increase in peptide concentrations, biofilm formation became compromised. Similar research has shown that cathelicidin, antimicrobial polypeptides found in lysosomes of macrophages and polymorphonuclear

leukocytes, and keratinocytes were able to inhibit both the attachment potency and development of biofilms by Staphylococci (Overhage *et al.*, 2008b).

The effect of sub-MIC doses of magainin II on the growth cycle of bacteria and viability were measured by counting CFU. These experiments showed that none of the tested sub-MIC doses of antimicrobial peptide displayed cell killing or growth arresting properties but that they only interfere with biofilm forming ability. Lactoferrin is an iron-binding glycoprotein present in milk and, to a lesser extent, in exocrine fluids such as bile and tears. It was similarly shown to have anti-biofilm activity at concentrations below those that were cytotoxic and growth arresting for bacteria (Singh *et al.*, 2002).

All these observations suggest that all the tested sub-MIC doses of magainin are potentially capable of inhibiting the microbial biofilm formation without damaging the microbial cell itself.

Although it was identified that the antimicrobial peptide, magainin II, attenuates biofilm development, the fundamental mechanisms of this biofilm inhibition has not been investigated. It is hypothesized that magainin II may interfere with those proteins and enzymes which are essential for microbial adherence, or formation of quorum sensing that resulted in the reduction of biofilm formation by the bacteria in the presence of peptide. Further researches are requisite to interpret the underlying mechanisms of microbial biofilm inhibition by using magainin II.

Exploring new agents that can attenuate microbial biofilm formation may shed light on therapeutic strategies for infections with microbes such as *P. aeruginosa* whose pathogenic potential strongly depends on successful biofilm formation within the host. In this event, antimicrobial peptides could be the next generation of antibiotics for contending multi-drug resistant and biofilm forming bacterial infections. Even though there are obstacles to the administration of peptides as therapeutics, such as low bioavailability, cytotoxicity and high costs, these complications may be overcome since a huge effort is being carried out to bypass the complications related to various approaches. These findings suggest that magainin II holds promise to counteract biofilm formation. Therefore, it is estimated that antimicrobial peptides will be considered the drugs of choice for emerging bacterial infections in the following years.

Chapter 6

General Discussion

6.1 Overview

Bacterial pathogens such as *S. aureus*, *P. aeruginosa* and *H. influenzae* are notorious for causing infections in CF patients which lead to health complexities, and even to death. The number of effective treatments remains insufficient. It is essential to study the interactions between bacterial pathogens and epithelial cells of the respiratory tract. Another factor to take into account is the presence of *Acanthamoeba* in the respiratory tract that could contribute to exacerbation of pulmonary infections.

Similar projects showed that *S. aureus* and *P. aeruginosa* adhere, invade and survive within epithelial cells (Ichikawa *et al.*, 2000). Virulence factors of *S. aureus* (Gordon & Lowy, 2008) and *P. aeruginosa* play key roles in binding to and invading host cells (Ben Haj Khalifa *et al.,* 2011).

To identify if bacteria interact with epithelial cells and *Acanthamoeba,* interaction assays were performed. Interaction assays consist of association, invasion and survival assays. In association the attachment of bacteria on the host cell was examined. In invasion assays, the number of bacteria invading the host cell was identified and in survival assays, the number of bacteria surviving overnight after invading the host cell was tested. For these purposes six types of bacteria (pathogenic MSSA*, H. influenzae, Str. pneumoniae, P. aeruginosa, non-pathogenic S. epidermidis* and *E. coli*), isolated from CF patients, human epithelial lung carcinoma (A549) cell line and *Acanthamoeba* trophozoites were studied. The results clearly showed that *S. epidermidis*, MSSA, *E. coli* and *P. aeruginosa* have the ability to associate, invade and survive within A549 cells.

Acanthamoeba was identified as able to host *Escherichia coli* (Alsam *et al.*, 2006), *P. aeruginosa* (Michel *et al.*, 1995) and MRSA (Cardas *et al.*, 2012a) protecting them from extracellular undesirable functions.

Findings of this study indicate that *Acanthamoeba* (genotype T4), was able to host *S. epidermidis*, MSSA, *E. coli* and *P. aeruginosa*. This is interesting as *Acanthamoeba* could play an additional role in pulmonary exacerbated infections associated with CF. Moreover, bacteria can remain and multiply inside *Acanthamoeba* within a harsh environment that may lead to distribution of bacterial pathogens to susceptible hosts and also to reinfection.

However, *H. influenzae and Str. pneumoniae* exhibited very low ability to invade and even no ability to survive within both A549 cells and *Acanthamoeba* trophozoites. This could be because of the presence of bacterial capsules, which prevent phagocytosis.

Both *H. influenzae and Str. pneumoniae* have encapsulated and non-encapsulated phenotypes. Bacterial capsule possession is one of the key virulent factor which prevents host phagocytosis (Merino & Tomás, 2015). In similar study it was shown that the *H. influenzae* capsule inhibits ingestion by macrophages (Noel *et al.*, 1992). *Str. pneumoniae* polysaccharide capsules were able to resist host phagocytosis (Hyams *et al.*, 2010).
In order to identify the presence of capsules in *H. influenzae* and *Str.pneumoniae,* PCR and Electron Microscopy Imaging assays were performed. Consistent with both results it was identified that both strains are encapsulated.

The presence of antimicrobial substances in microorganisms is a fact and they are accepted to be an ancient defence system against other microorganisms. The predatory role of *Acanthamoeba* in the regulation of bacterial populations in the ecosystem as well as the capability of the amoeba to perform as a "Trojan horse" of the microbial world suggests that bacteria-amoeba are involved in complex communications (Jousset *et al*., 2010). The exact nature of such complicated interactions remains unclear, but it is identified in this project that both bacteria and amoebae are competent to counter-attack one another and the overwhelming micro-organismal populations by discharging antimicrobial substances. Proteases produced by *Acanthamoeba* are used as significant markers to differentiate between pathogenic and non-pathogenic *Acanthamoeba* species (Khan *et al.*, 2000; Sissons *et al.*, 2006; Dudley *et al.*, 2008). *Acanthamoeba* proteases have antimicrobial effects against different types of bacteria including MRSA and *P. aeruginosa* (Iqbal *et al.*, 2014). Among six bacteria tested, it is attention-grabbing that ACM exhibited selective and effective activity against *Str. pneumoniae,* which is a pathogen of acute infections, many of which may possess strong epidemiological consequences, such as meningitis, otitis media, pneumonia, and sinusitis. Simultaneously some clones of *Str. pneumoniae* may be implicated in initial stages of lung inflammation in CF patients (Rosenfeld *et al*., 2003). The selective and potent nature of ACM against *Str. pneumoniae* and partially against MSSA and *P. aeruginosa*, but not against *S. epidermidis, E. coli* and *H. influenzae* should be further studied.

A great quantity of microorganisms has been examined for the isolation of hypothetically advantageous antimicrobials, while there has been effort in searching for new antibiotics from other sources (Iqbal *et al*., 2014).

It is possible that bacteria resistant to ACM and *Acanthamoeba* develop strategies to protect themselves and withstand amoebic attack (Pantosti & Venditti, 2009). Still to come are examinations where ACM will be tested against a broad range of bacterial pathogens to identify broad-spectrum or species-specific antibacterial properties. The detection of antibacterial compounds from *Acanthamoeba* will perhaps lead to ideas for future investigations in other free-living protists as a prospective source of antibiotics for the treatment of bacterial pathogens. Due to emerging tendencies in antibiotic resistance in various pathogenic bacteria (Alanis, 2005), the current findings in this study show some promise in our future ability to fight infections caused by bacteria.

Correspondingly, among various bacterial strains studied, BCM of *Str. pneumoniae, P. aeruginosa and H. influenzae* displayed quantifiable amoebistatic and amoebicidal activities.

Upcoming investigations will determine the fact of whether these BCM possess selective effects on *Acanthamoeba* or possess broad-spectrum activities on diverse protist pathogens.

Surprisingly, neither ACM nor BCM were significantly cytotoxic to adenocarcinomic human alveolar basal epithelial cells *in vitro*.

The current findings may lead to identifying complementary sources of antimicrobials with novel modes of action to challenge pulmonary infections. As antimicrobial resistance of pathogens rises, discovery of novel antimicrobial agents is essential.

In recent years, the rapid increase in multi-resistant bacteria has prompted a growing interest in antimicrobial peptides as an alternative to conventional antibiotics. Compared with conventional antibiotics, which act by interfering with a specific biochemical reaction inside the cell, antimicrobial peptides are believed to disrupt the bacterial membrane in a non-receptor-mediated fashion. The exact mechanism of action has not yet been fully described.

This is a first study to examine antimicrobial effects of magainin II peptide against *Acanthamoeba castellanii* and internalized bacteria associated with CF.

In this study, various time intervals along with different concentrations of magainin II were found to demonstrate antimicrobial efficacy *in vitro*. Similar studies revealed that magainins had been shown to be effective against *Acanthamoeba polyphaga* (Schuster & Jacob, 1992), *Paramecium caudatum* (Zasloff *et al*., 1988) *Leishmania* (Guerrero *et al*., 2004)*, Tryponosoma cruzi, and Entamoeba histolytica* (Huang *et al*., 1990).

On the other hand, as cationic antimicrobial peptides show broad-range activity against Gram-positive and Gram-negative bacteria (Hancock & Sahl, 2006), magainin II was tested against bacteria employed in this study and it displayed significant antibacterial potency. Recently, a study quantified magainin II activity within 3 hrs, and increasing peptide concentration positively correlated with the extent of MRSA and *E. coli* membrane disruption (O'Brien-Simpson *et al.*, 2016).

Similar studies showed magainin II inhibited the growth of *P. aeruginosa* (Dosler & Karaaslan, 2014), *S. epidermidis* (Imura *et al.*, 2007)*, Str. pneumoniae* (Kovács *et al.*, 2006)*,* and *H. influenzae* (Bishop-Hurley *et al.*, 2005).

Bacteria able to survive could contribute to reinfections. It is crucial to be able to eradicate intracellular bacteria without harming host cells. In this case, it was A549 cells. Magainin II in sub-cytotoxic concentrations was not able to eradicate intracellular bacteria, but could do so when used in combination with other antimicrobials such as silver nitrate, vancomycin and ciprofloxacin.

In the current project, by combining magainin II with silver nitrate, an effective and well-tolerated antimicrobial reagent (Unger & Luck, 2012), greater activity was seen in the experiments against intracellular bacteria. This allowed use of magainin II and silver nitrate at concentrations lower than minimum cytotoxic concentrations, with continuous activity. The reasons for the greater efficacy of silver when used in combination with magainin II may be associated with influx into the *Acanthamoeba* or A549 cell line cytoplasm as a result of increased membrane permeability. This hypothesis can be supported by results obtained in this study, where sub-lethal doses of antibiotics such as vancomycin and ciprofloxacin were combined with magainin II.

Magainin II was found to be cytotoxic to the A549 cell line. This is consistent with another study (Pascariu *et al*., 2012). The greater cytotoxicity could be related to the outer negative charge of cancerous cells. Exposure of the negatively charged phosphatidylserine (PS) on the surface of cancerous and other pathological cells (Zwaal *et al.*, 2005), as well

as apoptotic cells (Martin *et al.*, 1995) and thus PS represents a promising target for positively charged magainin II.

It is fundamental to generate model media to mimic those in the CF lung (Behrends *et al.*, 2013). Conventional media such as LB, BHI and RPMI support growth of bacteria, but the issue remains how the nutritional environment affects the growth and interaction of bacteria with host cells.

All bacteria employed in this study were able to exhibit growth potential in ASM. The ionic components of artificial sputum such as sodium, chloride, potassium, calcium, ammonium, and magnesium (Sanders *et al.*, 2006) had no inhibiting effect on growth of bacteria.

Acanthamoeba is an opportunistic protist that is universally dispersed in the environment. The routes of access into human organism for *Acanthamoeba* include the respiratory tract (Siddiqui & Khan, 2012). Moreover, *Acanthamoeba* feeds on bacteria (Martinez & Visvesvara, 1997). These data lead to the importance of studying bacteria-*Acanthamoeba* interactions in ASM. In the conditions similar to CF sputum, *S. epidermidis,* MSSA*, E. coli and P. aeruginosa* demonstrate ability to survive intracellularly. These bacteria were able not only to survive but also to multiply within host cells such as *Acanthamoeba* and A549 cells.

Magainin II was shown to express a bactericidal effect on *Burkholderia cepacia*, which is associated with chronic opportunistic lung infections of CF patients (Thwaite *et al.*, 2009). In order to analyse the antimicrobial effect of Magainin II, antimicrobial assays were performed. The cationic peptide was identified as bactericidal against *S. epidermidis,* MSSA, *Str. pneumoniae, E. coli, P. aeruginosa, H. influenzae* as well as amoebicidal against *A. castellanii* trophozoites. In earlier studies Magainin II has been shown to have antimicrobial potency against pathogenic *S. aureus* and *P. aeruginosa* (Giacometti *et al.*, 2005).

Interestingly, the present work has demonstrated that magainin II activity was inhibited (2-fold) when used in ASM compared to conventional laboratory media. This could be because of the increased salt concentration in ASM (Gilljam *et al.*, 1989). Magainins have been reported to be salt sensitive (Lee *et al.*, 1997). The less salt sensitive pleurocidin (Goldman *et al.*, 1997b) was examined for antimicrobial potency in ASM. Consistent with previous studies in this project pleurocidin, unlike magainin II, was

equally effective in all media without significant difference, and showed salt-insensitivity properties.

Cytotoxic effects of magainin II on Normal human urothelial cells (TERT-NHUC) were tested to compare with A549 cells. Magainin II was less cytotoxic against TERT-NHUC (at least 2 fold) in comparison with A549 cells. Higher pleurocidin concentrations did not exert cytotoxic effects on TERT-NHUC cells and exhibited no significant difference in either media including ASM. The antiamoebic effect of magainins has been previously established (Feldman *et al.*, 1991). Schuster *et al* showed that magainin peptide in combination with silver nitrate or other antimicrobial agents have *in vitro* activity against *Acanthamoeba* trophozoites and cysts (Schuster & Jacob, 1992b).

Acanthamoeba infections are threatening and commonly noncompliant to antimicrobial treatment. An additional aspect of drug response to take into account is the differential sensitivity of trophozoites and cysts, the former being more sensitive than the latter (Osato *et al.*, 1991). Cysticidal dosages for various antimicrobial agents range from 10-1000 times the doses effective against trophozoites of *Acanthamoeba* (Nagington & Richards, 1976).

Consistent with similar studies, a transmission electron microscope examination in the current project has revealed both structural and cytoplasmic membrane damage to *A. castellanii* (T4 genotype) cysts when treated with higher concentrations of magainin II alone or in combination with silver nitrate.

The lung of CF patients is in a chronic inflammatory state and responds inadequately to antimicrobial treatments. This deficiency in treatment efficiency might be due in part to the formation of antibiotic resistant biofilms forming in the CF lung (Moreau-Marquis *et al.*, 2008c). The occurrence of quorum-sensing molecules in CF sputum, utilized by *P. aeruginosa*, *S. aureus* and *H. influenzae* for cell to cell communication *in vitro* (Fuqua *et al.*, 2001) has been reported as indication that *P. aeruginosa* in the CF lung produce biofilms (Singh *et al.*, 2000).

In the current study the antibiofilm activity of magainin II against *P. aeruginosa*, *H. influenzae*, *E. coli*, MSSA, *Str. pneumoniae* and *S. epidermidis* was examined. Artificial sputum medium was used to mimic physiological circumstances in the CF lung. The results show that all the tested sub MIC doses of magainin II for all six types of strains demonstrated significant biofilm attenuation compared to controls where bacteria were grown and allowed to form biofilm in the absence of magainin II.

Previously, magainin II showed antibiofilm activity against *Streptococcus mutans* (Wei *et al.*, 2006). It has been reported that a human cationic host immunity peptide, LL-37, exhibited a potent inhibitory activity in biofilm formation at low concentrations against *P. aeruginosa* biofilm (Moreau-Marquis *et al.*, 2008c).

All the observations under the microscope with sub MIC doses of magainin II showed ability to considerably decrease microbial attachment to the well surface compared to the controls. It was observed that in the control sets, where the bacteria were untreated, a dense microbial biofilm developed over the well surface. Similar research has shown that cathelicidin of macrophages are able to inhibit both the attachment potency and development of biofilms by Staphylococci (Overhage *et al.*, 2008b).

None of the tested sub MIC doses of antimicrobial peptide displayed cell killing or growth arresting properties but that they only interfere with biofilm forming ability similar to lactoferrin which was shown to have antibiofilm activity at concentrations below those that were cytotoxic (Singh *et al.*, 2002).

All these observations suggest that all the tested sub MIC doses of magainin II are potentially capable of inhibiting the microbial biofilm formation.

Although it was demonstrated that the antimicrobial peptide, magainin II, attenuates biofilm development, the fundamental mechanisms of this biofilm inhibition have not been investigated. It is hypothesized that magainin II may interfere with those proteins and enzymes which are essential for microbial adherence, or formation of quorum sensing that resulted in the reduction of biofilm formation by the bacteria in the presence of peptide.

The overuse and misuse of antibiotics result in mutations of bacteria, which lead to antibiotic resistance. For this reason, antimicrobial peptides could be the next generation of antibiotics for contending multi-drug resistant and biofilm forming bacterial infections. Even though there are obstacles to the administration of peptides as therapeutics, such as low bioavailability, cytotoxicity and high costs, these complications may be overcome since a huge effort is being carried out to bypass the complications related to various approaches. Findings in this project suggest that magainin II holds promise to counteract pulmonary infections associated with CF. Therefore, it is estimated that antimicrobial peptides will be considered the drugs of choice for emerging bacterial infections in the near future.

6.2 Limitations of the study

The use of two types of cell lines, *Acanthamoeba*, three Gram-positive (MSSA, *S. epidermidis, Str. pneumoniae*) and three Gram-negative (*P. aeruginosa*, *E. coli, H. influenzae*) bacteria was a unique feature of this study. Several techniques were used in this project including interaction assays, antimicrobial activity (MBC and MIC), LDH cytotoxicity colorimetric assays, electron microscopy, flow cytometry, PCR and bioimaging. On the other hand, there were limitations of this study.

One of the limitations was that the A549 epithelial cell line was derived from a cancerous tissue and does not represent the epithelial tissue of a healthy individual. This cell line may not express the same receptors as normal healthy epithelial cells, therefore the interaction of bacteria with the A549 epithelial cell line may not be representative of the interaction which would take place in a healthy individual.

Clinical isolates of MSSA, *S. epidermidis, Str. pneumoniae, P. aeruginosa, E. coli* and *H. influenzae* were used in this study. The virulence factors of individual strains have not been characterized. To study individual virulence factors, inhibitors or mutant strains lacking one or more virulence factors could be used.

Acanthamoeba employed in this study was not isolated from CF patients. It was planned to investigate the lower respiratory sputum samples for the presence of *Acanthamoeba*. Despite all efforts of the author to get ethical approval for isolation of *Acanthamoeba* from lower respiratory tract of CF patients due to bureaucratic barriers it was not possible to obtain permission to perform experiments in Colchester General Hospital, Microbiology Laboratory.

6.3 Recommendations for future work

The findings presented in this thesis highlighted some aspects of an *Acanthamoeba* role in pulmonary infections in CF lung, along with the pathogenesis of MSSA, *Str. pneumoniae, H. influenzae* and *P. aeruginosa*, and the promise of using antimicrobial cationic peptide magainin II as an alternative therapeutic method. There are, however, needs for further investigations to give a better picture for *Acanthamoeba's* role in pulmonary infections as well as use of magainin derivatives as an antibiotic.

1. Experiments for the identification of *Acanthamoeba* presence in CF sputum are essential. These will include: Ethical approvals application, collecting sputum from CF patients, grow and isolate *Acanthamoeba* and genotype identification.
2. Epidemiological retrospective study of last 10 years to identify the incidence of AGE among CF patients is essential.
3. Further investigations of the virulence factors most responsible for cytotoxicity could be worth studying. To assess that, a few cell types derived from healthy human lung epithelial tissue are required. In addition, different bacterial strains lacking one or more of the virulence factors could be used on the above-mentioned cells. Many techniques would be helpful to assess that: for the level of proteins, flow cytometry, fluorescence microscopy, and western blot using antibodies; and at the gene level, such as PCR, sequencing and bioinformatics. Regarding BCM, Nuclear Magnetic Resonance technique could be employed to identify lactate or other by products of fermentative growth.
4. Conditioned media of *Acanthamoeba* and bacteria employed in this project were found to have antimicrobial properties. Further investigations will determine the fact of whether these ACM/BCM possess selective effects on microorganisms or possess broad-spectrum activities on diverse microbial pathogens.
5. Here, it was shown that the antimicrobial peptide, magainin II, attenuates biofilm development, and the fundamental mechanisms of this biofilm inhibition has to be further investigated. Magainin II may interfere with those proteins and enzymes, which are essential for microbial adherence, or formation of quorum sensing.

References

Abd H, Saeed A, Weintraub A, Sandström G (2009) Vibrio cholerae O139 requires neither capsule nor LPS O side chain to grow inside Acanthamoeba castellanii. *Journal of medical microbiology*, **58**, 125–31.

Abd H, Valeru SP, Sami SM, Saeed A, Raychaudhuri S, Sandström G (2010) Interaction between Vibrio mimicus and Acanthamoeba castellanii. *Environmental microbiology reports*, **2**, 166–171.

Accurso FJ, Moss RB, Wilmott RW et al. (2011) Denufosol tetrasodium in patients with cystic fibrosis and normal to mildly impaired lung function. *American journal of respiratory and critical care medicine*, **183**, 627–34.

Albiol Matanic VC, Castilla V (2004) Antiviral activity of antimicrobial cationic peptides against Junin virus and herpes simplex virus. *International journal of antimicrobial agents*, **23**, 382–9.

Alsam S, Jeong SR, Sissons J, Dudley R, Kim KS, Khan NA (2006) Escherichia coli interactions with Acanthamoeba: a symbiosis with environmental and clinical implications. *Journal of medical microbiology*, **55**, 689–94.

Andrä J, Gutsmann T, Garidel P, Brandenburg K (2006) Mechanisms of endotoxin neutralization by synthetic cationic compounds. *Journal of endotoxin research*, **12**, 261–77.

Andreu D, Rivas L (1998) Animal antimicrobial peptides: an overview. *Biopolymers*, **47**, 415–33.

Andreu D, Ubach J, Boman A, Wåhlin B, Wade D, Merrifield RB, Boman HG (1992) Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity. *FEBS letters*, **296**, 190–4.

Andrushchenko V V, Aarabi MH, Nguyen LT, Prenner EJ, Vogel HJ (2008) Thermodynamics of the interactions of tryptophan-rich cathelicidin antimicrobial peptides with model and natural membranes. *Biochimica et biophysica acta*, **1778**, 1004–14.

Bear CE, Li CH, Kartner N, Bridges RJ, Jensen TJ, Ramjeesingh M, Riordan JR (1992) Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). *Cell*, **68**, 809–18.

Bechinger B (2009) Rationalizing the membrane interactions of cationic amphipathic antimicrobial peptides by their molecular shape. *Current Opinion in Colloid & Interface Science*, **14**, 349–355.

Behrends V, Geier B, Williams HD, Bundy JG (2013) Direct Assessment of Metabolite

Utilization by Pseudomonas aeruginosa during Growth on Artificial Sputum Medium. *Applied and Environmental Microbiology*, **79**, 2467–2470.

Beintema JJ (1994) Structural features of plant chitinases and chitin-binding proteins. *FEBS letters*, **350**, 159–63.

Belas R, Manos J, Suvanasuthi R (2004) Proteus mirabilis ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides. *Infection and immunity*, **72**, 5159–67.

Bernard E, Rolain T, Courtin P, Guillot A, Langella P, Hols P, Chapot-Chartier M-P (2011) Characterization of O-acetylation of N-acetylglucosamine: a novel structural variation of bacterial peptidoglycan. *The Journal of biological chemistry*, **286**, 23950–8.

Bevers EM, Comfurius P, Zwaal RF (1996) Regulatory mechanisms in maintenance and modulation of transmembrane lipid asymmetry: pathophysiological implications. *Lupus*, **5**, 480–7.

Bilton D, Robinson P, Cooper P et al. (2011) Inhaled dry powder mannitol in cystic fibrosis: an efficacy and safety study. *The European respiratory journal*, **38**, 1071–80.

Bilton D, Bellon G, Charlton B et al. (2013) Pooled analysis of two large randomised phase III inhaled mannitol studies in cystic fibrosis. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, **12**, 367–76.

Birket SE, Chu KK, Liu L et al. (2014) A functional anatomic defect of the cystic fibrosis airway. *American journal of respiratory and critical care medicine*, **190**, 421–32.

Bishop-Hurley SL, Schmidt FJ, Erwin AL, Smith AL (2005) Peptides selected for binding to a virulent strain of Haemophilus influenzae by phage display are bactericidal. *Antimicrobial agents and chemotherapy*, **49**, 2972–8.

Bjarnsholt T, Ciofu O, Molin S, Givskov M, Høiby N (2013) Applying insights from biofilm biology to drug development — can a new approach be developed? *Nature Reviews Drug Discovery*, **12**, 791–808.

Bobone S, Roversi D, Giordano L et al. (2012) The lipid dependence of antimicrobial peptide activity is an unreliable experimental test for different pore models. *Biochemistry*, **51**, 10124–6.

Bobone S, Gerelli Y, De Zotti M et al. (2013) Membrane thickness and the mechanism of action of the short peptaibol trichogin GA IV. *Biochimica et biophysica acta*, **1828**, 1013–24.

Boman HG (1995) Peptide antibiotics and their role in innate immunity. *Annual review of*

immunology, **13**, 61–92.

Boohaker RJ, Lee MW, Vishnubhotla P, Perez JM, Khaled AR (2012) The use of therapeutic peptides to target and to kill cancer cells. *Current medicinal chemistry*, **19**, 3794–804.

Boucher RC (2004) New concepts of the pathogenesis of cystic fibrosis lung disease. *The European respiratory journal*, **23**, 146–58.

Bowdish DME, Davidson DJ, Lau YE, Lee K, Scott MG, Hancock REW (2005) Impact of LL-37 on anti-infective immunity. *Journal of leukocyte biology*, **77**, 451–9.

Bowdish DME, Davidson DJ, Hancock REW (2006) Immunomodulatory properties of defensins and cathelicidins. *Current topics in microbiology and immunology*, **306**, 27–66.

Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature reviews. Microbiology*, **3**, 238–50.

Bulet P, Hetru C, Dimarcq JL, Hoffmann D (1999) Antimicrobial peptides in insects; structure and function. *Developmental and comparative immunology*, **23**, 329–44.

Burman R, Gunasekera S, Strömstedt AA, Göransson U (2014) Chemistry and biology of cyclotides: circular plant peptides outside the box. *Journal of natural products*, **77**, 724–36.

del Campo R, Morosini M-I, de la Pedrosa EG-G et al. (2005) Population Structure, Antimicrobial Resistance, and Mutation Frequencies of Streptococcus pneumoniae Isolates from Cystic Fibrosis Patients. *Journal of Clinical Microbiology*, **43**, 2207–2214.

Cantin AM, Hartl D, Konstan MW, Chmiel JF (2015) Inflammation in cystic fibrosis lung disease: Pathogenesis and therapy. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, **14**, 419–30.

Caporale LH (1999) Chance favors the prepared genome. *Annals of the New York Academy of Sciences*, **870**, 1–21.

Cardas M, Khan NA, Alsam S (2012a) Staphylococcus aureus exhibit similarities in their interactions with Acanthamoeba and ThP1 macrophage-like cells. *Experimental parasitology*, **132**, 513–8.

Cardas M, Khan NA, Alsam S (2012b) Staphylococcus aureus exhibit similarities in their interactions with Acanthamoeba and ThP1 macrophage-like cells. *Experimental parasitology*, **132**, 513–8.

Castellani C, Cuppens H, Macek M et al. (2008) Consensus on the use and interpretation of cystic fibrosis mutation analysis in clinical practice. *Journal of cystic fibrosis : official*

journal of the European Cystic Fibrosis Society, **7**, 179–96.

Chakraborty K, Ghosh S, Koley H et al. (2008) Bacterial exotoxins downregulate cathelicidin (hCAP-18/LL-37) and human beta-defensin 1 (HBD-1) expression in the intestinal epithelial cells. *Cellular microbiology*, **10**, 2520–37.

Chan WC, Leyland M, Clark J et al. (1996) Structure-activity relationships in the peptide antibiotic nisin: antibacterial activity of fragments of nisin. *FEBS letters*, **390**, 129–32.

Chen Y, Mant CT, Farmer SW, Hancock REW, Vasil ML, Hodges RS (2005) Rational design of alpha-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index. *The Journal of biological chemistry*, **280**, 12316–29.

Chen C, Fan H, Huang Y, Peng F, Fan H, Yuan S, Tong Y (2014) Recombinant lysostaphin protects mice from methicillin-resistant Staphylococcus aureus pneumonia. *BioMed research international*, **2014**, 602185.

Chen X, Yan CC, Zhang X, Zhang X, Dai F, Yin J, Zhang Y (2016) Drug-target interaction prediction: databases, web servers and computational models. *Briefings in bioinformatics*, **17**, 696–712.

Ciofu O, Hansen CR, Høiby N (2013) Respiratory bacterial infections in cystic fibrosis. *Current opinion in pulmonary medicine*, **19**, 251–8.

Clara A, Manjramkar DD, Reddy VK (2004) Preclinical evaluation of magainin-A as a contraceptive antimicrobial agent. *Fertility and sterility*, **81**, 1357–65.

Clement JL, Jarrett PS (1994) Antibacterial silver. *Metal-based drugs*, **1**, 467–82.

Clifton LA, Sanders MR, Hughes A V, Neylon C, Frazier RA, Green RJ (2011) Lipid binding interactions of antimicrobial plant seed defence proteins: puroindoline-a and β-purothionin. *Physical chemistry chemical physics : PCCP*, **13**, 17153–62.

Cole AM, Darouiche RO, Legarda D, Connell N, Diamond G (2000) Characterization of a fish antimicrobial peptide: gene expression, subcellular localization, and spectrum of activity. *Antimicrobial agents and chemotherapy*, **44**, 2039–45.

Corsaro D, Walochnik J, Köhsler M, Rott MB (2015) Acanthamoeba misidentification and multiple labels: redefining genotypes T16, T19, and T20 and proposal for Acanthamoeba micheli sp. nov. (genotype T19). *Parasitology research*, **114**, 2481–90.

Costerton JW (1999) Introduction to biofilm. *International journal of antimicrobial agents*, **11**, 217-21–9.

Costerton JW, Irvin RT, Cheng KJ (1981) The bacterial glycocalyx in nature and disease. *Annual review of microbiology*, **35**, 299–324.

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science (New York, N.Y.)*, **284**, 1318–22.

Cotter PD, Hill C, Ross RP (2005) Bacteriocins: developing innate immunity for food. *Nature reviews. Microbiology*, **3**, 777–88.

Coulon C, Collignon A, McDonnell G, Thomas V (2010) Resistance of Acanthamoeba cysts to disinfection treatments used in health care settings. *Journal of clinical microbiology*, **48**, 2689–97.

Cox NS, Alison JA, Holland AE (2013) Interventions for promoting physical activity in people with cystic fibrosis. *The Cochrane database of systematic reviews*, CD009448.

Crossley KB, Jefferson KK, Archer GL, Fowler VG (eds.) (2009) *Staphylococci in Human Disease*. Wiley-Blackwell, Oxford, UK.

Dalemans W, Barbry P, Champigny G et al. (1991) Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. *Nature*, **354**, 526–8.

Daniels R (2011) Surviving the first hours in sepsis: getting the basics right (an intensivist's perspective). *The Journal of antimicrobial chemotherapy*, **66 Suppl 2**, ii11-23.

Dashper SG, Liu S-W, Walsh KA et al. (2013) Streptococcus mutans biofilm disruption by κ-casein glycopeptide. *Journal of dentistry*, **41**, 521–7.

Dathe M, Nikolenko H, Meyer J, Beyermann M, Bienert M (2001) Optimization of the antimicrobial activity of magainin peptides by modification of charge. *FEBS letters*, **501**, 146–50.

Dauletbaev N, Fischer P, Aulbach B et al. (2009) A phase II study on safety and efficacy of high-dose N-acetylcysteine in patients with cystic fibrosis. *European journal of medical research*, **14**, 352–8.

Davis GS, Sandstedt SA, Patel M, Marrs CF, Gilsdorf JR (2011) Use of bexB to detect the capsule locus in Haemophilus influenzae. *Journal of clinical microbiology*, **49**, 2594–601.

Dempsey CE (1990) The actions of melittin on membranes. *Biochimica et biophysica acta*, **1031**, 143–61.

Deslouches B, Phadke SM, Lazarevic V, Cascio M, Islam K, Montelaro RC, Mietzner TA (2005) De novo generation of cationic antimicrobial peptides: influence of length and tryptophan substitution on antimicrobial activity. *Antimicrobial agents and chemotherapy*, **49**, 316–22.

Donaldson SH, Bennett WD, Zeman KL, Knowles MR, Tarran R, Boucher RC (2006) Mucus clearance and lung function in cystic fibrosis with hypertonic saline. *The New England journal of medicine*, **354**, 241–50.

Dong N, Ma Q, Shan A, Lv Y, Hu W, Gu Y, Li Y (2012) Strand length-dependent antimicrobial activity and membrane-active mechanism of arginine- and valine-rich β-hairpin-like antimicrobial peptides. *Antimicrobial agents and chemotherapy*, **56**, 2994–3003.

Donlan RM (2002) Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases*, **8**, 881–890.

Dosler S, Karaaslan E (2014) Inhibition and destruction of Pseudomonas aeruginosa biofilms by antibiotics and antimicrobial peptides. *Peptides*, **62**, 32–37.

Dosler S, Karaaslan E, Alev Gerceker A (2015) Antibacterial and anti-biofilm activities of melittin and colistin, alone and in combination with antibiotics against Gram-negative bacteria. *Journal of chemotherapy (Florence, Italy)*, 1973947815Y0000000004.

Easton DM, Nijnik A, Mayer ML, Hancock REW (2009) Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends in Biotechnology*, **27**, 582–590.

Ecevit IZ, McCrea KW, Pettigrew MM, Sen A, Marrs CF, Gilsdorf JR (2004) Prevalence of the hifBC, hmw1A, hmw2A, hmwC, and hia Genes in Haemophilus influenzae Isolates. *Journal of clinical microbiology*, **42**, 3065–72.

Edmondson C, Davies JC (2016) Current and future treatment options for cystic fibrosis lung disease: latest evidence and clinical implications. *Therapeutic advances in chronic disease*, **7**, 170–83.

Ehrenstein G, Lecar H (1977) Electrically gated ionic channels in lipid bilayers. *Quarterly reviews of biophysics*, **10**, 1–34.

Ehsan Z, Wetzel JD, Clancy JP (2014) Nebulized liposomal amikacin for the treatment of Pseudomonas aeruginosa infection in cystic fibrosis patients. *Expert opinion on investigational drugs*, **23**, 743–9.

Eisenberg D (1984) Three-dimensional structure of membrane and surface proteins. *Annual review of biochemistry*, **53**, 595–623.

Elkins MR, Robinson M, Rose BR et al. (2006) A controlled trial of long-term inhaled hypertonic saline in patients with cystic fibrosis. *The New England journal of medicine*, **354**, 229–40.

Epand RM, Epand RF (2011) Bacterial membrane lipids in the action of antimicrobial agents. *Journal of peptide science : an official publication of the European Peptide Society*, **17**, 298–305.

Epand RM, Vogel HJ (1999) Diversity of antimicrobial peptides and their mechanisms of action. *Biochimica et biophysica acta*, **1462**, 11–28.

Ericksen B, Wu Z, Lu W, Lehrer RI (2005) Antibacterial activity and specificity of the six human {alpha}-defensins. *Antimicrobial agents and chemotherapy*, **49**, 269–75.

Ewald A, Glückermann SK, Thull R, Gbureck U (2006) Antimicrobial titanium/silver PVD coatings on titanium. *Biomedical engineering online*, **5**, 22.

Falla TJ, Karunaratne DN, Hancock RE (1996) Mode of action of the antimicrobial peptide indolicidin. *The Journal of biological chemistry*, **271**, 19298–303.

Feldman ST, Speaker M, Cleveland P (1991) Effect of magainins on Acanthamoeba castellanii. *Reviews of infectious diseases*, **13 Suppl 5**, S439.

Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO (2000) A mechanistic study of the antibacterial effect of silver ions on Escherichia coli and Staphylococcus aureus. *Journal of biomedical materials research*, **52**, 662–8.

Fernández-Vidal M, Jayasinghe S, Ladokhin AS, White SH (2007) Folding amphipathic helices into membranes: amphiphilicity trumps hydrophobicity. *Journal of molecular biology*, **370**, 459–70.

Fjell CD, Jenssen H, Hilpert K, Cheung WA, Panté N, Hancock REW, Cherkasov A (2009) Identification of novel antibacterial peptides by chemoinformatics and machine learning. *Journal of medicinal chemistry*, **52**, 2006–15.

Fjell CD, Jenssen H, Cheung WA, Hancock REW, Cherkasov A (2011) Optimization of antibacterial peptides by genetic algorithms and cheminformatics. *Chemical biology & drug design*, **77**, 48–56.

Fouhy Y, Scanlon K, Schouest K et al. (2007) Diffusible signal factor-dependent cell-cell signaling and virulence in the nosocomial pathogen Stenotrophomonas maltophilia. *Journal of bacteriology*, **189**, 4964–8.

Fox JL (2013) Antimicrobial peptides stage a comeback. *Nature biotechnology*, **31**, 379–82.

Fuchs HJ, Borowitz DS, Christiansen DH et al. (1994) Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group. *The New England journal of medicine*, **331**, 637–42.

Funderburg N, Lederman MM, Feng Z et al. (2007) Human -defensin-3 activates professional antigen-presenting cells via Toll-like receptors 1 and 2. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 18631–5.

Fuqua C, Parsek MR, Greenberg EP (2001) Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annual review of genetics*, **35**, 439–68.

Ganz T (2003) Defensins: antimicrobial peptides of innate immunity. *Nature reviews. Immunology*, **3**, 710–20.

García-Castillo M, Morosini MI, Valverde A, Almaraz F, Baquero F, Cantón R, del Campo R (2007) Differences in biofilm development and antibiotic susceptibility among Streptococcus pneumoniae isolates from cystic fibrosis samples and blood cultures. *The Journal of antimicrobial chemotherapy*, **59**, 301–4.

Gayvallet-Montredon N, Sauvestre C, Bergeret M, Gendrel D, Raymond J (1998) [Bacteriologic surveillance of nosocomial septicemia and bacteremia in a pediatric hospital]. *Archives de pédiatrie : organe officiel de la Sociéte française de pédiatrie*, **5**, 1216–20.

Ghosh A, Kar RK, Jana J et al. (2014) Indolicidin targets duplex DNA: structural and mechanistic insight through a combination of spectroscopy and microscopy. *ChemMedChem*, **9**, 2052–8.

Giacometti A, Cirioni O, Del Prete MS, Barchiesi F, Fortuna M, Drenaggi D, Scalise G (2000a) In Vitro Activities of Membrane-Active Peptides Alone and in Combination with Clinically Used Antimicrobial Agents against Stenotrophomonas maltophilia. *Antimicrobial Agents and Chemotherapy*, **44**, 1716–1719.

Giacometti A, Cirioni O, Prete MS Del, Barchiesi F, Paggi AM, Petrelli E, Scalise G (2000b) Comparative activities of polycationic peptides and clinically used antimicrobial agents against multidrug-resistant nosocomial isolates of Acinetobacter baumannii. *Journal of Antimicrobial Chemotherapy*, **46**, 807–810.

Giacometti A, Cirioni O, Barchiesi F, Scalise G (2000c) *In-vitro activity and killing effect of polycationic peptides on methicillin-resistant Staphylococcus aureus and interactions with clinically used antibiotics*, Vol. 38. 115-118 pp.

Giacometti A, Cirioni O, Del Prete MS, Barchiesi F, Fortuna M, Drenaggi D, Scalise G (2000d) In Vitro Activities of Membrane-Active Peptides Alone and in Combination with Clinically Used Antimicrobial Agents against Stenotrophomonas maltophilia.

Antimicrobial Agents and Chemotherapy, **44**, 1716–1719.

Giacometti A, Cirioni O, Kamysz W et al. (2005) In vitro activity of amphibian peptides alone and in combination with antimicrobial agents against multidrug-resistant pathogens isolated from surgical wound infection. *Peptides*, **26**, 2111–2116.

Gilljam H, Ellin A, Strandvik B (1989) Increased bronchial chloride concentration in cystic fibrosis. *Scandinavian journal of clinical and laboratory investigation*, **49**, 121–4.

Giovannini MG, Poulter L, Gibson BW, Williams DH (1987) Biosynthesis and degradation of peptides derived from Xenopus laevis prohormones. *The Biochemical journal*, **243**, 113–20.

Giudici M, Pascual R, de la Canal L, Pfüller K, Pfüller U, Villalaín J (2003) Interaction of viscotoxins A3 and B with membrane model systems: implications to their mechanism of action. *Biophysical journal*, **85**, 971–81.

Giuliani A, Pirri G, Nicoletto S (2007) Antimicrobial peptides: an overview of a promising class of therapeutics. *Open Life Sciences*, **2**, 1–33.

Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM (1997a) Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell*, **88**, 553–60.

Goldman MJ, Anderson GM, Stolzenberg ED, Kari UP, Zasloff M, Wilson JM (1997b) Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis. *Cell*, **88**, 553–60.

Goossens H (2003) Susceptibility of multi-drug-resistant Pseudomonas aeruginosa in intensive care units: results from the European MYSTIC study group. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, **9**, 980–3.

Gopal R, Seo CH, Song PI, Park Y (2013) Effect of repetitive lysine-tryptophan motifs on the bactericidal activity of antimicrobial peptides. *Amino acids*, **44**, 645–60.

Gordon RJ, Lowy FD (2008) Pathogenesis of methicillin-resistant Staphylococcus aureus infection. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, **46 Suppl 5**, S350-9.

Greub G, Raoult D (2004) Microorganisms resistant to free-living amoebae. *Clinical microbiology reviews*, **17**, 413–33.

Gross E, Morell JL (1971) The structure of nisin. *Journal of the American Chemical Society*, **93**, 4634–5.

Gruenheid S, Le Moual H (2012) Resistance to antimicrobial peptides in Gram-negative bacteria. *FEMS microbiology letters*, **330**, 81–9.

Haardt M, Benharouga M, Lechardeur D, Kartner N, Lukacs GL (1999) C-terminal truncations destabilize the cystic fibrosis transmembrane conductance regulator without impairing its biogenesis. A novel class of mutation. *The Journal of biological chemistry*, **274**, 21873–7.

Ben Haj Khalifa A, Moissenet D, Vu Thien H, Khedher M [Virulence factors in Pseudomonas aeruginosa: mechanisms and modes of regulation]. *Annales de biologie clinique*, **69**, 393–403.

Hall-Stoodley L, Stoodley P (2009) Evolving concepts in biofilm infections. *Cellular microbiology*, **11**, 1034–43.

Hallström T, Riesbeck K (2010) Trends In Mircrobiology (review) Haemophilus influenzae and the complement system.

Hancock REW, Sahl H-G (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature biotechnology*, **24**, 1551–7.

Harrison MJ, McCarthy M, Fleming C et al. (2014) Inhaled versus nebulised tobramycin: a real world comparison in adult cystic fibrosis (CF). *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, **13**, 692–8.

Hase K, Murakami M, Iimura M et al. (2003) Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against Helicobacter pylori. *Gastroenterology*, **125**, 1613–25.

Hasper HE, Kramer NE, Smith JL et al. (2006) An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science (New York, N.Y.)*, **313**, 1636–7.

He J, Starr CG, Wimley WC (2015) A lack of synergy between membrane-permeabilizing cationic antimicrobial peptides and conventional antibiotics. *Biochimica et biophysica acta*, **1848**, 8–15.

Heijerman H (2005) Infection and inflammation in cystic fibrosis: a short review. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, **4 Suppl 2**, 3–5.

Hein-Kristensen L, Franzyk H, Holch A, Gram L (2013) Adaptive evolution of Escherichia coli to an α-peptide/β-peptoid peptidomimetic induces stable resistance. *PloS one*, **8**, e73620.

Heltshe SL, Saiman L, Popowitch EB et al. (2015) Outcomes and Treatment of Chronic

Methicillin-Resistant Staphylococcus aureus Differs by Staphylococcal Cassette Chromosome mec (SCCmec) Type in Children With Cystic Fibrosis. *Journal of the Pediatric Infectious Diseases Society*, **4**, 225–31.

Hewer SL (2012) Inhaled antibiotics in cystic fibrosis: what's new? *Journal of the Royal Society of Medicine*, S19-24.

Hillman JD, Novák J, Sagura E et al. (1998) Genetic and biochemical analysis of mutacin 1140, a lantibiotic from Streptococcus mutans. *Infection and immunity*, **66**, 2743–9.

Hilpert K, Elliott MR, Volkmer-Engert R et al. (2006) Sequence requirements and an optimization strategy for short antimicrobial peptides. *Chemistry & biology*, **13**, 1101–7.

Hoffman LR, D'Argenio DA, MacCoss MJ, Zhang Z, Jones RA, Miller SI (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature*, **436**, 1171–5.

Hojo K, Nagaoka S, Ohshima T, Maeda N (2009) Bacterial interactions in dental biofilm development. *Journal of dental research*, **88**, 982–90.

Horswill AR, Stoodley P, Stewart PS, Parsek MR (2007) The effect of the chemical, biological, and physical environment on quorum sensing in structured microbial communities. *Analytical and bioanalytical chemistry*, **387**, 371–80.

Hritonenko V, Stathopoulos C (2007) Omptin proteins: an expanding family of outer membrane proteases in Gram-negative Enterobacteriaceae. *Molecular membrane biology*, **24**, 395–406.

Hsu C-H, Chen C, Jou M-L et al. (2005) Structural and DNA-binding studies on the bovine antimicrobial peptide, indolicidin: evidence for multiple conformations involved in binding to membranes and DNA. *Nucleic acids research*, **33**, 4053–64.

Huang HW (2006) Molecular mechanism of antimicrobial peptides: the origin of cooperativity. *Biochimica et biophysica acta*, **1758**, 1292–302.

Huang Y, Wiradharma N, Xu K et al. (2012) Cationic amphiphilic alpha-helical peptides for the treatment of carbapenem-resistant Acinetobacter baumannii infection. *Biomaterials*, **33**, 8841–8847.

Hwang T-C, Sheppard DN (2009) Gating of the CFTR Cl- channel by ATP-driven nucleotide-binding domain dimerisation. *The Journal of physiology*, **587**, 2151–61.

Hyams C, Camberlein E, Cohen JM, Bax K, Brown JS (2010) The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infection and immunity*, **78**, 704–15.

Ichikawa JK, Norris A, Bangera MG, Geiss GK, van 't Wout AB, Bumgarner RE, Lory S (2000) Interaction of pseudomonas aeruginosa with epithelial cells: identification of differentially regulated genes by expression microarray analysis of human cDNAs. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 9659–64.

Ilg K, Endt K, Misselwitz B, Stecher B, Aebi M, Hardt W-D (2009) O-antigen-negative Salmonella enterica serovar Typhimurium is attenuated in intestinal colonization but elicits colitis in streptomycin-treated mice. *Infection and immunity*, **77**, 2568–75.

Ilić N, Novković M, Guida F, Xhindoli D, Benincasa M, Tossi A, Juretić D (2013) Selective antimicrobial activity and mode of action of adepantins, glycine-rich peptide antibiotics based on anuran antimicrobial peptide sequences. *Biochimica et biophysica acta*, **1828**, 1004–12.

Imura Y, Nishida M, Matsuzaki K (2007) Action mechanism of PEGylated magainin 2 analogue peptide. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, **1768**, 2578–2585.

Iwasaki T, Ishibashi J, Tanaka H, Sato M, Asaoka A, Taylor D, Yamakawa M (2009) Selective cancer cell cytotoxicity of enantiomeric 9-mer peptides derived from beetle defensins depends on negatively charged phosphatidylserine on the cell surface. *Peptides*, **30**, 660–8.

Iwata H, Sawada R, Mizutani S, Kotera M, Yamanishi Y (2015) Large-Scale Prediction of Beneficial Drug Combinations Using Drug Efficacy and Target Profiles. *Journal of chemical information and modeling*, **55**, 2705–16.

Jacob L, Zasloff M (1994) Potential therapeutic applications of magainins and other antimicrobial agents of animal origin. *Ciba Foundation symposium*, **186**, 197-216–23.

Jahnsen RD, Frimodt-Møller N, Franzyk H (2012) Antimicrobial activity of peptidomimetics against multidrug-resistant Escherichia coli: a comparative study of different backbones. *Journal of medicinal chemistry*, **55**, 7253–61.

Jansen G, Lee AY, Epp E et al. (2009) Chemogenomic profiling predicts antifungal synergies. *Molecular systems biology*, **5**, 338.

Jansen G, Mahrt N, Tueffers L et al. (2016) Association between clinical antibiotic resistance and susceptibility of Pseudomonas in the cystic fibrosis lung. *Evolution, medicine, and public health*.

Javadpour MM, Juban MM, Lo WC et al. (1996) De novo antimicrobial peptides with low

mammalian cell toxicity. *Journal of medicinal chemistry*, **39**, 3107–13.

Jenssen H, Hamill P, Hancock REW (2006) Peptide antimicrobial agents. *Clinical microbiology reviews*, **19**, 491–511.

José Maschio V, Corção G, Rott MB (2015) IDENTIFICATION OF Pseudomonas spp. AS AMOEBA-RESISTANT MICROORGANISMS IN ISOLATES OF Acanthamoeba. *Revista do Instituto de Medicina Tropical de São Paulo*, **57**, 81–83.

Junker LM, Clardy J (2007) High-throughput screens for small-molecule inhibitors of Pseudomonas aeruginosa biofilm development. *Antimicrobial agents and chemotherapy*, **51**, 3582–90.

Juretić D, Vukicević D, Ilić N, Antcheva N, Tossi A (2009) Computational design of highly selective antimicrobial peptides. *Journal of chemical information and modeling*, **49**, 2873–82.

Kagan BL, Selsted ME, Ganz T, Lehrer RI (1990) Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proceedings of the National Academy of Sciences of the United States of America*, **87**, 210–4.

Kanelis V, Hudson RP, Thibodeau PH, Thomas PJ, Forman-Kay JD (2010) NMR evidence for differential phosphorylation-dependent interactions in WT and DeltaF508 CFTR. *The EMBO journal*, **29**, 263–77.

Kerem B, Rommens JM, Buchanan JA et al. (1989) Identification of the cystic fibrosis gene: genetic analysis. *Science (New York, N.Y.)*, **245**, 1073–80.

Kerem E, Corey M, Kerem BS et al. (1990) The relation between genotype and phenotype in cystic fibrosis--analysis of the most common mutation (delta F508). *The New England journal of medicine*, **323**, 1517–22.

Khandelia H, Kaznessis YN (2007) Cation-pi interactions stabilize the structure of the antimicrobial peptide indolicidin near membranes: molecular dynamics simulations. *The journal of physical chemistry. B*, **111**, 242–50.

Khardori N, Yassien M (1995) Biofilms in device-related infections. *Journal of industrial microbiology*, **15**, 141–7.

Kilvington S, Price J (1990) Survival of Legionella pneumophila within cysts of Acanthamoeba polyphaga following chlorine exposure. *The Journal of applied bacteriology*, **68**, 519–25.

Kollberg H, Carlander D, Olesen H, Wejåker P-E, Johannesson M, Larsson A (2003) Oral

administration of specific yolk antibodies (IgY) may prevent Pseudomonas aeruginosa infections in patients with cystic fibrosis: a phase I feasibility study. *Pediatric pulmonology*, **35**, 433–40.

Kondejewski LH, Jelokhani-Niaraki M, Farmer SW et al. (1999) Dissociation of antimicrobial and hemolytic activities in cyclic peptide diastereomers by systematic alterations in amphipathicity. *The Journal of biological chemistry*, **274**, 13181–92.

Konstan MW, Ratjen F (2012) Effect of dornase alfa on inflammation and lung function: potential role in the early treatment of cystic fibrosis. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, **11**, 78–83.

Konstan MW, Flume PA, Kappler M et al. (2011) Safety, efficacy and convenience of tobramycin inhalation powder in cystic fibrosis patients: The EAGER trial. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, **10**, 54–61.

Kovács M, Halfmann A, Fedtke I et al. (2006) A functional dlt operon, encoding proteins required for incorporation of d-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in Streptococcus pneumoniae. *Journal of bacteriology*, **188**, 5797–805.

Kumar Pandian SR, Deepak V, Kalishwaralal K, Viswanathan P, Gurunathan S (2010) Mechanism of bactericidal activity of silver nitrate - a concentration dependent bi-functional molecule. *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]*, **41**, 805–9.

Lacombe C, Cifuentes-Diaz C, Dunia I, Auber-Thomay M, Nicolas P, Amiche M (2000) Peptide secretion in the cutaneous glands of South American tree frog Phyllomedusa bicolor: an ultrastructural study. *European journal of cell biology*, **79**, 631–41.

Lamothe J, Thyssen S, Valvano MA (2004) Burkholderia cepacia complex isolates survive intracellularly without replication within acidic vacuoles of Acanthamoeba polyphaga. *Cellular Microbiology*, **6**, 1127–1138.

Landers P, Kerr KG, Rowbotham TJ, Tipper JL, Keig PM, Ingham E, Denton M (2000) Survival and growth of Burkholderia cepacia within the free-living amoeba Acanthamoeba polyphaga. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*, **19**, 121–3.

Landini P, Antoniani D, Burgess JG, Nijland R (2010) Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. *Applied microbiology and*

biotechnology, **86**, 813–23.

Langton Hewer SC, Smyth AR (2014) Antibiotic strategies for eradicating Pseudomonas aeruginosa in people with cystic fibrosis. *The Cochrane database of systematic reviews*, CD004197.

Lau YE, Rozek A, Scott MG, Goosney DL, Davidson DJ, Hancock REW (2005) Interaction and cellular localization of the human host defense peptide LL-37 with lung epithelial cells. *Infection and immunity*, **73**, 583–91.

Laver DR (1994) The barrel-stave model as applied to alamethicin and its analogs reevaluated. *Biophysical journal*, **66**, 355–9.

Lawrence ER, Griffiths DB, Martin SA, George RC, Hall LMC (2003) Evaluation of semiautomated multiplex PCR assay for determination of Streptococcus pneumoniae serotypes and serogroups. *Journal of clinical microbiology*, **41**, 601–7.

LeBel G, Piché F, Frenette M, Gottschalk M, Grenier D (2013) Antimicrobial activity of nisin against the swine pathogen Streptococcus suis and its synergistic interaction with antibiotics. *Peptides*, **50**, 19–23.

Lee IH, Cho Y, Lehrer RI (1997) Effects of pH and salinity on the antimicrobial properties of clavanins. *Infection and immunity*, **65**, 2898–903.

de Leeuw E, Rajabi M, Zou G, Pazgier M, Lu W (2009) Selective arginines are important for the antibacterial activity and host cell interaction of human alpha-defensin 5. *FEBS letters*, **583**, 2507–12.

de Leeuw E, Li C, Zeng P et al. (2010) Functional interaction of human neutrophil peptide-1 with the cell wall precursor lipid II. *FEBS letters*, **584**, 1543–8.

Lehár J, Krueger AS, Avery W et al. (2009) Synergistic drug combinations tend to improve therapeutically relevant selectivity. *Nature biotechnology*, **27**, 659–66.

Lehmann J, Retz M, Sidhu SS et al. (2006) Antitumor activity of the antimicrobial peptide magainin II against bladder cancer cell lines. *European urology*, **50**, 141–7.

Levy SB, Marshall B (2004) Antibacterial resistance worldwide: causes, challenges and responses. *Nature medicine*, **10**, S122-9.

Lewis K (2001) Riddle of biofilm resistance. *Antimicrobial agents and chemotherapy*, **45**, 999–1007.

Li W-F, Ma G-X, Zhou X-X (2006) Apidaecin-type peptides: biodiversity, structure-function relationships and mode of action. *Peptides*, **27**, 2350–9.

Liau SY, Read DC, Pugh WJ, Furr JR, Russell AD (1997) Interaction of silver nitrate with

readily identifiable groups: relationship to the antibacterial action of silver ions. *Letters in applied microbiology*, **25**, 279–83.

Linares JF, Gustafsson I, Baquero F, Martinez JL (2006) Antibiotics as intermicrobial signaling agents instead of weapons. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 19484–9.

Lipuma JJ (2010) The changing microbial epidemiology in cystic fibrosis. *Clinical microbiology reviews*, **23**, 299–323.

López-Causapé C, Rojo-Molinero E, Macià MD, Oliver A (2015) The problems of antibiotic resistance in cystic fibrosis and solutions. *Expert Review of Respiratory Medicine*, **9**, 73–88.

Lyczak JB, Cannon CL, Pier GB (2002) Lung infections associated with cystic fibrosis. *Clinical microbiology reviews*, **15**, 194–222.

Macneill S (2015) *Hodson and Geddes' cystic fibrosis. Epidemiology of cystic fibrosis.*, 4th ed. edn. CRC Press.

Marciano-Cabral F, Cabral G (2003) Acanthamoeba spp. as agents of disease in humans. *Clinical microbiology reviews*, **16**, 273–307.

Marion D, Bakan B, Elmorjani K (2007) Plant lipid binding proteins: properties and applications. *Biotechnology advances*, **25**, 195–7.

Marolda CL, Hauröder B, John MA, Michel R, Valvano MA (1999) Intracellular survival and saprophytic growth of isolates from the Burkholderia cepacia complex in free-living amoebae. *Microbiology (Reading, England)*, 1509–17.

Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM, Green DR (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *The Journal of experimental medicine*, **182**, 1545–56.

Martinez AJ, Visvesvara GS (1997) Free-living, amphizoic and opportunistic amebas. *Brain pathology (Zurich, Switzerland)*, **7**, 583–98.

Mason AJ (2005) The antibiotic and DNA-transfecting peptide LAH4 selectively associates with, and disorders, anionic lipids in mixed membranes. *The FASEB Journal*.

Mason AJ, Chotimah INH, Bertani P, Bechinger B (2006a) A spectroscopic study of the membrane interaction of the antimicrobial peptide Pleurocidin. *Molecular membrane biology*, **23**, 185–94.

Mason AJ, Gasnier C, Kichler A, Prévost G, Aunis D, Metz-Boutigue M-H, Bechinger B

(2006b) Enhanced membrane disruption and antibiotic action against pathogenic bacteria by designed histidine-rich peptides at acidic pH. *Antimicrobial agents and chemotherapy*, **50**, 3305–11.

Mason AJ, Chotimah INH, Bertani P, Bechinger B (2006c) A spectroscopic study of the membrane interaction of the antimicrobial peptide Pleurocidin. *Molecular Membrane Biology*, **23**, 185–194.

Mason AJ, Moussaoui W, Abdelrahman T et al. (2009) Structural determinants of antimicrobial and antiplasmodial activity and selectivity in histidine-rich amphipathic cationic peptides. *The Journal of biological chemistry*, **284**, 119–33.

Matsuzaki K (2009) Control of cell selectivity of antimicrobial peptides. *Biochimica et biophysica acta*, **1788**, 1687–92.

Matsuzaki K, Harada M, Funakoshi S, Fujii N, Miyajima K (1991) Physicochemical determinants for the interactions of magainins 1 and 2 with acidic lipid bilayers. *Biochimica et biophysica acta*, **1063**, 162–70.

McCoy KS, Quittner AL, Oermann CM, Gibson RL, Retsch-Bogart GZ, Montgomery AB (2008) Inhaled aztreonam lysine for chronic airway Pseudomonas aeruginosa in cystic fibrosis. *American journal of respiratory and critical care medicine*, **178**, 921–8.

McCrea KW, Wang ML, Xie J et al. (2010) Prevalence of the sodC gene in nontypeable Haemophilus influenzae and Haemophilus haemolyticus by microarray-based hybridization. *Journal of clinical microbiology*, **48**, 714–9.

Mcdaniel CT, Panmanee W, Hassett DJ (2015) An Overview of Infections in Cystic Fibrosis Airways and the Role of Environmental Conditions on Pseudomonas aeruginosa Biofilm Formation and Viability.

McIlwaine MP, Alarie N, Davidson GF et al. (2013) Long-term multicentre randomised controlled study of high frequency chest wall oscillation versus positive expiratory pressure mask in cystic fibrosis. *Thorax*, **68**, 746–51.

McKone EF, Emerson SS, Edwards KL, Aitken ML (2003) Effect of genotype on phenotype and mortality in cystic fibrosis: a retrospective cohort study. *Lancet (London, England)*, **361**, 1671–6.

Mendell JT, Dietz HC (2001) When the message goes awry: disease-producing mutations that influence mRNA content and performance. *Cell*, **107**, 411–4.

Merino S, Tomás JM (2015) Bacterial Capsules and Evasion of Immune Responses. In: *eLS*, pp. 1–10. John Wiley & Sons, Ltd, Chichester, UK.

Michel R, Burghardt H, Bergmann H (1995) [Acanthamoeba, naturally intracellularly infected with Pseudomonas aeruginosa, after their isolation from a microbiologically contaminated drinking water system in a hospital]. *Zentralblatt für Hygiene und Umweltmedizin = International journal of hygiene and environmental medicine*, **196**, 532–44.

Mogayzel PJ, Naureckas ET, Robinson KA et al. (2014) Cystic Fibrosis Foundation pulmonary guideline. pharmacologic approaches to prevention and eradication of initial Pseudomonas aeruginosa infection. *Annals of the American Thoracic Society*, **11**, 1640–50.

Mojsoska B, Jenssen H (2015) Peptides and Peptidomimetics for Antimicrobial Drug Design. *Pharmaceuticals (Basel, Switzerland)*, **8**, 366–415.

Monroe D (2007) Looking for chinks in the armor of bacterial biofilms. *PLoS biology*, **5**, e307.

Moore DL, MacDonald NE, Canadian Paediatric Society, Infectious Diseases and Immunization Committee (2015) Preventing ophthalmia neonatorum. *The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie médicale / AMMI Canada*, **26**, 122–5.

Moreau-Marquis S, Stanton BA, O'Toole GA (2008a) Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. *Pulmonary pharmacology & therapeutics*, **21**, 595–9.

Moreau-Marquis S, Stanton BA, O'Toole GA (2008b) Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. *Pulmonary Pharmacology & Therapeutics*, **21**, 595–599.

Moreau-Marquis S, Stanton BA, O'Toole GA (2008c) Pseudomonas aeruginosa biofilm formation in the cystic fibrosis airway. *Pulmonary pharmacology & therapeutics*, **21**, 595–9.

Mowat E, Paterson S, Fothergill JL et al. (2011) Pseudomonas aeruginosa population diversity and turnover in cystic fibrosis chronic infections. *American journal of respiratory and critical care medicine*, **183**, 1674–9.

Murphy TF (2000) Haemophilus influenzae in chronic bronchitis. *Seminars in respiratory infections*, **15**, 41–51.

Nagington J, Richards JE (1976) Chemotherapeutic compounds and Acanthamoebae from eye infections. *Journal of clinical pathology*, **29**, 648–51.

Nawrocki KL, Crispell EK, McBride SM (2014) Antimicrobial Peptide Resistance Mechanisms of Gram-Positive Bacteria. *Antibiotics (Basel, Switzerland)*, **3**, 461–92.

Noel GJ, Hoiseth SK, Edelson PJ (1992) Type b capsule inhibits ingestion of Haemophilus influenzae by murine macrophages: studies with isogenic encapsulated and unencapsulated strains. *The Journal of infectious diseases*, **166**, 178–82.

Nomura I, Goleva E, Howell MD et al. (2003) Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *Journal of immunology (Baltimore, Md. : 1950)*, **171**, 3262–9.

O'Brien-Simpson NM, Pantarat N, Attard TJ, Walsh KA, Reynolds EC (2016) A Rapid and Quantitative Flow Cytometry Method for the Analysis of Membrane Disruptive Antimicrobial Activity. *PloS one*, **11**, e0151694.

Ong PY, Ohtake T, Brandt C et al. (2002) Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *The New England journal of medicine*, **347**, 1151–60.

Orioni B, Bocchinfuso G, Kim JY et al. (2009) Membrane perturbation by the antimicrobial peptide PMAP-23: a fluorescence and molecular dynamics study. *Biochimica et biophysica acta*, **1788**, 1523–33.

Osato MS, Robinson NM, Wilhelmus KR, Jones DB (1991) In vitro evaluation of antimicrobial compounds for cysticidal activity against Acanthamoeba. *Reviews of infectious diseases*, **13 Suppl 5**, S431-5.

Ouellette AJ, Selsted ME (1996) Paneth cell defensins: endogenous peptide components of intestinal host defense. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, **10**, 1280–9.

Overhage J, Campisano A, Bains M, Torfs ECW, Rehm BHA, Hancock REW (2008a) Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infection and immunity*, **76**, 4176–82.

Overhage J, Campisano A, Bains M, Torfs ECW, Rehm BHA, Hancock REW (2008b) Human host defense peptide LL-37 prevents bacterial biofilm formation. *Infection and immunity*, **76**, 4176–82.

Pace CN, Scholtz JM (1998) A helix propensity scale based on experimental studies of peptides and proteins. *Biophysical journal*, **75**, 422–7.

Pag U, Oedenkoven M, Sass V et al. (2008) Analysis of in vitro activities and modes of action of synthetic antimicrobial peptides derived from an alpha-helical "sequence template". *The Journal of antimicrobial chemotherapy*, **61**, 341–52.

Pai R, Gertz RE, Beall B (2006) Sequential multiplex PCR approach for determining capsular serotypes of Streptococcus pneumoniae isolates. *Journal of clinical microbiology*, **44**, 124–31.

Palmer J (2006) Bacterial biofilms in chronic rhinosinusitis. *The Annals of otology, rhinology & laryngology. Supplement*, **196**, 35–9.

Palmer KL, Aye LM, Whiteley M (2007) Nutritional Cues Control Pseudomonas aeruginosa Multicellular Behavior in Cystic Fibrosis Sputum. *Journal of Bacteriology*, **189**, 8079–8087.

Pardi A, Zhang XL, Selsted ME, Skalicky JJ, Yip PF (1992) NMR studies of defensin antimicrobial peptides. 2. Three-dimensional structures of rabbit NP-2 and human HNP-1. *Biochemistry*, **31**, 11357–64.

Park CB, Kim MS, Kim SC (1996) A novel antimicrobial peptide from Bufo bufo gargarizans. *Biochemical and biophysical research communications*, **218**, 408–13.

Park IY, Cho JH, Kim KS, Kim Y-B, Kim MS, Kim SC (2004) Helix stability confers salt resistance upon helical antimicrobial peptides. *The Journal of biological chemistry*, **279**, 13896–901.

Park S-C, Park Y, Hahm K-S (2011) The role of antimicrobial peptides in preventing multidrug-resistant bacterial infections and biofilm formation. *International journal of molecular sciences*, **12**, 5971–92.

Pasupuleti M, Schmidtchen A, Malmsten M (2012) Antimicrobial peptides: key components of the innate immune system. *Critical reviews in biotechnology*, **32**, 143–71.

Pathak N, Salas-Auvert R, Ruche G, Janna MH, McCarthy D, Harrison RG (1995) Comparison of the effects of hydrophobicity, amphiphilicity, and alpha-helicity on the activities of antimicrobial peptides. *Proteins*, **22**, 182–6.

Pazgier M, Hoover DM, Yang D, Lu W, Lubkowski J (2006) Human beta-defensins. *Cellular and molecular life sciences : CMLS*, **63**, 1294–313.

Perron GG, Zasloff M, Bell G (2006) Experimental evolution of resistance to an antimicrobial peptide. *Proceedings. Biological sciences / The Royal Society*, **273**, 251–6.

Piers KL, Hancock RE (1994) The interaction of a recombinant cecropin/melittin hybrid peptide with the outer membrane of Pseudomonas aeruginosa. *Molecular microbiology*, **12**, 951–8.

Pitts B, Hamilton MA, Zelver N, Stewart PS (2003) A microtiter-plate screening method for biofilm disinfection and removal. *Journal of microbiological methods*, **54**, 269–76.

Plant BJ, Goss CH, Plant WD, Bell SC (2013) Management of comorbidities in older patients with cystic fibrosis. *The Lancet. Respiratory medicine*, **1**, 164–74.

Pokorny A, Birkbeck TH, Almeida PFF (2002) Mechanism and kinetics of delta-lysin interaction with phospholipid vesicles. *Biochemistry*, **41**, 11044–56.

Pollard JE, Snarr J, Chaudhary V et al. (2012) In vitro evaluation of the potential for resistance development to ceragenin CSA-13. *The Journal of antimicrobial chemotherapy*, **67**, 2665–72.

Porter EM, van Dam E, Valore E V, Ganz T (1997) Broad-spectrum antimicrobial activity of human intestinal defensin 5. *Infection and immunity*, **65**, 2396–401.

Pütsep K, Carlsson G, Boman HG, Andersson M (2002) Deficiency of antibacterial peptides in patients with morbus Kostmann: an observation study. *Lancet (London, England)*, **360**, 1144–9.

Quinton PM (1990) Cystic fibrosis: a disease in electrolyte transport. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, **4**, 2709–17.

Raghuraman H, Chattopadhyay A (2007) Melittin: a membrane-active peptide with diverse functions. *Bioscience reports*, **27**, 189–223.

Ramsey BW, Davies J, McElvaney NG et al. (2011) A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. *The New England journal of medicine*, **365**, 1663–72.

Reddy KVR, Yedery RD, Aranha C (2004) Antimicrobial peptides: premises and promises. *International journal of antimicrobial agents*, **24**, 536–47.

Reen FJ, Flynn S, Woods DF et al. (2016) Bile signalling promotes chronic respiratory infections and antibiotic tolerance. *Scientific Reports*, **6**, 29768.

Rich DP, Anderson MP, Gregory RJ et al. (1990) Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature*, **347**, 358–63.

Riordan JR (2008) CFTR function and prospects for therapy. *Annual review of biochemistry*, **77**, 701–26.

Riordan JR, Rommens JM, Kerem B et al. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science (New York, N.Y.)*, **245**,

1066–73.

Rogers LA (1928) THE INHIBITING EFFECT OF STREPTOCOCCUS LACTIS ON LACTOBACILLUS BULGARICUS. *Journal of bacteriology*, **16**, 321–5.

Rogers GB, Hart CA, Mason JR, Hughes M, Walshaw MJ, Bruce KD (2003) Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. *Journal of clinical microbiology*, **41**, 3548–58.

Rogers GB, Shaw D, Marsh RL, Carroll MP, Serisier DJ, Bruce KD (2015) Respiratory microbiota: addressing clinical questions, informing clinical practice. *Thorax*, **70**, 74–81.

Rollins-Smith LA (2009) The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, **1788**, 1593–1599.

Römling U, Fiedler B, Bosshammer J, Grothues D, Greipel J, von der Hardt H, Tümmler B (1994) Epidemiology of chronic Pseudomonas aeruginosa infections in cystic fibrosis. *The Journal of infectious diseases*, **170**, 1616–21.

Roversi D, Luca V, Aureli S, Park Y, Mangoni ML, Stella L (2014) How many antimicrobial peptide molecules kill a bacterium? The case of PMAP-23. *ACS chemical biology*, **9**, 2003–7.

Rowe S, Miller S, E S (2005) Cystic Fibrosis. *N Engl J Med*.

Russell AD, Hugo WB (1994) Antimicrobial activity and action of silver. *Progress in medicinal chemistry*, **31**, 351–70.

Sai KP, Reddy PN, Babu M (1995) Investigations on wound healing by using amphibian skin. *Indian journal of experimental biology*, **33**, 673–6.

Saiman L, Anstead M, Mayer-Hamblett N et al. (2010) Effect of azithromycin on pulmonary function in patients with cystic fibrosis uninfected with Pseudomonas aeruginosa: a randomized controlled trial. *JAMA*, **303**, 1707–15.

Samuelsson G, Jayawardene AL (1974) Isolation and characterization of viscotoxin 1-Ps from Viscum album L. ssp. austriacum (Wiesb.) vollmann, growing on Pinus silvestris. *Acta pharmaceutica Suecica*, **11**, 175–84.

Sanders NN, Franckx H, De Boeck K, Haustraete J, De Smedt SC, Demeester J (2006) Role of magnesium in the failure of rhDNase therapy in patients with cystic fibrosis. *Thorax*, **61**, 962–966.

Sasirekha B, M. Megha D, S. Sharath M, Soujanya R (2015) Study on Effect of Different Plant Extracts on Microbial Biofilms. *Asian Journal of Biotechnology*, **7**, 1–12.

Sass V, Pag U, Tossi A, Bierbaum G, Sahl H-G (2008) Mode of action of human beta-defensin 3 against Staphylococcus aureus and transcriptional analysis of responses to defensin challenge. *International journal of medical microbiology : IJMM*, **298**, 619–33.

Sass V, Schneider T, Wilmes M et al. (2010) Human beta-defensin 3 inhibits cell wall biosynthesis in Staphylococci. *Infection and immunity*, **78**, 2793–800.

Sawyer JG, Martin NL, Hancock RE (1988) Interaction of macrophage cationic proteins with the outer membrane of Pseudomonas aeruginosa. *Infection and immunity*, **56**, 693–8.

Schibli DJ, Hunter HN, Aseyev V et al. (2002) The solution structures of the human beta-defensins lead to a better understanding of the potent bactericidal activity of HBD3 against Staphylococcus aureus. *The Journal of biological chemistry*, **277**, 8279–89.

Schuster FL, Jacob LS (1992a) Effects of magainins on ameba and cyst stages of Acanthamoeba polyphaga. *Antimicrobial agents and chemotherapy*, **36**, 1263–71.

Schuster FL, Jacob LS (1992b) Effects of magainins on ameba and cyst stages of Acanthamoeba polyphaga. *Antimicrobial agents and chemotherapy*, **36**, 1263–71.

Schuster A, Haliburn C, Döring G, Goldman MH, Freedom Study Group (2013) Safety, efficacy and convenience of colistimethate sodium dry powder for inhalation (Colobreathe DPI) in patients with cystic fibrosis: a randomised study. *Thorax*, **68**, 344–50.

Schuster ' FL, Jacob2 LS (1992) Effects of Magainins on Ameba and Cyst Stages of Acanthamoeba polyphaga. *ANTIMICROBIAL AGENTS AND CHEMOTHERAPY*, **36**, 1263–1271.

Scocchi M, Pallavicini A, Salgaro R, Bociek K, Gennaro R (2009) The salmonid cathelicidins: a gene family with highly varied C-terminal antimicrobial domains. *Comparative biochemistry and physiology. Part B, Biochemistry & molecular biology*, **152**, 376–81.

Scocchi M, Tossi A, Gennaro R (2011) Proline-rich antimicrobial peptides: converging to a non-lytic mechanism of action. *Cellular and molecular life sciences : CMLS*, **68**, 2317–30.

Seigneuret M, Devaux PF (1984) ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proceedings*

of the National Academy of Sciences of the United States of America, **81**, 3751–5.

Selsted ME, Ouellette AJ (2005) Mammalian defensins in the antimicrobial immune response. *Nature immunology*, **6**, 551–7.

Sengupta D, Leontiadou H, Mark AE, Marrink S-J (2008) Toroidal pores formed by antimicrobial peptides show significant disorder. *Biochimica et biophysica acta*, **1778**, 2308–17.

Severini C, Improta G, Falconieri-Erspamer G, Salvadori S, Erspamer V (2002) The tachykinin peptide family. *Pharmacological reviews*, **54**, 285–322.

Shafer WM, Qu X, Waring AJ, Lehrer RI (1998) Modulation of Neisseria gonorrhoeae susceptibility to vertebrate antibacterial peptides due to a member of the resistance/nodulation/division efflux pump family. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 1829–33.

Shepherd CM, Schaus KA, Vogel HJ, Juffer AH (2001) Molecular dynamics study of peptide-bilayer adsorption. *Biophysical journal*, **80**, 579–96.

Siddiqui R, Khan NA (2012) Biology and pathogenesis of Acanthamoeba. *Parasites & vectors*, **5**, 6.

Simmaco M, Mignogna G, Barra D (1998) Antimicrobial peptides from amphibian skin: what do they tell us? *Biopolymers*, **47**, 435–50.

Singh PK, Schaefer AL, Parsek MR, Moninger TO, Welsh MJ, Greenberg EP (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. *Nature*, **407**, 762–4.

Singh PK, Parsek MR, Greenberg EP, Welsh MJ (2002) A component of innate immunity prevents bacterial biofilm development. *Nature*, **417**, 552–5.

Soehnlein O, Kai-Larsen Y, Frithiof R et al. (2008) Neutrophil primary granule proteins HBP and HNP1-3 boost bacterial phagocytosis by human and murine macrophages. *The Journal of clinical investigation*, **118**, 3491–502.

Sriramulu DD (2005) Microcolony formation: a novel biofilm model of Pseudomonas aeruginosa for the cystic fibrosis lung. *Journal of Medical Microbiology*, **54**, 667–676.

Sriramulu DD, Lünsdorf H, Lam JS, Römling U (2005) Microcolony formation: a novel biofilm model of Pseudomonas aeruginosa for the cystic fibrosis lung. *Journal of medical microbiology*, **54**, 667–76.

Starner TD, Zhang N, Kim G, Apicella MA, McCray PB (2006) Haemophilus influenzae forms biofilms on airway epithelia: implications in cystic fibrosis. *American journal of*

respiratory and critical care medicine, **174**, 213–20.

Stec B (2006) Plant thionins--the structural perspective. *Cellular and molecular life sciences : CMLS*, **63**, 1370–85.

Steiner H (1982) Secondary structure of the cecropins: antibacterial peptides from the moth Hyalophora cecropia. *FEBS letters*, **137**, 283–7.

Steinert M, Birkness K, White E, Fields B, Quinn F (1998) Mycobacterium avium bacilli grow saprozoically in coculture with Acanthamoeba polyphaga and survive within cyst walls. *Applied and environmental microbiology*, **64**, 2256–61.

Stotz HU, Thomson JG, Wang Y (2009) Plant defensins: defense, development and application. *Plant signaling & behavior*, **4**, 1010–2.

Strother T, Hamers RJ, Smith LM (2000) Covalent attachment of oligodeoxyribonucleotides to amine-modified Si (001) surfaces. *Nucleic acids research*, **28**, 3535–41.

Stuart Elborn J, Geller DE, Conrad D et al. (2015) A phase 3, open-label, randomized trial to evaluate the safety and efficacy of levofloxacin inhalation solution (APT-1026) versus tobramycin inhalation solution in stable cystic fibrosis patients. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, **14**, 507–14.

Sutherland I (2001) Biofilm exopolysaccharides: a strong and sticky framework. *Microbiology (Reading, England)*, **147**, 3–9.

Tam JP, Lu Y-A, Yang J-L (2002) Correlations of cationic charges with salt sensitivity and microbial specificity of cystine-stabilized beta -strand antimicrobial peptides. *The Journal of biological chemistry*, **277**, 50450–6.

Tanabe H, Ayabe T, Maemoto A et al. (2007) Denatured human alpha-defensin attenuates the bactericidal activity and the stability against enzymatic digestion. *Biochemical and biophysical research communications*, **358**, 349–55.

Tängdén T (2014) Combination antibiotic therapy for multidrug-resistant Gram-negative bacteria. *https://mc.manuscriptcentral.com/ujms*.

Tappenden P, Harnan S, Uttley L, Mildred M, Carroll C, Cantrell A (2013) Colistimethate sodium powder and tobramycin powder for inhalation for the treatment of chronic Pseudomonas aeruginosa lung infection in cystic fibrosis: systematic review and economic model. *Health technology assessment (Winchester, England)*, **17**, v–xvii, 1-181.

Thwaite JE, Humphrey S, Fox MA et al. (2009) The cationic peptide magainin II is

antimicrobial for Burkholderia cepacia-complex strains. *Journal of Medical Microbiology*, **58**, 923–929.

Trapnell BC, McColley SA, Kissner DG et al. (2012) Fosfomycin/tobramycin for inhalation in patients with cystic fibrosis with pseudomonas airway infection. *American journal of respiratory and critical care medicine*, **185**, 171–8.

Travis SM, Anderson NN, Forsyth WR et al. (2000) Bactericidal activity of mammalian cathelicidin-derived peptides. *Infection and immunity*, **68**, 2748–55.

Tullis DE, Burns JL, Retsch-Bogart GZ, Bresnik M, Henig NR, Lewis SA, Lipuma JJ (2014) Inhaled aztreonam for chronic Burkholderia infection in cystic fibrosis: a placebo-controlled trial. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society*, **13**, 296–305.

Uttley L, Harnan S, Cantrell A, Taylor C, Walshaw M, Brownlee K, Tappenden P (2013) Systematic review of the dry powder inhalers colistimethate sodium and tobramycin in cystic fibrosis. *European respiratory review : an official journal of the European Respiratory Society*, **22**, 476–86.

Vogel HJ, Schibli DJ, Jing W, Lohmeier-Vogel EM, Epand RF, Epand RM (2002) Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides. *Biochemistry and cell biology = Biochimie et biologie cellulaire*, **80**, 49–63.

Wagner VE, Iglewski BH (2008a) P. aeruginosa Biofilms in CF Infection. *Clinical reviews in allergy & immunology*, **35**, 124–34.

Wagner VE, Iglewski BH (2008b) P. aeruginosa Biofilms in CF Infection. *Clinical reviews in allergy & immunology*, **35**, 124–34.

Wang G (2013) Database-Guided Discovery of Potent Peptides to Combat HIV-1 or Superbugs. *Pharmaceuticals (Basel, Switzerland)*, **6**, 728–58.

Wang Z, Wang G (2004) APD: the Antimicrobial Peptide Database. *Nucleic acids research*, **32**, D590-2.

Wang G, Li X, Wang Z (2009a) APD2: the updated antimicrobial peptide database and its application in peptide design. *Nucleic acids research*, **37**, D933-7.

Wang C-W, Yip B-S, Cheng H-T, Wang A-H, Chen H-L, Cheng J-W, Lo H-J (2009b) Increased potency of a novel D-beta-naphthylalanine-substituted antimicrobial peptide against fluconazole-resistant fungal pathogens. *FEMS yeast research*, **9**, 967–70.

Wang KF, Nagarajan R, Camesano TA (2014) Antimicrobial peptide alamethicin insertion

into lipid bilayer: A QCM-D exploration. *Colloids and Surfaces B: Biointerfaces*, **116**, 472–481.

Warhurst D (1968) Study of a Small Amoeba from Mammalian Cell Cultures Infected with "Ryan Virus." *J. gen. Microbiol*, **50**, 207–2.

Waters CM, Bassler BL (2005a) Quorum sensing: cell-to-cell communication in bacteria. *Annual review of cell and developmental biology*, **21**, 319–46.

Waters CM, Bassler BL (2005b) Quorum sensing: cell-to-cell communication in bacteria. *Annual review of cell and developmental biology*, **21**, 319–46.

Waters VJ, Ratjen FA (2014) Is there a role for antimicrobial stewardship in cystic fibrosis? *Annals of the American Thoracic Society*, **11**, 1116–9.

Wei G-X, Campagna AN, Bobek LA (2006) Effect of MUC7 peptides on the growth of bacteria and on Streptococcus mutans biofilm. *The Journal of antimicrobial chemotherapy*, **57**, 1100–9.

Wei G, de Leeuw E, Pazgier M et al. (2009) Through the looking glass, mechanistic insights from enantiomeric human defensins. *The Journal of biological chemistry*, **284**, 29180–92.

Welch WJ (2004) Role of quality control pathways in human diseases involving protein misfolding. *Seminars in cell & developmental biology*, **15**, 31–8.

Wertheim HFL, Vos MC, Ott A et al. (2004) Risk and outcome of nosocomial Staphylococcus aureus bacteraemia in nasal carriers versus non-carriers. *Lancet (London, England)*, **364**, 703–5.

Whiteley M, Bangera MG, Bumgarner RE, Parsek MR, Teitzel GM, Lory S, Greenberg EP (2001) Gene expression in Pseudomonas aeruginosa biofilms. *Nature*, **413**, 860–4.

Wieprecht T, Dathe M, Krause E, Beyermann M, Maloy WL, MacDonald DL, Bienert M (1997) Modulation of membrane activity of amphipathic, antibacterial peptides by slight modifications of the hydrophobic moment. *FEBS letters*, **417**, 135–40.

Wimley WC (2010) Describing the mechanism of antimicrobial peptide action with the interfacial activity model. *ACS chemical biology*, **5**, 905–17.

Wiradharma N, Khan M, Yong L-K, Hauser CAE, Seow SV, Zhang S, Yang Y-Y (2011) The effect of thiol functional group incorporation into cationic helical peptides on antimicrobial activities and spectra. *Biomaterials*, **32**, 9100–9108.

Xu Z, Zhong Z, Huang L, Peng L, Wang F, Cen P (2006) High-level production of bioactive human beta-defensin-4 in Escherichia coli by soluble fusion expression. *Applied*

microbiology and biotechnology, **72**, 471–9.

Yakandawala N, Gawande P V, Lovetri K, Madhyastha S (2007) Effect of ovotransferrin, protamine sulfate and EDTA combination on biofilm formation by catheter-associated bacteria. *Journal of applied microbiology*, **102**, 722–7.

Yang L, Harroun TA, Weiss TM, Ding L, Huang HW (2001) Barrel-stave model or toroidal model? A case study on melittin pores. *Biophysical journal*, **81**, 1475–85.

Yeaman MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance. *Pharmacological reviews*, **55**, 27–55.

Yeung ATY, Gellatly SL, Hancock REW (2011) Multifunctional cationic host defence peptides and their clinical applications. *Cellular and molecular life sciences : CMLS*, **68**, 2161–76.

Yin LM, Edwards MA, Li J, Yip CM, Deber CM (2012) Roles of hydrophobicity and charge distribution of cationic antimicrobial peptides in peptide-membrane interactions. *The Journal of biological chemistry*, **287**, 7738–45.

Yousuf FA, Siddiqui R, Khan NA (2013) Acanthamoeba castellanii of the T4 genotype is a potential environmental host for Enterobacter aerogenes and Aeromonas hydrophila. *Parasites & vectors*, **6**, 169.

Zanetti M (2004) Cathelicidins, multifunctional peptides of the innate immunity. *Journal of leukocyte biology*, **75**, 39–48.

Zasloff M (1987a) Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 5449–53.

Zasloff M (1987b) Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 5449–53.

Zasloff M (2002a) Antimicrobial peptides of multicellular organisms. *Nature*, **415**, 389–95.

Zasloff M (2002b) Antimicrobial peptides of multicellular organisms. *Nature*, **415**, 389–95.

Zasloff M, Martin B, Chen HC (1988) Antimicrobial activity of synthetic magainin peptides and several analogues. *Proceedings of the National Academy of Sciences of the United States of America*, **85**, 910–3.

Zhao H, Sood R, Jutila A, Bose S, Fimland G, Nissen-Meyer J, Kinnunen PKJ (2006) Interaction of the antimicrobial peptide pheromone Plantaricin A with model membranes: implications for a novel mechanism of action. *Biochimica et biophysica acta*, **1758**, 1461–74.

Zimmermann GR, Lehár J, Keith CT (2007) Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discovery Today*, **12**, 34–42.

De Zotti M, Biondi B, Peggion C, Formaggio F, Park Y, Hahm K-S, Toniolo C (2012) Trichogin GA IV: a versatile template for the synthesis of novel peptaibiotics. *Organic & biomolecular chemistry*, **10**, 1285–99.

Zumla AI, Gillespie SH, Hoelscher M et al. (2014) New antituberculosis drugs, regimens, and adjunct therapies: needs, advances, and future prospects. *The Lancet Infectious Diseases*, **14**, 327–340.

Zwaal RFA, Comfurius P, Bevers EM (2005) Surface exposure of phosphatidylserine in pathological cells. *Cellular and molecular life sciences : CMLS*, **62**, 971–88.

Appendices

Appendix 1: List of reagents and chemicals

7.1 List of reagents and chemicals

Dulbecco's Modified Eagle's Medium (DMEM)	(GibcoTM 61965026)
MEM Non-Essential Amino Acid Solution (100×)	(Sigma 7145)
Fetal Bovine Serum	(Fisher Scientific10117272)
Phosphate buffered saline (PBS)	(Sigma P4417)
Bacteriological agar	(Sigma A5306)
CLED Agar	(Sigma 55420)
Ringers solution	(Sigma 96724)
D-(+)-Glucose	(Sigma G8270)
Peptone Water	(Sigma 70179)
Yeast Extract	(Sigma 92144)
Trypsin-EDTA solution	(Sigma T4174)
Sodium chloride (NaCl)	(Sigma S7653)
Tryptone	(Sigma T7293)
Accutase® solution	(Sigma A6964)
Magnesium sulfate heptahydrate	(Sigma M1880)
Calcium chloride dihydrate	(Sigma 223506)

7.1.1 Antibiotics and antimicrobial agents

Silver nitrate (AgNO3)	(Sigma 792276)
Pleurocidin	(AnaSpec ANA64981)
Magainin 2	(AnaSpec ANA20639)
Vancomycin	(Sigma V2002)
Ciprofloxacin	(Sigma17850)

7.1.2 Reagent for Electron Microscopy

Sodium phosphate monobasic	(Sigma 74092)
Sodium phosphate dibasic	(Sigma 71653)
Glutaraldehyde solution	(Sigma G7526)
Sodium cacodylate trihydrate	(Sigma C0250)

7.1.3 Reagent for Fluorescence Microscopy

Rose Bengal	(Sigma 198250)
Lysotracker Green	(ThermoFisher L7526)

7.1.4 Kits

QIAamp DNA Micro Kit	(QIAGEN 56304)
QIAquick Gel Extraction Kit	(QIAGEN 28704)
Cytotoxicity Detection Kit (LDH)	(ROCHE 11644793001)

7.1.5 Reagents for flow cytometry

Annexin V-FITC	(BioLegend 640906)
Annexin V Binding Buffer	(BioLegend 422201)
Cell staining buffer	(BioLegend 420201)
Zombie NIR	(BioLegend 423106)

7.1.6 Reagents for PCR and gel electrophoresis

Agarose	(Fisher Scientific 10776644)
dNTP Mix	(Thermo ScientificTM R0241)
Taq DNA Polymerase	(Thermo ScientificTM EP0401)
SafeView Nucleic Acid Stain	(NBS-Biologicals NBS-SV1)
GeneRuler 1 kb DNA Ladder	(Thermo ScientificTM SM0311)

7.1.7 General reagents

Ethylenediaminetetraacetic acid (EDTA) (10×)	(Sigma E9884)
Acetone	(Fisher Scientific 10045800)
CelLytic™ M	(Sigma C2978)
Virkon	(Fisher Scientific 12358667)
Ethanol	(Fisher Scientific BP2818)
Dimethyl Sulfoxide (DMSO)	(Sigma D2650)

Appendix 2: List and preparation of buffers and cultured medium

7.2 List and preparation of buffers and cultured medium

7.2.1 Saline

7.2.1.1 Page's Amoeba Saline:

Solution1:

Sodium phosphate dibasic (Na_2HPO_4)	0.142 g
Potassium phosphate monobasic (KH_2PO_4)	0.136 g
DI Water	500.0 ml

- Autoclave at 121°C for 15 min

Solution2:

Magnesium Sulfate Heptahydrate ($MgSO_4.7H_2O$)	4.0 mg
Calcium chloride dehydrate ($CaCl_2.2H_2O$)	4.0 mg
Sodium chloride (NaCl)	0.120 g
Distilled Water	500.0 ml

- Autoclave at 121°C for 15 min
- Combine solutions 1 and 2 when cooled to room temperature.

7.2.1.2 Ringers solution 1/4 strength tablets: One tablet is sufficient for 500ml contained:

Calcium chloride	0.12 g/l
Potassium chloride	0.105 g/l
Sodium bicarbonate	0.05 g/l
Sodium chloride	2.25 g/l

Phosphate buffered saline (PBS) tablets: One tablet dissolved in 200 ml of distilled water giving 0.01 M phosphate buffer contained:

Potassium chloride	0.0027 M
Sodium chloride	0.137 M

- Autoclave at 121°C for 15 min. pH 7.4, at 25 °C

7.2.2 Human cell, amoebal, and bacterial culture medium

DMEM for A549 cells (v/v): DMEM 80 %, and FBS 10 %.

PYG (w/v): proteose peptone 0.75 %, yeast extract 0.75 %, and glucose 1.5 % distilled water prior to autoclaving 121°C for 15 min.

Luria Bertani broth (w/v): 10 g tryptone, 5 g yeast extract and 10 g NaCl in distilled water to a final volume of 1000 ml, adjusting the pH to 7.5 before autoclaving at 121°C for 15 min.

CLED (w/v): 36.15 g of CLED agar dissolved up to 1000 ml distilled water prior to autoclaving 121°C for 15 min.

Nutrient agar (w/v): 23 g of nutrient agar dissolved up to 1000 ml distilled water prior to autoclaving 121°C for 15 min.

Non-nutrient agar (w/v): 30 g bacteriological agar dissolved in to 1000 ml Ringers solution, or in Page amoeba saline prior to autoclaving 121°C for 15 min.

Trypsin 1x (v/v): 10 ml up to100 ml PBS.

10 % SDS (w/v): 10 g dissolved up to 100 ml distilled water.

7.2.3 Antibiotics and antimicrobial peptides

Vancomycin (w/v): stock solution: 1 mg/ml dissolved in distilled water

Ciprofloxacin (w/v): stock solution: 1 mg/ml dissolved in distilled water with 0.05mM HCl

Magainin II (w/v): stock solution: 1 mg/ml dissolved in distilled water.

Pleurocidin (w/v): stock solution: 1 mg/ml dissolved in distilled water.

Silver nitrate (w/v): stock solution: 10 mg/ml dissolved in distilled water.

7.2.4 Primers preparation

- Spin the tubes at top speed for 10 minutes to ensure that there are no lyophilized primers stuck to the cap.
- To make a typical 100 mM (100X) stock concentration of primers, dissolve the primers in a volume of sterile distilled water that is 10X the amount of nmoles in the tube, using µL of water. This value is printed on the side of the tube. For example, if your tube contains 53.4 nmoles of primer, then you would dissolve using 534 µL of water. This will now be at a 100 µM concentration.

i.e. 53.4 nmoles of primer dissolve in 534 µl of water =(53.4 nmoles/l) (x moles/534 x 10-6 l) = 0.0001 mole/l = 0.0001 M = 0.1 mM=100 µM 3. To prepare primers for use: Dilute this stock 1:10, to give a concentration of 10 µM.

4. Store all sets of primers at -20°C.
5. Typically use 0.1-1.0 µM final concentrations in a PCR mix. i.e. if your reaction volume is 25 mL, then using 2.5 µL for each separate primer, will give you a final concentration of 1µM.

7.2.5 Disinfections used in the laboratory:

1 % virkon (w/v): 1 mg of virkon powder in 100 ml PBS

70 % ethanol (v/v): 70 ml of concentrated ethanol in 100 ml H_2O

Appendix 3: List of cell lines; microorganisms; storage and thawing methods

7.3 List of cell lines; microorganisms; storage and thawing methods

7.3.1 List of Cell lines

A549 cells (adenocarcinomic human alveolar basal epithelial cells)

TERT-NHUC (hTERT-immortalised normal human urothelial cells)

7.3.2 *Acanthamoeba* strain

Acanthamoeba castellanii T4 (ATCC 30234)

7.3.3 Storage method for cells and *Acanthamoeba*

Cells and *Acanthamoeba* were stored as axenic cultures at -80 °C and liquid nitrogen. Briefly, growing cells or amoebae were re-suspended at a density of 1-5 x 10^6 cells/ml in the freezing medium [90 % FBS, and 10 % DMSO (v/v) for human cells; 90 % PYG, and 10 % DMSO (v/v) for *Acanthamoeba*] and placed into a cryotube. The freezing process were done gradually in ice, then -20 °C for 60 minutes followed by their storage at -80°C freezer. Some of cryotubes were transferred after overnight incubation in -80 °C to store in liquid nitrogen.

7.3.4 Thawing method for cells and *Acanthamoeba*

Thawing and recovery of stored cells and *Acanthamoeba* from both liquid nitrogen and -80 °C freezer was done quickly. The cryotubes were placed immediately in the wormer incubator 37 °C for 3 minutes after taken out from liquid nitrogen or -80 °C freezer. Cells or *Acanthamoeba* were transferred into a 15 ml tube with warmed medium (DMEM for A549 cell line, KGM for TERT-NHUC, and PYG for *Acanthamoeba*) and centrifuged at 2000 x *g* for 5 minutes. Next, supernatants were discarded, then pellets were resuspended in owned medium (DMEM for A549 cell line, KGM for TERT-NHUC, and PYG for *Acanthamoeba*) and transferred into a T25 tissue flasks. Flasks were incubated at 37 °C, 5 % CO_2 and 95 % humidity for A549 and TERT-NHUC cells or 30 °C air incubator for *Acanthamoeba* until the cells reached an enough number to passage.

7.3.5 Bacterial strains

Clinical isolates of MSSA, *S. epidermidis, Str. pneumoniae, E. coli, H. influenzae* and *P. aeruginosa* were generously provided by Dr Tony Elston from Colchester General Hospital.

7.3.6 Storage of bacterial cultures

By using a disposable sterile loop, about 20-30 isolated colonies of bacteria were picked up from the agar plate and inoculated in a 10 ml of bacterial freezing medium [70 % Luria-Bertani broth and 30 % glycerol (v/v)]. Then, the mixture of freezing medium with bacteria were divided into cryotubes final volume of each tube was 0.5 ml and stored in a -20 °C freezer.

7.3.7 Thawing and re-culturing bacteria

To re-culture bacteria, a disposable sterile loop was inserted into the frozen cryotube and then streaked on fresh agar plate and incubated at 37 °C air incubator overnight. After the incubation, the plate was carefully examined if the colonies have similar morphologies such as textures, colors and border shapes and stored at 4 °C for up to a month.

Poster and Oral Presentations

The Microbiology Society Annual Conference 2016, Liverpool, 21–24 March. Poster presentation: “An investigation of the effects of an antimicrobial peptide on the survival of *Acanthamoeba* and intracellular bacteria associated with Cystic Fibrosis” B. Tashmukhambetov, G. Mitchell and S. Alsam

3Rd Research Forum For Central Asia 2016, University of Cambridge, 11 March. Oral presentation: “Complications in genetic diseases such as Cystic Fibrosis “ B. Tashmukhambetov, G. Mitchell and S. Alsam

The Molecular and Cellular Bioscience Group (MCBG) Seminar 2015, 9 July. Oral presentation: “ Using antimicrobial peptides to fight bacterial infections in Cystic Fibrosis lung” B. Tashmukhambetov, G. Mitchell and S. Alsam

13th Annual Graduate Forum 2014, Essex University, 10th September. Oral presentation: “Interaction of *Acanthamoeba* and bacteria associated with CF” B. Tashmukhambetov, G. Mitchell and S. Alsam

Bolashak Educational Forum 2014, University of Cambridge, 29 April. Oral presentation: “Contemporary issues within the educational system in Kazakhstan” B. Tashmukhambetov