Unravelling a food derived bioactive peptide with dual functionality of antimicrobial and immunomodulatory capabilities

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Declaration

I, Niamh Máire Monica Mohan, certify that the experiments recorded herein represent my own work, unless otherwise stated in the text, and have not been previously presented for a higher degree at this or any other University. This thesis will remain on stay until 2025 after which time it may be lent at the discretion of the Librarian at Trinity College Dublin.

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Summary

In this thesis, a computational data-mining and feature based approach to unravel the abundance of unexplored peptides from nature and to identify potent, novel antimicrobial peptides (AMPs) from phyto-proteins is proposed. In the first section of this thesis, we explored *Pisum sativum* hydrolysed (PSH) protein and evaluated its potential as a bio-preservative. As the "clean label" revolution takes hold, paired with increased consumer demand for natural products, alternative preserving solutions are needed to maintain food safety and limit spoilage. PSH offers an attractive plant-based solution, capable of reducing disease causing pathogens while being economically viable, and environmentally friendly. Expanding on the potential of the PSH, we identified NuriPep 1653, an individual peptide component of the hydrolysate with remarkable antimicrobial activity. AMPs are an integral part of every species contributing to host defence and have been lauded as contenders in combatting the antibiotic crisis. Despite immense scientific efforts to bring antibiotic alternatives to the market, very few have been approved for market sale as they have yet to surpass the activity of traditional therapies. Paired with the reluctance from the pharmaceutical industry to financially support antimicrobial research, this means that the need for novel antimicrobial compounds has never been greater. We have explored the therapeutic potential of NuriPep 1653 against clinically relevant pathogens and highlighted its appeal for the pharmaceutical sector given its limited propensity for inducing resistance, rapid kill kinetics, non-toxic nature, ability to reverse resistance and synergise with conventional antibiotic therapies. Moreover, NuriPep 1653 reduced the activation of pathways and cytokines associated with pro-inflammatory response indicating its abilities to modulate the host immune response. This vastly expands the therapeutic potential of the peptide. Overall, we describe a novel discovery approach to identify AMPs encrypted within phyto-proteins. Through PSH and NuriPep 1653, we hope to expand on the current knowledge related to protein hydrolysates and AMPs and inch closer to providing novel antimicrobial solutions across the food and pharmaceutical industries.

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Publications and Conferences

Publications

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List of Abbreviations

°C	Degrees Celsius
3D	3 Dimensional
AMPs	Antimicrobial Peptides
ATCC	American Tissue Culture Collection
a_w	Water Activity
BARDA	Biomedical Advanced Research and Development Authority
BCA	Bicinchoninic acid
BHI	Brain Heart Infusion
BSA	Bovine serum Albumin
$CaCl_2$	Calcium Chloride
CAMP	Collection of Anti-Microbial Peptides
CARB-X	Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator
CE	Complete Elimination
CFU	Colony Forming Unit
CLSI	Clinical Laboratory Standards Institute
ColRAB	Colistin Resistant *Acinetobacter baumannii*
ColSAB	Colistin Susceptible *Acinetobacter baumannii*
DH	Degree of Hydrolysis
dH2O	Distilled Water
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide.
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
ESKAPE	*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter species*
EtOH	Ethanol
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FBS	Foetal Bovine Serum

FDA	Food and Drug Administration
FFC	Foods with Functional Claims
FHI	Food for Health Ireland
FIC	Fractional Inhibitory Concentration
Fig	Figure
Fmoc	9-fluorenylmethoxycarbonyl
FOSHU	Food for Specified Health Use
FSC	Forward Scatter
FWHM	Full Width Half Maximum
GFP	Green Fluorescent Protein
GI	Gastrointestinal
GLP-1	Glucagon Like Peptide - 1
GRAS	Generally Regarded as Safe
HBD1/2/3	Human Beta Defensin 1/2/3
HDMS	Hexamethyldisilisane
HPLC	High Performance Liquid Chromatography
HUS	Haemolytic Urea Syndrome
IEC	Ion Exchange Chromatography
IFN-γ	Interferon gamma
IL	Interleukin
IMI	Innovative Medicines Initiative
I-TASSER	Iterative Threading ASSEmbly Refinement
IκB	Inhibitor kappa-light-chain-enhancer of activated B-cells
JPIARM	Joint Programming Initiative on Antimicrobial Resistance
kDa	Kilodalton
kV	Kilovolts
LC	Liquid Chromatography
LPS	Lipopolysaccharide
MAST	Motif Alignment Search Tool
MBC	Minimum Bactericidal Concentration
MDR	Multi Drug Resistant
MHB	Mueller Hinton Broth
MHC	Major Histocompatibity Complex
MIC	Minimum Inhibitory Concentration
mm-Hg	milligrams of Mercury

MOI	Multiplicity of Infection
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor
MWCO	Molecular Weight Cut Off
NaCl	Sodium Chloride
NB	Nutrient Broth
NCBI	National Centre for Biotechnology Information
ND4BB	New Drugs for Bad Bugs
NF-κB	Nuclear Factor Kappa B
NK	Natural Killer
NT	Not Tested
OD	Optical Density
P:L	Peptide to Lipid Ratio
PBNS	Phosphate Buffer No Salt
PBS	Phosphate Buffer
PDR	Pan Drug Resistant
pH	Potential Hydrogen
Pi	Propidium Iodide
PI	Isoelectric Point
PMA	phorbol 12-myristate 13-acetate
PRR	Pattern Recognition Receptors
PSH	*Pisum sativum* Hydrolysate
PTMs	Post Translational Modifications
QSAR	Quantitative Structure Activity Relationship
R	Resistant
RBC	Red Blood Cells
REMC	Replica-Exchange Monte Carlo simulation
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute
rPsd1	Recombinant *Pisum sativum* defensin1
SD	Standard Deviation
SEAP	Secreted Embryonic Alkaline Phosphatase
SEC	Size Exclusion Chromatography

SEM	Scanning Electron Microscopy
SPE	Solid Phase Extraction
SSC	Side Scatter
SVM	Support Vector Machine
Tc	Cytotoxic T cells
Th	Helper T cells
TLR	Toll Like Receptor
TNF-α	Tumour Necrosis factor alpha
Treg	T regulatory cells
t-SNE	t-Distributed Stochastic Neighbour Embedding
UF	Ultra Filtration
USD	United States Dollars
V/V	Volume by volume
W/V	Weight by volume
WHO	World Health Organisation
XDR	Extensively Drug Resistant
ZOI	Zone of Inhibition

List of Amino Acids

Amino Acid	3-Letter Abbreviation	1-Letter Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Alanine	Ala	A
Arginine	Arg	R

Chapter 1

General Introduction

1.1 *Protein Hydrolysates*

1.1.1 Background and existing state of the art

Proteins have an essential role as a macronutrient in the diet of humans and many animals, providing essential amino acids for basic nutrition and energy in the body. Additionally, proteins can be a source of physiologically active components and encrypted bioactive peptides which are contained within their amino acid sequence (Chalamaiah, Yu, and Wu 2018). These peptides, 3-50 residues in length, are dormant in their intact form within the parent protein, however can exhibit vast functionalities which are beneficial for health when cleaved and activated by chemical/physical hydrolysis, microbial fermentation and/or digestion by endogenous proteases (Cesar Lemes *et al*. 2016; Bhat, Kumar, and Bhat 2015; Garcia *et al*. 2013). When compared to whole proteins, protein hydrolysates which contain a complex mixture of low molecular weight oligopeptides, peptides and free amino acids, are more digestible and bioavailable with anticancer, antihypertensive, antioxidant, opiate, antimicrobial and immunomodulatory activities widely reported (Chalamaiah, Yu, and Wu 2018; Bhat, Kumar, and Bhat 2015; M. Nasri 2017).

1.1.1.1 Chemical/Physical Hydrolysis

Peptides consist of two or more amino acids linked by peptide bonds, which form by the removal of one water molecule. In the case of chemical hydrolysis, these bonds are cleaved with acid or alkali solutions. Proteins from feather, bristles, horns and beaks are commonly hydrolysed using acidic or alkaline processes due to their tough keratin composition (V. K. Pasupuleki and Braun 2010). The adverse effects of the chemicals involved make this non-environmentally friendly hydrolysis method unfavourable. In addition, reproducibility can be difficult with this nonspecific treatment and it can result in highly variable cleavage products with reduced activity (M. Nasri 2017). Physical hydrolysis can be achieved with heat treatment leading to random protein hydrolysis, similar to chemical hydrolysis. Amino acids such as cysteine and methionine are sensitive to oxidation and strong acids can damage serine and threonine residues (Bucci and Unlu 2000). These are some of the reasons that limit the application of chemical hydrolysis.

1.1.1.2 Microbial Hydrolysis - Fermentation

In microbial hydrolysis, bacteria, yeasts or moulds are grown on protein substrates, and as they create biomass, they secrete proteolytic enzymes to breakdown the proteins into fragments, *i.e.* peptides. Microbial fermentation is common in the dairy industry as the microorganisms used in the production of cheese and milk, for example are highly proteolytic (M. Nasri 2017; Hannu Korhonen and Pihlanto 2006). By varying the strain used, the protein source and the length of microbial fermentation, as well as other environmental parameters (potential hydrogen (pH), temperature, dissolved oxygen, agitation), the level of hydrolysis and functionalities of the products can differ significantly (Banan-Mwine Daliri, Oh, and Lee 2017). In one study, it was shown that when milk was fermented with 37 different *Lactobacillus helveticus* sub strains, each sample yielded different peptide contents and degrees of hydrolysis after fermentation (Y. Chen *et al.* 2014). Microbial hydrolysis with bacterial combinations have also been proposed for process optimisation (Chaves-López *et al*. 2014). Microbial fermentation is attractive as it can generate hydrolysates at an industrial scale and the peptides can be purified without further hydrolysis (Duarte, Vinderola, Ritz, Perdigón, and Matara 2006; M. Nasri 2017). The main disadvantages of the process is the low yield of product which can end up as a carbon/nitrogen source for microbial growth during the fermentation process (M. Nasri 2017).

1.1.1.3 Enzymatic Hydrolysis

Enzymatic hydrolysis with exogenous enzymes is the most convenient and widely used method to produce protein hydrolysates with biological functions from animal products (*e.g*. casein and meat) and plant ingredients (*e.g*. soy, wheat and pea) (V. K. Pasupuleki, Holmes, and Demain 2010; Kristinsson and Rasco 2000). The pH and temperatures used during enzymatic hydrolysis are less harsh than the ones used in chemical production, therefore the process can be better controlled from start to finish in an ecologically friendly way making it suitable for food and pharmaceutical applications (S. W. Kim and Wijesekara 2010). Other benefits of this method include short reaction time, high efficiency, low complexity, ease of scalability, predictability and reproducibility based on specific cleavage, as the cutting patterns of various proteases and enzymes are known (Tu *et al*. 2018). The choice of enzyme is a very important consideration as shown by Zhang *et al*. where rice bran hydrolysed with subtilisin showed improved activity over cysteine endopeptidase, papain and pepsin (H. Zhang, Yokoyama, and Zhang 2012). Several findings suggest that peptides > 10 kilodalton

(kDa) are likely to have better functionalities as antioxidants and antihypertensives (García-Tejedor *et al.* 2014; Fernández- Musoles *et al*. 2013; Wattanasiritham, L Theerakulkait, C Wickramasekara, Maier, and Stevens 2016). Compared with well-studied non-ribosomally synthesised bioactive peptides (Cesar Lemes *et al*. 2016), food derived protein hydrolysates show more diversity in structure, sequence and mode of action (M. Nasri 2017). In this way, enzymatic hydrolysis can lead to the production of novel bioactive peptides and facilitate non-traditional discovery of bioactive compounds. As this is the method used for the production of hydrolysates and bioactive peptides in this work, it will be discussed in further detail below.

1.1.2 Factors Influencing Enzymatic Hydrolysis

The main variables influencing the potency and activities of enzymatically hydrolysed peptides are: the nature of the protein source; the specificity of the cutting patterns; and the hydrolysis procedure (Li Chan 2015; van der Ven *et al.* 2002). These are discussed in detail below.

1.1.2.1 Protein Source

Animal, plant and marine sources have all been explored as sources of protein hydrolysates (Bhat, Kumar, and Bhat 2015; He, Liu, and Ma 2013). Among the most investigated protein substrates are milk, whey, egg, fish, corn, soy and pea (Chalamaiah, Yu, and Wu 2018). Recently, there has been a surge in "greener" sources, *i.e.* using waste by-products from agri-industries, such as microalgae (Reyna-Martinez, R. Gomez-Flores, López-Chuken, U. Quintanilla-Licea, R. Caballero-Hernandez, and Rodríguez-Padilla, C. Beltrán-Rocha, J.C. Tamez-Guerra 2018), soy meal (Rayaprolu, S.J. Hettiarachchy *et al*. 2013), fish waste (Slizyte *et al*. 2016) and feathers (Fontoura *et al*. 2014) to replace non-renewable sources of protein for hydrolysis. These proteinaceous waste products are normally discarded or used in low economic value animal feeds. Instead, value added products could be created by hydrolysing these under-used sources and discovering peptide hydrolysates with health promoting properties and various other functionalities (M. Nasri 2017).

1.1.2.2 Choice of Proteolytic Enzyme

The second most important consideration in the production of protein hydrolysates is the choice of exogenous enzyme and its specificity. The enzyme cutting pattern directly affects the length of the peptides as well as the amino acid composition, which are both essential aspects influencing bioactivity. The more specific an enzyme, the longer the peptide fragment released. A series of enzymes are available from plant, animal and microbial sources and the choice should depend on the final application, as only certain enzymes are permitted if the hydrolysate is to be consumed (Simpson *et al*. 1998). Understanding enzyme-cutting patterns is essential in order to predict where proteolysis will occur in the protein and the peptides that will be released as a consequence. The cleavage patterns of a few enzymes are well understood. For example, the gastrointestinal (GI) enzyme trypsin specifically cuts at the carboxyl-terminal of arginine and lysin residues while chymotrypsin cleaves on the carboxyl end of aromatic and hydrophobic side chains such as phenylalanine, tyrosine, and tryptophan (M. Nasri 2017). Similarly, Alcalase® is frequently employed in the production of protein hydrolysates and it also cuts peptide bonds on the C-terminal of hydrophobic residues (M. Nasri 2017). Combining enzyme treatments with different specificities will cleave at various sequence sites of the protein, releasing a mixture of peptides of varying length and sequences. Given that protein hydrolysates contain a mixture of sometimes thousands of peptides, it can often be that the activities observed are a result of synergies within the matrix. In this context, when papain and trypsin were combined to generate egg white lysozyme hydrolysates, the bioactivity of the product was greater than hen produced with either enzyme alone (Memarpoor-Yazdi, Asoodeh, and Chamani 2012). A number of bio-informatics *in silico* digestion tools such as PeptideCutter[1], BIOPEP[2] and Enzyme Predictor[3] are being designed in order to aid the prediction of potential cleavage sites of proteases in a given protein sequence (Tu *et al*. 2018). The role of bioinformatics in the future of protein hydrolysates will be discussed in a later section.

[1] https://web.expasy.org/peptide_cutter/

[2] http://www.uwm.edu.pl/biochemia/index.php/en/biopep

[3] http://bioware.ucd.ie/~enzpred/Enzpred.php

1.1.2.3 Hydrolysis Conditions

There are a number of parameters during the hydrolysis process, such as the reaction time, temperature, pH for optimal enzyme activity and the enzyme to substrate ratio, which influence the degree of hydrolysis (DH). The DH ultimately alters hydrolysate activity as it affects the length and amino acid sequence of the peptides. Increasing the enzyme to substrate ratio accelerates the hydrolysis process which in turn produces shorter peptide fragments (M. Nasri 2017). Having a stringent control on the hydrolysis conditions is therefore essential for the production of reproducible hydrolysates while retaining bioactivity (R. Nasri *et al.* 2014).

A general hydrolysis process is outlined below in Figure (Fig) 1.1. The hydrolysis time can vary from 4 – 48 hours after which time the enzyme(s) are inactivated by heat treatment or pH variation to terminate the reaction (Hou *et al.* 2017). The resulting protein hydrolysates are comprised of a complex mixture of peptides with varying length and amino acid composition. As a result, hydrolysates often present multi-functional activity. Post hydrolysis, the insoluble portion is removed *via* centrifugation or filtration (Pasupuleti and Braun, 2008). This process can be repeated multiple times to purify the protein hydrolysate. Following this, salts may be removed using solid phase extraction (SPE), ion exchange chromatography (IEC), desalting columns or ultrafiltration (UF).

Specific individual peptides, which are present in low concentrations, are often responsible for imparting beneficial physiological activities. These bioactive sequences can undergo fractionation based on molecular weight, charge and hydrophobicity to concentrate the functional portion (Lafarga and Hayes 2016). Techniques employed to purify individual peptides or to enrich the hydrolysate mixture include: size exclusion chromatography (SEC); IEC; liquid chromatography (LC); and UF using different molecular weight cut off (MWCO) membranes. The final hydrolysate can be pasteurised to limit microbial spoilage and then either spray or freeze dried. Mass Spectrometry (MS) are then commonly used to identify and confirm the presence of the bioactive peptides within the mixture (Swietlow and Lax 2004).

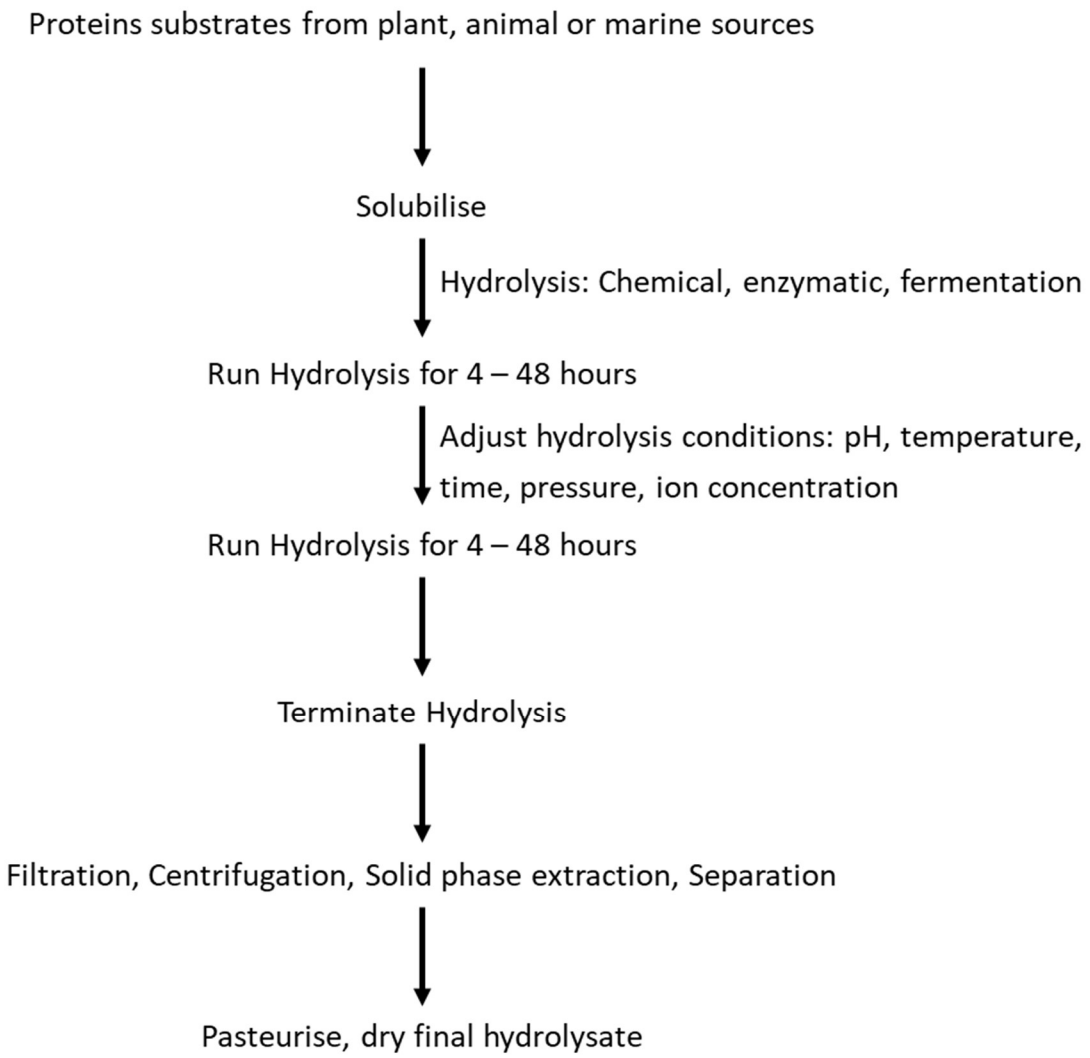


Figure 1.1 Hydrolysis process. General procedures for the generation of peptides from plant, animal or marine sources through chemical, enzymatic, or microbial hydrolysis. The hydrolysis process may need to be modified for peptide production, depending on the sources of the protein and on product specifications (Adapted from Hou *et al.* 2017).

1.1.3 Applications of Protein Hydrolysates

The diverse mixture of peptides contained within the hydrolysed proteins of plants and animals have been extensively researched and described to exhibit an array of physiological and regulatory functions (López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar 2014). These bioactive peptides can aid human health across a spectrum of disorders from obesity, to infectious disease, cancer and diabetes. Banan-Mwine Daliri *et al* reviewed the physiological effects of protein hydrolysates across various diseases and highlight examples of specific sequences, unlocked from parent proteins which exert potent activities (Banan-Mwine Daliri, Oh, and Lee 2017).

Beyond this, given their proteinaceous nature, hydrolysates provide a rich source of amino acids for basic nutrition and metabolism in the body. They have been beneficial in maintaining the nutritional status of individuals or patients whose needs are not met by conventional foods in their daily diet (Clemente 2000). Many milk protein hydrolysates such as casein and whey feature in sports nutrition as supplements to maximise skeletal muscle protein anabolism (Manninen 2009). When used in animal feed, protein hydrolysates were shown to significantly impact the intestinal health, growth and production performance of the animals (McCalla, Waugh, and Lohry 2010). This way attributed to the rate of absorption and catabolism of small peptides; the balanced composition of amino acids in the portal vein; and the physiological bioactivities the sequences can possess, as previously described.

The biological functions of hydrolysates may span beyond human health. Hydrolysates with antimicrobial activity have the potential to be used as alternatives to chemical antimicrobial agents in controlling the spoilage of food. Osman *et al.* describe the production of lupin protein hydrolysates generated by Alcalase® and its potential use as a bio-preservative in an infected minced meat model (Osman, El-Araby, and Taha 2016). This is the focus of a chapter in this work and therefore will be discussed in more detail at a later stage.

1.1.4 Protein Hydrolysates – Application in this Study

In this study we used the common pea (*Pisum sativum*) as a protein substrate. Peas are one of the most widely consumed legumes in the world. In the last 10 years, pulses have gained significant attention as sources of non-bitter bioactive hydrolysates (Humiski and Aluko 2007; Luna-Vital *et al.* 2015). As pea storage proteins are responsible for the generation of many host defence peptides (Meneguetti *et al*. 2017), it is not surprising that some of the most potent AMPs identified in this study were from the P54 storage pea protein. Moreover, it has been shown that pea storage proteins can have a non-allergenic character and good functional properties, therefore these are a major source of interest for generating bioactive compounds (Barac *et al*. 2012).

Breakthroughs in antimicrobial research and the discovery of novel natural bactericidal compounds need to be established quickly if the next generation are to withstand the mounting threat of multi drug resistant (MDR) pathogens. These "superbugs" have emerged through the uncontrolled use and abuse of antibiotics and represent a danger to human health in the form of infectious disease, impacting the host immune system, as well as through contamination in cosmetics, pharmaceuticals and the food industry (Sandberg 2011). Numerous groups have documented the successful generation of antimicrobial hydrolysates and peptides from sources such as beans (Salas *et al.* 2015), haemoglobin (Nedjar-Arroume *et al*. 2006), chicken lysozyme (Pellegrini *et al*. 1997), whey (Demers-Mathieu *et al.* 2013) and fish (Guinane *et al.* 2015). In many cases, the peptides linked with conferring bioactivity exhibit properties such as positive charge, medium length, hydrophobic residues and ability to adopt an alpha-helix structure (M. Nasri 2017). These features will be described in more detail in the following section.

Given their potential bioactivities beyond basic nutrition, natural, cheap sustainable source, environmentally friendly process, and general safety profile, pea hydrolysates and peptides may have value across a number of industries such as a bio-preservative against pathogens and spoilage organisms in food (Sandberg 2011). The MDR epidemic must be solved with a modern-day solution, therefore the use of computational approaches and *in silico* tools may help to revolutionise drug discovery and maximise our knowledge and realise the potential of natural compounds around us, how they affect bacteria, and how they can influence the host immune system (Sandberg 2011).

1.2 Antimicrobial Peptides

1.2.1 History of Antimicrobial Peptides

AMPs can be found across the evolutionary spectrum and are essential for proper functioning in all classes of life (Yeaman and Yount 2003a). The activity of the first human antimicrobial enzyme, lysozyme, was shown almost 100 years ago by Alexander Fleming (Nakatsuji and Gallo 2012). In 1928, his work inadvertently led to the discovery of penicillin which was further developed by Florey, Chain and Heatley into the first commercial antibiotic which was instrumental in saving lives during World War II. Interestingly, the ancient Egyptians had a practise whereby infected wounds were treated with a poultice of mouldy bread (American Chemical Society International Historic Chemical Landmarks 2017). It is likely that they were aware of the antimicrobial properties of the common bread mould long before Fleming, however it was he who was accredited with the discovery of penicillin. Over the next 20 years, the antibiotic era flourished. It wasn't until the emergence of multidrug resistant bacteria in the late 1960's that interest in studying host defence molecules as clinically useful antimicrobials was revived. As shown in Fig. 1.2, research into the effects of AMP as powerful compounds in controlling microbial populations has progressively increased for the last decade.

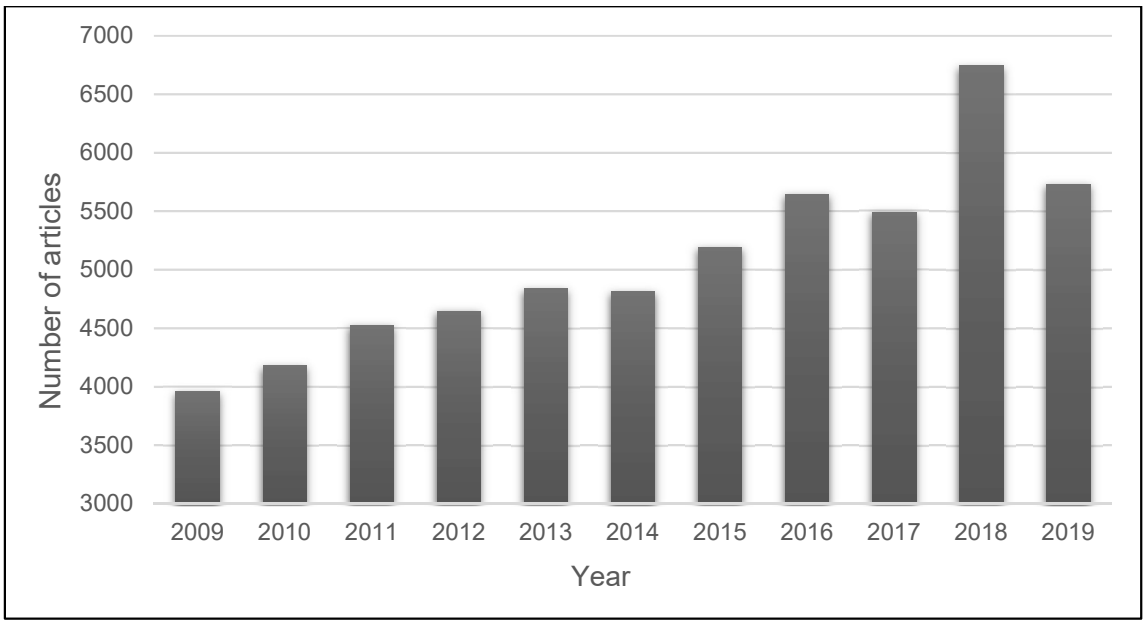


Figure 1.2 Published research on AMPs identified from 2009 until September 2019. Article counts were carried out after searching in PubMed using the following key words: "antimicrobial peptides", "AMPs", and/or "host defence peptides". The search results demonstrate that in the last decade the AMP research field has progressively expanded as represented by the continuous increase in the number of published articles. Note: The search for 2019 is inclusive of January – September, given the date of submission. (Search conduced by NM. Mohan).

Plants, frogs and moths were the main sources of initial AMP discoveries (Hultmark *et al.* 1980; Michael Zasloff 1987). Tissues and organs such as the skin are a rich source of peptides as they are the primary barrier to airborne pathogens and biological threats (Bahar and Ren 2013). AMPs have evolved as integral components in recruiting and promoting aspects of the innate immune system involved in anti- and pro-inflammatory action (A. L. Hilchie, Wuerth, and Hancock 2013). This will be discussed in a later section. To date, there are more than 3,088 AMP entries from 6 kingdoms in the Antimicrobial Peptide Database[4] however; thousands remain unidentified as this list corresponds to peptides found in only a tiny proportion of the antimicrobial proteins found in nature so far. Furthermore, while thousands of AMPs have been characterised with diverse sequences, these peptides have been grouped into only a few classes. This suggests that a bias may exist within the structure-

[4] http://aps.unmc.edu/AP/main.php

activity relationships when classifying AMPs into families (Yeaman and Yount 2003a). This is most prevalent among plant AMPs where defining features separating families are few. It may be the case that this "tunnel vision" regarding certain features is the reason behind that so few AMPs from plants have been identified to date. With divergent thinking and advances in technology, many new families of peptides may be discovered in the future.

1.2.2 Characteristics and Physicochemical Properties of Antimicrobial Peptides

The AMPs described in literature are generally hydrophobic, have between 2 and 50 amino acids in their sequence and typically have a global positive charge. AMPs are diverse in nature and this umbrella term covers a wide range of peptides that have one main common factor, they have been shown to inhibit the growth or completely kill a microorganism. Structural classification of AMPs includes 3 main groups: α-helical, which are abundant in extracellular fluids and only adopt their conformation when in contact with hydrophobic bacterial membranes; β-sheets, which usually contain disulphide bonds and an amphipathic composition with distinct hydrophilic and hydrophobic portions in their sequence; and extended peptides, which rather than a structural motif, are enriched in specific amino acids such as histidine, arginine, glycine or tryptophan (LJ Zhang and Gallo 2016).

Outside structural characterisation, AMPs can be described according to their charge as cationic or anionic; by their radial composition as aromatic; by their global hydrophobicity or based on their synthesis, ribosomally or non-ribosomally. Ribosomal peptides are synthesised by translation of messenger ribonucleic acid (mRNA) and are often proteolytically cleaved from larger precursor proteins to release the bioactive form. Bacteriocins are one such example. Unlike non-ribosomal peptides, usually, they cannot contain residues outside the 20 proteinogenic amino acids utilised by the ribosome since they are translated. However, they can contain post-translational modifications (McIntosh, Donia, and Schmidt 2009). Some AMP are constitutively expressed, such as the human beta defensin 1 (HBD1), while others are induced by chemical, biological of physical stresses, such as HBD2 and HBD3 (LJ Zhang and Gallo 2016). Non-ribosomal peptides are secondary metabolites assembled by enzymes such as the commercial antibiotic, polymyxin.

At present, little is known on if relationships exist between structural groups of AMPs and their mode of action or range of targeted cells (Jenssen, Hamill, and Hancock 2006). Buforin and magainin 2 are not dissimilar in structure, however, while the former targets deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), the latter acts on the cell

membrane (Kobayashi et al. 2000; Park *et al.* 2000). Regardless of class and structural diversity, AMPs share a few important physicochemical properties contributing to their bioactivity namely, size, charge, hydrophobicity and amphipathicity and solubility (Tossi, Sandri, and Giangaspero 2000).

1.2.2.1 Length

The length of a peptide can have a direct correlation with its 3 dimensional (3D) structure and activity as 7-8 residues are required for the formation of amphipathic peptide structures with alternating hydrophobic and hydrophilic amino acids (Bahar and Ren 2013). Peptides classed as β-sheets need at least 8 amino acids for this structure to form and approximately 22 amino acids are required before an AMP can penetrate the bacterial lipid bi-layer (Westerhoff *et al.* 1989). AMP length can also determine its cytotoxicity. Often "daughter" peptides, which are shorter analogues of the original sequence, can be less toxic. This is the case for a fragment of the bee venom peptide melittin, which is 11 residues shorter than its parent sequence, yet is 300 times less haemolytic (Subbalakshmi, Nagaraj, and Sitaram 1999). With that said, the melittin analogue displayed antibacterial activity around 5-7 times less than that of the longer AMP. Therefore, care should be taken to ensure a balance exists between bioactivity and toxicity.

1.2.2.2 Net Charge

Net charge is the sum of the charges of the ionizable groups in the structure. This property has been well described as essential in the action of cationic AMPs (charged 2-9) as it governs the initial electrostatic interactions between the positively charged peptide and the negatively charged phospholipid bacterial membranes (Sánchez-gómez *et al*. 2015). Altering the charge of a peptide can massively influence bioactivity and the impact on host cells. It has been suggested that a limit exists beyond which increasing the net charge of the peptide no longer confers increased bioactivity. In the case of magainin 2, increasing the charge beyond +5 significantly increased the haemolytic activity and had adverse effects on antimicrobial selectivity, despite other structural motifs being conserved (Dathe *et al*. 2001). Other studies have reported similar findings (Jiang *et al*. 2008). Reduced activity may result from over adherence of the peptide to the phospholipid portion of the membrane, preventing further antimicrobial action (Yeaman and Yount 2003a).

1.2.2.3 Helix Secondary Structure

Helix secondary structure relates to the spin structure of a peptide. While this physicochemical property is not massively associated with conferring activity, it is imperative in cytotoxicity (Y. Huang, Huang, and Chen 2010). By replacing L amino acids with D versions in a peptide, helix conformation is diminished. This can reduce haemolysis and enhance stability without affecting bioactivity (Bahar and Ren 2013; Oren and Shai 1996). During membrane insertion, peptides often change their conformation. Proline and glycine can limit the helicity and flexibility of a peptide therefore, amino acid composition in the primary sequence can affect the mechanism of action (Pace and Scholtz 1998).

1.2.2.4 Hydrophobicity

Hydrophobicity is the total percentage of hydrophobic amino acid groups in a sequence and is an important physicochemical property which facilitates peptide-membrane interactions and lipid bilayer insertion (Yeaman and Yount 2003a). Around 50% of residues in AMPs are hydrophobic, making them rich in leucine, isoleucine, valine, phenylalanine and tryptophan. Either an increase or a decrease of hydrophobicity near the charged amino acids can cause positively or negatively influence activity, respectively (Y. Huang, Huang, and Chen 2010). However, it should be noted that a hydrophobic saturation effect exists, after which, activity is compromised and eukaryotic cell toxicity ensues (Bahar and Ren 2013). It has also been proposed that hydrophobicity might be a factor in expanding the range of target cells, therefore it can be worthwhile identifying the optimal hydrophobicity of a peptide (Kustanovich *et al.* 2002).

1.2.2.5 Amphipathicity

Amphipathicity refers to both the polar and non-polar domains in a peptide. Amphipathic peptides are accommodated within the dispersed polar gradient in the microbial phospholipid membrane (Dempsey *et al.* 2010). One measure of amphipathicity is the hydrophobic moment, through which the permeabilisation and haemolytic activities of a peptide can be amplified (Yeaman and Yount 2003a). The presence of a positively charged residue on the non-polar domain can interrupt amphipathicity and widen the therapeutic window – a measure between activity and toxicity (Dempsey *et al.* 2010). The polar angle is a measure of the proportion of polar and non-polar surfaces corresponding to the

amphipathic helix (Yeaman and Yount 2003a). The smaller the polar angle, the greater the global stability and hydrophobic exterior which is linked with improved membrane disruption (Dathe and Wieprecht 1999).

1.2.2.6 Solubility

The solubility of AMPs in aqueous environments dictates their ability to act on microbial membranes. Aggregation can therefore be responsible for poor bioactivity. Amino acid substitutions can be employed as a method to improve solubility for example, by inhibiting dimerisation in hybrid AMPs (Y. Chen *et al*. 2005). The solubility of AMPs is often pH sensitive as shown for modified versions of the bacteriocin nisin where solubility was highest at low pH values and decreased drastically at neutral pH (Rollema *et al*. 1995). The relationship between solubility, pH and activity is important for AMPs. Based on the isoelectric point (PI), (the pH at which a molecule is electrically neutral) of peptides, altering the pH can therefore increase the overall peptide charge, subsequently enhancing activity and solubility. For example, histidine carries no net electrical charge at physiological pH but becomes positively charged at low pH, significantly boosting the potential electrostatic interactions with anionic membranes under acid conditions (Kacprzyk *et al.* 2007).

1.2.3 Plant Antimicrobial Peptides

Beyond the physicochemical properties and structural characterisation classes described above, in the 1990's, plant AMP were characterised into families of their own, including thionins, defensins, cyclotides, Hevin-like and snakins, among more (Tam *et al.* 2015). PhytAMP[5] is a comprehensive database of 271 plant AMPs, however this represents less than 10% of the total number of peptides reported from all species. The majority described are from the extensively studied *Viola* and *Arabidopsis* families. Literature describes a number of defining features for most plant peptides emphasising an average sequence length of 30-50 amino acids, a size of 2-6 kDa, up to six intramolecular disulphide bonds, net charges 0 - +10 with an average of +4.6, cysteine rich motifs and basic side chains with 4-12 hydrophobic residues (Tam *et al.* 2015). Combined, these features are important in their antimicrobial mechanism and ability to traverse bacterial membranes. Approximately half of the peptides reported have had their activity confirmed, one third of which were described

[5] http://phytamp.pfba-lab-tun.org/statistics.php

as antimicrobial (Cândido *et al.* 2011). While members of the plant AMP families such as the defensins all have a similar structure with an α-helix followed by 2-3 β-strands stabilised by 3-4 disulphide bridges, functionality and host range can differ significantly, indicating that structure and activity cannot be directly linked (Pelegrini *et al.* 2011). While certain peptides may exhibit activity and have high thermal, chemical and enzymatic stability, they still may still struggle to be developed as commercial drugs. At present, none of the peptides which are currently authorised by the U.S. Food and Drug Administration (FDA) as approved drugs have plant origins and instead, are largely bacterially derived (Usmani *et al.* 2017). This may stem from the limited number of plant species which have been thoroughly investigated as sources of AMPs combined with the potential bias in only searching for peptides with the above-mentioned features (Tam *et al*. 2015). *In silico* methods to discover peptides are helping to overcome this and identify sequences, which fall outside these structural boundaries.

1.2.4 Production of Antimicrobial Peptides

The majority of well-described AMPs are secondary metabolites. The classical approach to study these peptides involves purification from the substrate and conducting antimicrobial susceptibility testing *in vitro* (Haney, Mansour, and Hacock 2017). While these peptides can be extremely potent antimicrobials requiring only very small concentrations to kill bacteria, they can also be highly toxic, have complex structures and on top of that, are usually secreted in amounts that are not significant or profitable for research or commercial application by the producer organism. There are two ways to produce pure peptides at scale; chemical synthesis and microbial synthesis with engineered strains.

1.2.4.1 Chemical Synthesis of Antimicrobial Peptides

In the case of synthetic chemical production, two main methods exist, solution phase and solid phase. The latter is preferred as it allows for better final yields, reduced by-product production and decreased the amount of toxic chemicals required (A Zambrowicz *et al.* 2013). Despite this, the peptides produced in either way have limited economic viability. The high production costs and time-consuming process restricts the economic viability of many synthetically produced peptides and reserves their application as drugs for the pharmaceutical industry or for diagnostic purposes.

1.2.4.2 Microbial Synthesis of Antimicrobial Peptides

Microbial peptide synthesis using modified bacteria can be a fast and efficient production system. Peptides can be either multimerised and expressed in a bacterium such as *Escherichia coli* BL21 or a recombinant protein can be expressed in the cells which can be hydrolysed by microbial proteinase (A. Zambrowicz *et al.* 2013). However, using prokaryotic heterologous expression is not without its flaws. In many cases, the overexpressed peptide has lethal effect of the microbial cell itself based on its inherent antimicrobial action. This can sometimes be circumvented by attaching a large fusion protein to the peptide to inhibit toxic effects within the bacteria, however this may influence the expression yield (Haney, Mansour, and Hacock 2017; Gauri *et al*. 2011). Importantly, producing peptides recombinantly requires neutralisation of the carboxyl terminus and sequences are limited to those containing only naturally occurring amino acids as microbial cells are not capable of producing post translational modifications (PTMs), with the exception of lantibiotics (Haney, Mansour, and Hacock 2017; Pelegrini *et al*. 2011). Eukaryotic expression in organisms such as methylotrophic yeasts, plants and *Pichia pastoris* may represent better systems, as shown for recombinant *P. sativum* defensin 1 (rPsd1) (Bowdish, Davidson, and Hancock 2005; Almeida *et al.* 2002). There may be a future for transgenic plant bio-factories in producing large quantities of peptide antibiotics, including those with PTMs, at low cost, however at present, no AMPs in pre-clinical drug development are being produced in this way (Haney, Mansour, and Hacock 2017; Pelegrini *et al*. 2011). Biological and/or transgenic expression systems are largely undesirable in certain industries such as in food production. In 2010, the US Food Safety Modernisation Act was passed which addressed labelling issues for foods containing ingredients produced by genetically modified organisms (Cortez 2016). Regulation hasn't evolved as fast as advances in technology, however many industries, in anticipation of heightened consumer demand for clean labels and stricter laws, are moving away from recombinant production of food ingredients meaning advances in this area may be reserved for pharmaceuticals.

1.2.4.3 Truncated Peptide Synthesis

Another way to produce and study AMPs is though testing truncated versions of a longer peptide and determining if bioactivity is affected. For example, the first three amino acids at the N-terminal of magainin 2 can be removed without significantly affecting activity however, when an analogue without the fourth lysine residue was synthesised, peptide potency was severely affected (M Zasloff, Martin, and Chen 1988). Narrowing down the residues, which are important in conferring activity, can help reduce the cost of the peptide synthesis. Conversely, often, large amounts of synthetic peptide analogues need to be synthesised and tested with sometimes only a modest, or no increase in the observed activity.

1.2.4.4 Computer Aided Peptide Synthesis

Ideally, researchers want to identify as many promising candidates as possible while reducing the number of peptides, which need to be synthesised to validate the result. *In silico* methods encompassing genome and DNA sequence screening are becoming increasingly popular (J. Wang *et al.* 2011). Also, as mentioned above with protein hydrolysates, the bioactivity of peptides can be predicted prior to synthesis using methods such as Quantitative Structure Activity Relationship (QSAR) modelling which relates measured activity to the structural characteristics of the sequence using physicochemical descriptors, thereby saving valuable resources (Haney, Mansour, and Hacock 2017; Cherkasov 2005).

1.2.5 Mechanism of Action of Antimicrobial Peptides

Most AMPs are cationic and amphipathic which facilitate their primary mode of action - initial attraction to microbial membranes. Various events post membrane-binding are involved in antimicrobial mechanisms and insertion into the anionic membrane interior (Bahar and Ren 2013). While some AMPs have potent lytic effects on microbial membranes resulting in cell death, others can act within the cytoplasm inhibiting cell wall biosynthesis, cell energetics and division. Furthermore, some AMPs can enter the cell itself and interfere with intracellular functions involving DNA, RNA and proteins (Haney, Mansour, and Hacock 2017). AMPs activity is dependent on their amino acid sequence and 3D structure. The details of their antimicrobial mechanisms of action are described in more detail in the following section.

1.2.5.1 Peptide Interactions with Membrane Targets

1.2.5.1.1 Electrostatic Interactions

It is well documented that electrostatic interactions between cationic AMPs and negatively charged membrane components, such as anionic phospholipids, lipopolysaccharides (LPS) (Gram-negative bacteria) and teichoic acid (Gram-positive bacteria) are the primary step in the antimicrobial mechanism of the peptides. Numerous studies have confirmed the importance of this attraction and highlighted the correlation between charge and binding (Dathe *et al.* 2001; Menzel *et al*. 2016). Across the evolution of various phyla, positive charge in AMPs has been conserved indicating its importance as a biophysical driving force for microbial membranes (Yeaman and Yount 2003a).

1.2.5.1.2 Receptor Mediated Membrane Interactions

Numerous studies dating back 30 years suggested that both D- and L-isoforms of peptides exhibited equivalent activity, therefore these findings made a strong case for non-receptor mediated interactions with bacterial membranes (Wade *et al.* 1990; Bessalle *et al.* 1990). While this may often be the case, important exceptions exist such as the bacteriocin and food preservative nisin. This potent cyclotide specifically binds *via* a receptor like interaction to lipid II. Breukink and de Kruijff compared the increase in fluorescein leakage in bilayers lacking and containing lipid II. They reported that a 1000- fold increase was observed in the presence of the epitope indicating its importance as a target (Breukink and de Kruijiff 1999).

1.2.5.2 Events Post Membrane Binding

While initial membrane interactions are widely accepted as the initial step in microbial killing for AMPs, controversy arises around the subsequent actions of the peptide in permeabilising the membrane and inducing any intracellular effects. Various hypotheses exist, and these actions may vary among peptides. Fig. 1.3 illustrates some of these mechanisms which are associated with pore formation.

1.2.5.2.1 Threshold Concentration and Self-Aggregation

Following initial attraction to the biomembrane, peptides continue to interact with the host cell by accumulating on the surface until a threshold concentration is reached. During this phase, peptides traverse the membrane in various ways expanding their bioactivity intracellularly (Yeaman and Yount 2003a). Here, the peptides may self-associate and form complex structures within the membrane, which gives rise to specific activity. Various intrinsic and extrinsic factors are critical in influencing threshold concentrations such as peptide concentration, phospholipid bilayer components, fluidity and likelihood to aggregate (L. Yang *et al.* 2000). AMPs may function *via* multiple mechanisms depending on their physicochemical properties and target membrane.

1.2.5.2.2 Conformational Phase Transition

Post membrane attachment, peptides, particularly those with an α-helical conformation, undergo conformational phase transition. It has been shown that the vast majority of AMPs are unstructured in aqueous conditions displaying random coil or extended conformations, and only adopt highly ordered helical structures upon interaction with the phospholipid portions of the membrane (Bello, Bello, and Granados 1982). It is likely that their inherent and/or dynamic conformation phase transitions may be linked with their selective toxicity. Unger, Z and Shai (2001), compared the structure, bioactivity and membrane interaction of linear and cyclic analogues of melittin and magainin. They revealed that cyclisation did indeed affect binding, compared to the linear peptide versions. Furthermore, cyclic melittin showed improved antimicrobial activity and induced less haemolysis while the cyclic magainin performed oppositely. Their study concluded that conformation can indeed influence initial interactions and membrane disruption (Unger, Z, and Shai 2001; Yeaman and Yount 2003a).

1.2.5.2.3 Barrel-Stave Mechanism

The Barrel-Stave mechanism refers to the passage created in the membrane by peptide aggregates during this type of permeabilization. Here, peptides burrow a channel in a "barrel" ring position around an aqueous pore. Transmembrane beams or "staves" made up of individual peptides for inside the channel (Yeaman and Yount 2003a). In this model, the hydrophobic chains are positioned outwards whereas the hydrophilic chains face inwards towards the pore (Breukink and de Kruijiff 1999). Firstly, the AMPs must electrostatically bind to the membrane, then adopt a conformational shift whereby the positively charged residues locate near the phospholipid acyl-chains and the hydrophobic portion inserts into

and weakens the membrane (Pelegrini *et al*. 2011). At a threshold concentration, peptides multimerise and traverse deeper into the bilayer. The Barrel-Stave formation protects the hydrophilic portion of the peptide from the membrane (Shai 2002). Accumulation of more AMPs expands the pore and allows transportation intracellularly where the peptides can act *via* other mechanisms inside the cell.

1.2.5.2.4 Toroidal Pore Mechanism

A well-documented mechanism of AMP action is the toroidal pore. It differs from the Barrel-Stave in that membrane lipids are interposed with overlapping peptides inside the pore (Yeaman and Yount 2003a). This supramolecular assembly is composed of polar peptide residues and phospholipid groups along the length of the pore (L. Yang *et al*. 2001). Peptides adopt an α-helical parallel structure post electrostatic interaction with the hydrophobic microbial membrane. The attached peptides dislocate the polar head groups and form a curved channel in the hydrophobic region (Hara *et al*. 2001). The membrane continues to deteriorate and lose integrity increasing its susceptibility for AMP attack. It has been suggested that as the pore collapses, peptides can be shifted into the microbial cytoplasm where further antimicrobial targets may be accessed (Uematsu and Matsuzaki 2000).

1.2.5.2.5 Carpet Mechanism

The carpet mechanism has been compared to the action observed by detergents on microbial membranes. However, their mode of action is not indiscriminate. Similar to the other mechanisms, a mass of AMPs are attracted to the microbial surface of the target cell and mount up to a high density, carpeting the bilayer (Oren and Shai 1996). In this model, no specific structured channel forms, rather, the threshold concentration causes adverse effects on the cell energetics, increasing fluidity, eventually permeabilising the membrane barrier and causing cell death (Pelegrini *et al*. 2011). In this way, disruption ensues without the formation of pores, and peptides do not necessarily inset into the bilayer.

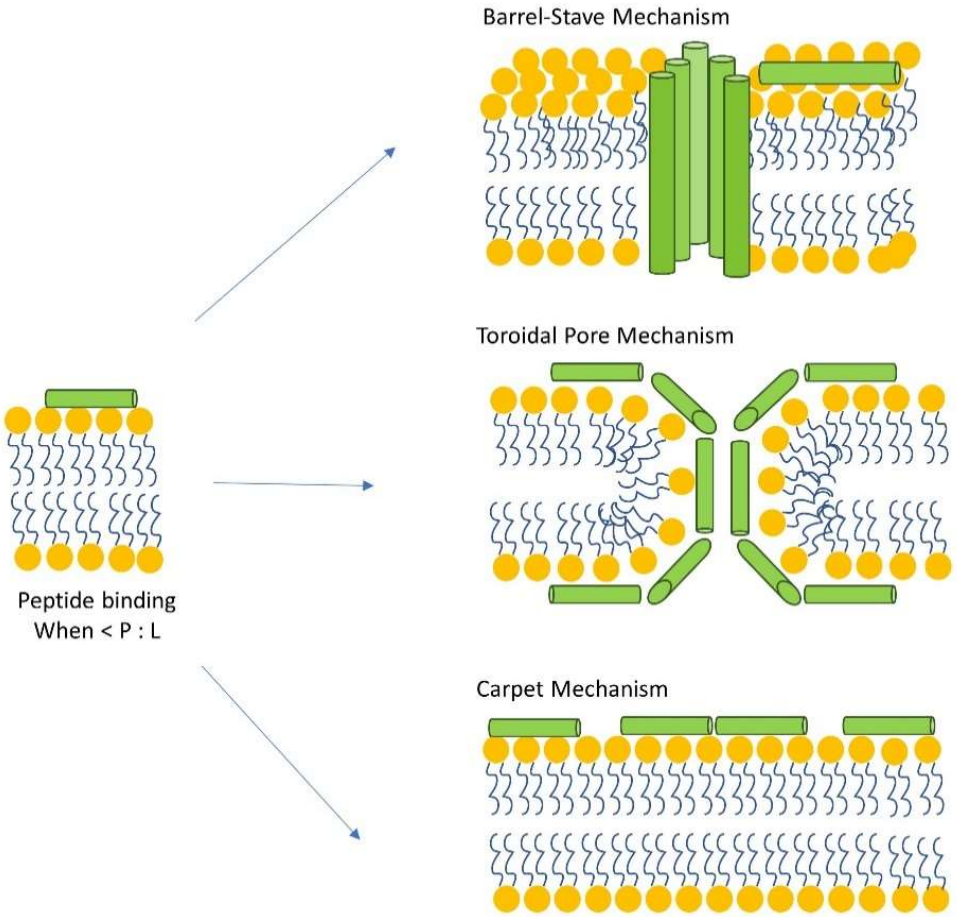


Figure 1.3 Pore forming mechanisms of AMPs. Peptides are electrostatically attracted to the microbial membrane where they aggregate until a certain P:L (peptide to lipid ratio) and threshold concentration is reached after which pores are formed *via* the Barrel-Stave, Toroidal pore or Carpet mechanism (Adapted from Yeaman and Yount 2003a).

1.2.5.3 Peptide Interactions with Intracellular Targets

In early AMP studies, membrane disruption was thought to be the only mechanism of killing. It was recommended to use AMPs at high concentrations in order to disrupt membrane integrity with multiple pores (Cudic and Otvis 2002). With more research, opinions began to change as some AMPs were found to induce permeabilisation at concentrations less than their minimum inhibitory concentrations (MIC), while for others, concentrations far above their MIC were needed. This meant that certain AMPs were selectively killing host targets without permeabilising their membrane, therefore indicating another mechanism of action (Bahar and Ren 2013). It may be the case that AMPs undergo cellular uptake either *via* direct penetration or endocytosis (Madani *et al*. 2011). Various peptides, indolicin for example, have now been confirmed as having an intracellular mechanism of microbial killing such as inhibiting: protein synthesis; ion channels; peptidoglycan synthesis and binding to DNA (Otvos, L 2005; Marchand *et al*. 2006; Nicolas 2009; Spelbrink *et al*. 2004).

1.2.5.4 Antimicrobial Peptide Target Specificity and Selective Toxicity

There are many AMPs which appear to have little or no relevance in host defence however, exhibit potent antimicrobial action *in vitro*. Contrastingly, peptides with diverse structures and sources can provide optimal host defence against microbial threats but function poorly *in vitro*. Given this, it is interesting how an AMP can distinguish between microbial and host cells. Inherent and fundamental differences in structure, functions and membrane composition help discriminate microbial and host cells (Yeaman and Yount 2003a). The phospholipid bilayer is a key constituent of all biomembranes however, important differences exist between those of prokaryotic and eukaryotic cells. The net charge of phospholipids in biomembranes represents a selective target of AMPs. Neutral sterols are found in bilayers of mammalian cells lending to a zwitterionic overall charge whereas bacterial cells are composed of phosphatidylglycerol and other negatively charged hydroxylated phospholipids. Moreover, the charge separation in the cytoplasm between intra- and extracellular components differs in mammalian and microbial cells. This may be another means of host cell selectivity.

1.2.6 Antimicrobial Peptides – Application in this Study

In this study, we used a computational approach (the methods of which are described in more detail later in the thesis) to screen and rank the peptides released post-hydrolysis of *P. sativum* based on features most associated with conferring antimicrobial functionality. The top scoring peptides were then validated experimentally against a range of bacteria, including the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, & *Enterobacter spp*.) pathogens. We focused on targeting these organisms, "red-flagged" by global healthcare bodies, as they represent a detrimental hazard to human health with remaining effective treatments waning.

Considering that the average approved drug reflects just one of thousands of screened compounds, decades of research, and millions of euro in order to reach the market, the challenges in therapeutic development mentioned here, are not exclusive for AMPs. Discovery of novel antimicrobial structures represents one of the biggest hurdles in medicine at present, as antibiotic backbones become exhausted. Here, we emphasise that billions of peptides remain unidentified in nature. One such example, NuriPep 1653, is described in detail and its bactericidal mechanism against a pan drug resistant (PDR) strain of *A. baumannii* is illustrated. Ultimately, plant peptides may be a fruitful source of novel compounds, which could help us remain one step ahead of bacteria in the battle against multidrug resistance.

1.3 Immunomodulatory Peptides

1.3.1 The Immune System – Native and Adaptive Immunity

The immune system is widely recognised as a key player in disease prevention and clearance as well as overall health and wellness but can be influenced by stress, unhealthy lifestyle, diet, infection and antigens (Segerstorn and Miller 2004). The immune system is a complex network of cells, tissues and organs which acts as a shield to protect and eliminate invading microbes (Chalamaiah, Yu, and Wu 2018). Native immunity is made up of multiple layers including; anatomic, physiologic, cellular and inflammatory components. Macrophages and B-cells and dendritic cells are instrumental in phagocytosis and presenting antigens to the T-cells of the immune system while natural killer (NK) cells do not require specific antigenic activation to detect and kill pathogens and respond to cancerous cells (RE Aluko 2008; Chalamaiah, Yu, and Wu 2018). Contrastingly, acquired immunity specifically targets foreign antigens mainly through B- and T-lymphocytes.

B-lymphocytes are responsible for the humoral response of the adaptive immune system by secreting antibodies upon contact with specific antigens labelling them for clearance by macrophages (Santiago-López *et al*. 2016). T-cells can be classified as helper T-cells (Th), cytotoxic T-cells (Tc) and T regulatory cells (Treg) cells (Chalamaiah, Yu, and Wu 2018). Th cells can only function when activated to become effector cells. They are activated on the surface of antigen-presenting cells, which mature during the innate immune responses triggered by infection. Th cells express CD4+, a surface protein, stimulating the release of cytokines such as interferon-gamma (IFN-γ), and various interleukin (IL) as messenger molecules to activate other immune cells (Broere *et al*. 2011). Similarly, Tc lymphocytes display CD8+, distinguishing antigens complexed with MHC I and have anti-cancer activities (Chalamaiah, Yu, and Wu 2018). As the name suggests, Treg cells maintain stasis by supressing self-attacking immune cells (Cano and Lopera 2013).

1.3.2 Immunomodulatory Peptides and Protein Hydrolysates

Through decades of research, progress has been made in understanding the complex interactions of the immune system and how to develop drugs or immunomodulators, which might help modulate host defence systems. However, many of the currently available drugs are prohibitively expensive and have severe side effects *e.g*. cyclosporine, an immunosuppressant used to prevent organ rejection which can cause infection, hypertension and kidney damage (Y. Wang *et al*. 2010). Certain food derived peptides which appear to

possess no antimicrobial activity *in vitro*, are in fact capable of boosting or dampening the immune system through pro- and anti-inflammatory cytokines, antibody production, lymphocyte proliferation and increasing phagocytic abilities (Chalamaiah, Yu, and Wu 2018; Duarte, Vinderola, Ritz, Perdigón, and Matara 2006; Marr, Gooderham, and Hancock 2006; Bowdish, Davidson, and Hancock 2005). All of these properties have contributed to the interest in peptides as natural immunomodulators.

1.3.2.1 Sources of Immunomodulatory Peptides and Protein Hydrolysates

Immunomodulatory protein hydrolysates and isolated peptides have been identified in a wide range of food sources (Chalamaiah, Yu, and Wu 2018; Duarte, Vinderola, Ritz, Perdigón, and Matara 2006; Y. Wang *et al.* 2010). Milk proteins, particularly casein and whey, have been the most extensively studied as sources of immunomodulators (Gill *et al*. 2000). In one example, whey was hydrolysed using trypsin and chymotrypsin and the immunomodulatory actions were measured on murine splenocytes with and without concanavalin A, a legume mitogen which stimulates T-cells (Saint-Sauveur *et al*. 2008). This study revealed that whey fractions encourage splenocyte proliferation and production of the cytokines IL-2 and IFN-γ. Another study revealed that kefir, a dairy product fermented using lactic acid bacteria, can impact host immune responses by enhancing the release of IL-4, IL-6 and IL-10 in the small intestine (C. Vinderola *et al*. 2005). Interesting, kefir up- and down-regulated various components thus acting as an immunoregulator, maintaining homeostasis (G. Vinderola *et al*. 2006). Slattery, Cotter, and O'Toole (2019) provide a detailed review on the array of health benefits associated with kefir, including antimicrobial activity; cholesterol metabolism; immunomodulation; anti-oxidative effects; anti-diabetic effects; anti-allergenic effects; and tumor suppression, which are attributable to the peptides released during fermentation. The beneficial health effects associated with fermented foods can also occur as a result of metabolites released during fermentation. Regarding other animal proteins, chicken eggs white ovalbumin and ovomucoid have featured as rich sources of immunoregulatory peptides and protein hydrolysates by enhancing the activity of macrophages (Memarpoor-Yazdi, Asoodeh, and Chamani 2012; Bhat, Kumar, and Bhat 2015).

Marine precursors have been another fruitful source of peptides with immunomodulatory activities (S. W. Kim and Wijesekara 2010; Ahn, Cho, and Je 2015). Seacure® is a commercially available fermented fish protein product which enhanced macrophage activity

and stimulated pro-inflammatory cytokines while maintaining a balanced composition in the intestines (Duarte, Vinderola, Ritz, Perdigón, and Matar 2006). Similarly, Lee *et al* describe an immunomodulatory peptide purified from clams and its inhibitory effects on nitric oxide production in LPS induced murine macrophages (S. Lee *et al*. 2012).

Legumes are a rich source of bioactive compounds, however have been understudied as precursors for immunoregulatory peptides when compared with animal and marine sources. Santiago-López *et al.* (2016) briefly reviews plant derived peptides from chickpea, wheat and rice and provides more detail on the sequences, effects on proliferation of immune cells and how phagocytic functions of macrophages can be enhanced by these peptides and protein fragments.

1.3.3 Mechanism of action of Immunomodulatory Peptides

Since their discovery, there has been a significant improvement in understanding the antimicrobial mechanisms of peptides. However, the detailed way in which peptides exert immunomodulatory actions, enhancing host defences without directly interacting with pathogens remains largely unknown.

1.3.3.1 Features of Immunomodulatory peptides

As with other activities, the immunological functions of peptides are dependent on certain amino acid composition, sequence and other features (Jacquot *et al*. 2010). In particular, immunomodulatory peptides from food sources are generally short, 3-10 amino acids in length and have over 50% hydrophobic residues such as glycine (Gly), valine (Val), leucine, (Leu), proline (Pro) and phenylalanine (Phe), in their sequences (Chalamaiah, Yu, and Wu 2018; Vo, Park, and Lee 2013; Ahn, Je, and Cho 2012). Various authors describe charge, molecular weight, and acidic residue content as important features in conferring immunomodulatory activity in food derived peptides to stimulate innate and adaptive immunity (Saint-Sauveur *et al*. 2008; Kong *et al*. 2008; Rodríguez-Carrio *et al*. 2014). In contrast to AMPs, little is known on the structural requirements and 3D conformations which confer immunological properties to the peptides. Unlike AMPs, there are multiple cellular targets for immunoregulatory peptides, making a specific structural template unlikely (Haney and Hancock 2013).

1.3.3.2 Actions of Peptides on Immunological Functions

Absorption occurs in the intestine *via* carrier mediated transport or passive diffusion after which the peptides interact directly with multiple signalling pathways and an array of targets including macrophages, NK cells, T- and B-cells and dendritic cells stimulating the production of cytokines and antibodies (Santiago-López *et al.* 2016). Known immunological effects occur through various processes some of which are summarised in Fig. 1.4. These include macrophage activation, increasing phagocytic action, immunoglobulin production, cytokine regulation, increased production of NK cells and splenocytes and activating transcription factor nuclear factor kappa B (NF-κB), and mitogen-activated protein kinase pathways, among more (Chalamaiah, Yu, and Wu 2018; Duarte, Vinderola, Ritz, Perdigón, and Matar 2006; Ahn, Cho, and Je 2015; D. Huang *et al.* 2014; Ling-Ling *et al.* 2017). Peptides with arginine and tryptophan residues can be recognised by, and bind to, specific opioid surface receptors activating T-cells and inducing the production of cytokines (Haque, Chand, and Kapila 2008). Beyond opioid systems, peptides released *via* gastric digestion of legumes were shown to have immunological effects by controlling the production of reactive oxygen species (ROS); modulating cell survival; performing anticancer functions and encouraging the proliferation of healthy cells (Hartmann and Meisel 2007).

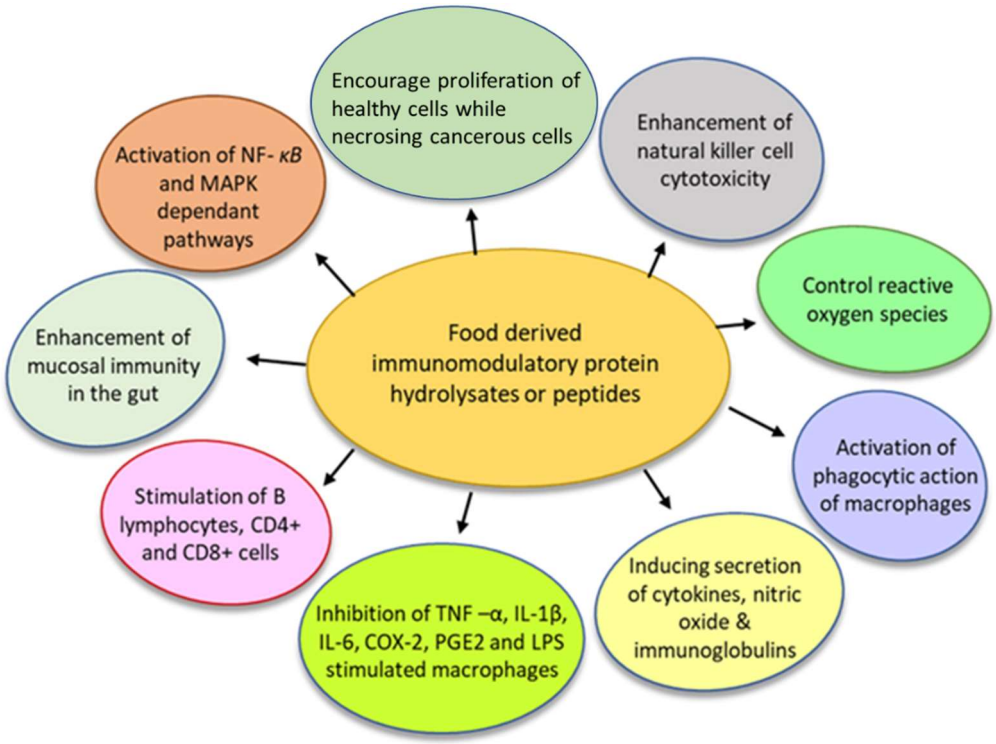


Figure 1.4 Overview of the mechanisms of action of immunomodulatory protein hydrolysates and peptides (Adapted from Chalamaiah, Yu, and Wu 2018).

1.3.4 Inflammation Related Immune Functions

Complimentary to the immunoregulatory effects of food derived peptides and protein hydrolysates are inflammation-related activities. A common *ex vivo* model for measuring anti-inflammatory potential involves using murine RAW or human THP-1 macrophage cells activated with toll-like receptor (TLR) ligands. Once stimulated, these cells release mediators promoting inflammation, such as NF-κB, IL-1β, IL-6, IL-8, and tumour necrosis factor alpha (TNF-α). Food derived AMPs can regulate inflammation-related immune functions by inhibiting these mediators (Chalamaiah, Yu, and Wu 2018; Ahn, Cho, and Je 2015; Karnjanapratum *et al*. 2016). As inflammation is paramount in the pathophysiology of hundreds of diseases from cancer to arthritis, peptides with multiple biological targets have extra ordinary potential.

1.3.5 Immunomodulatory peptides - Application in this Study

In this work, the effects of the lead AMP NuriPep 1653 on immune cells was evaluated. In this way, we determined if the peptide has a dual functionality, capable of directly targeting MDR pathogens while simultaneously enhancing the host immune system. We explored the inflammation-regulated immune functions of our natural food derived peptide and its ability to modulate various cytokines and inflammation mediators. This will help to build knowledge on not only the bactericidal mechanism, but on the immunological mechanism of NuriPep 1653. Through these studies, we aim to build a better profile of the therapeutic potential of NuriPep 1653 highlighting its multifaceted functionalities and beneficial effects for human health.

1.4 Aims of this Study

The primary aim of this study was to identify novel, natural, bioactive peptides embedded within the sequence of *P. sativum* protein and release these peptides by means of enzymatic hydrolysis.

The first experimental chapter of this study pertains to food safety. With regulations governing food ingredients becoming more stringent, paired with increased consumer appeal in natural products, we evaluated the potential of our *P. sativum* hydrolysate (PSH) as a plant-based preservative for the food industry to control populations of spoilage and pathogenic organisms in an infected lettuce leaf model.

In a continuation, the second part of this work employed a computational data mining strategy to sort and order the PSH products according to identifying features, which we classified as important in conferring antibacterial activity. The top scoring peptide, NuriPep 1653 was then synthesised and its bioactivity validated *in vitro*. While antibiotics were indeed one of the most remarkable medical interventions in the last century, their era has ended and the threat of multi drug resistance becomes more pertinent everyday. We investigated the potential of NuriPep 1653 as a natural anti-infective agent against an array of these resistant pathogens. We explored the therapeutic potential of the natural pea peptide by investigating its bactericidal mechanism; toxic profile to human cells; synergistic action with traditional antibiotics and its propensity to induce or limit the development of resistance in bacteria.

The final chapter expanded on the biological functions of NuriPep 1653 as we aimed to uncover if the peptide had a modulatory role on the immune system of the host. Many cationic AMPs have also been termed host defence peptides on account of their stimulatory actions on T- and B- cells; activation of the NF-κB pathway; and inhibition of various pro-inflammatory cytokines. Specifically, we aimed to explore the immunological cell response in infected and uninfected macrophages where the cells were exposed to the peptide both prophylactically and therapeutically. The inflammation regulated immune functions of NuriPep 1653 were then researched by measuring the inhibition of TNF-α in LPS stimulated human macrophages as well as by monitoring NF-κB modulation.

Ultimately, through this work and the publication we generated from it, we hope to have contributed to and expanded the realms of peptide discovery. By going back to nature, recognising the abundance of unexplored peptides and utilising cutting edge technology to mine for hits, we present a non-conventional strategy to discover novel peptides from plants. Moreover, our findings may help dilute existing bias relating to classifying features of plant peptide families. Finally, we presented a potential application for novel protein hydrolysates as a bio-preservative in the food sector and for peptides as promising lead drug candidates in pharmaceutical industry to directly and indirectly fight MDR bacteria.

Chapter 2

The potential of *Pisum sativum* protein hydrolysate as a bio-preservative to reduce the population of *Escherichia coli* O157:H7 in a lettuce leaf model

Note: This chapter is in submission:

Niamh M. Mohan, Amine Zorgani, Leah Earley, Sweeny Chauhan, Sanja Trajkovic, John Savage, Alessandro Adelfio, Nora Khaldi and Marta Martins. The potential of *Pisum sativum* protein hydrolysate as a bio-preservative to reduce the population of *Escherichia coli* O157:H7 in a lettuce leaf model. *International Food Science and Emerging Technologies*. 2019; (In submission).

2.1 Introduction

Increasingly health conscious, urban consumers are changing their buying habits. With convenience and nutritional value of paramount importance, products such as bagged lettuce and salads have become increasingly popular in the last 10 years as "healthy fast-food" (Rekhy and McConchie 2014; Koukkidis and Freestone 2018). The pH of lettuce (5.5-6.0) paired with its high water activity (a_w) value, provides optimal conditions for microbial growth (Tirpanalan *et al.* 2011). This is made worse in cut/shredded lettuce as the surface area is vastly increased making it a highly perishable food which is often implicated in outbreaks of food borne illness (Qadri, Yousuf, and Srivastava 2015). Therefore, in order to avoid these incidents, preservation and processing strategies have been implemented by the food industry to serve two purposes. Firstly, to delay enzymatic decay and oxidation which contributes to natural spoilage. The second, to control the proliferation of undesirable, pathogenic micro-organisms that can cause adverse organoleptic changes and often result in food poisoning.

Shiga-toxin producing *E. coli* O157:H7 poses one of the biggest threats as it is highly virulent with a reported infectious dose of only 10-100 cells (Greig *et al*. 2010). In 2018, romaine lettuce contaminated with *E. coli* O157:H7 was responsible for a multi-state outbreak of food poisoning infecting 210 people, 12% of whom developed haemolytic urea syndrome (HUS) after hospitalisation (CDC 2018). More recently, in early 2019, *E. coli* O157:H7 contaminated lettuce grown in California caused 62 illnesses over 16 states (CDC 2018). As technology in detection, surveillance and reporting develops, the management of these type of infections has indeed improved (Al-Qadiri *et al*. 2006). However, these very recent outbreaks highlight that current microbial control strategies are not always effective at eliminating the bacterial risks encountered from farm to fork.

Bacterial pathogens are commonly spread to lettuce and other vegetables when fields are irrigated with contaminated water. Processing techniques are therefore employed post-harvest to try and reduce pathogenic populations and extend the shelf life of processed products. Physical preservation treatments such as modified atmosphere packaging (Siroli 2014), edible coatings (Corbo *et al*. 2015) and irradiation (Rico *et al*. 2007; Misra *et al*. 2011) have all been investigated to date and may indeed be employed as hurdle technologies, however minimal processing such as washes are still favoured for lettuce. Chlorine is the most commonly used chemical wash to decontaminate fresh cut produce due to its efficacy, solubility, ease of use and inexpensive cost (Petri, Rodríguez, and García 2015). However, health and environmental-related concerns regarding the carcinogenic by-products of chlorine bound to organic matter such as chloroform, haloketones and trihalomethane have spurred research into alternative decontamination solutions (Rico *et al*. 2007; Abadias *et al.* 2011). Various other washing treatments have been proposed as safe and effective such as hydrogen peroxide, ozone, electrolysed water and organic acids. While some have been employed as surface decontaminants on packaging materials, none are currently permitted for use with food (Siroli 2014; Ölmez 2010; Rico *et al.* 2007). As the demand for natural ingredient flourishes and "clean label" in the food sector, it is likely that industry will move away from chemical means of food preservation and opt for other alternatives such as food-derived AMP and hydrolysed proteins (Carocho, Morales, and Ferreira 2015).

Protein plays a critical role in providing the body with essential amino acids for basic nutrition and energy. Additionally, proteins can be a source of physiologically active compounds and encrypted bioactive peptides which are contained within their amino acid sequence (Chalamaiah, Yu, and Wu 2018). These peptides remain dormant when within the parent protein, however can exhibit vast bioactivities when cleaved by acid treatment, protease addition, microbial fermentations or during food digestion by the action of gastric enzymes (Mora, Aristoy, and Toldrá 2019; Cesar Lemes *et al*. 2016; Bhat, Kumar, and Bhat 2015; Garcia *et al.* 2013). Given the body's natural biological effort to breakdown macromolecules into smaller absorbable units, protein hydrolysis, and therefore the production and uptake of bioactive peptides, has been happening for hundreds of years before any functionalities were established (Fitzgerald, Rai, and Marchbank 2005; Sato, Egashira, and Ono 2013). When compared to whole proteins, protein hydrolysates which contain a complex mixture peptides which are more digestible and bioavailable with anticancer, antihypertensive, antioxidant, opiate, antimicrobial and immunomodulatory activities widely reported (Chalamaiah, Yu, and Wu 2018; Bhat, Kumar, and Bhat 2015; M.

Nasri 2017). Enzymatically hydrolysing proteins outside of the GI tract allows for tighter control on the peptides released. These can then be studied individually or as a mixture for biological activities and potential uses which differ to that of the parent protein.

Animal protein sources, predominantly those from milk (León-calvijo *et al.* 2015), eggs (Mine, Ma, and Lauriau 2004), meat (Di Bernardini *et al.* 2011) and skin (Nalinanon *et al.* 2011), have been exploited as commercial protein hydrolysates. Numerous studies report the actions of antioxidant, antihypertensive, anti-cancer, immunomodulatory, and cholesterol lowering peptides which are released from proteins without those functions (Mine, Ma, and Lauriau 2004; Slizyte *et al.* 2016). Interestingly, consumption of vegetables and pulses has been associated with the prevention of chronic disease. The benefits to health are attributable to micronutrients, phytochemicals and recently, bioactive peptides (López-Barrios, Gutiérrez-Uribe, and Serna-Saldívar 2014). With the exception of a few food sources, such as soy and rice, protein hydrolysates from plants and pulses remain largely unexplored.

Computer-assisted approaches for predicting the location of bioactive peptides within known parent protein sequence have changed the way in which peptides are discovered. *In silico* data mining paired with protein hydrolysis and MS are, therefore, a method in which potent protein fragments can be identified from plants. In this study, we explore the potential of PSH as a bio-preservative to inhibit the growth of *E. coli* O157:H7 in an infected lettuce food model. As plant-based trends continue to flourish in the food sector, paired with their inexpensiveness, voluminous production, natural origins and history of safe consumption, plant protein hydrolysates and the AMPs contained within them are interesting candidates as alternatives to chemical preservatives with a "from food – for food" approach (Chakrabarti, Jahandideh, and Wu 2014; Aleksandra Zambrowicz *et al*. 2012; Schaafsma 2009; Hou *et al*. 2017).

2.2 Materials and Methods

2.2.1 Plant Material and Reagents

80% protein powder of *P. sativum* was purchased from a commercial supplier. Leucidal liquid was purchased from Active Micro Technologies (North Carolina, USA) and used as a positive control due to its known antimicrobial activity. This is a commercially available fermented hydrolysate bio-preservative which is based on an AMP originally derived from the lactic acid bacteria, *Leuconostoc kimchi*. The antimicrobial activity of Leucidal Liquid

against various Gram-negative and -positive pathogens is available online and so this was used in the infected leaf model as a comparative bench mark for PSH (Active Micro Technologies 2014). Vivaspin 500 Centrifugal Concentrator MWCO filters were purchased from Sigma Aldrich (United Kingdom). Oasis HLB 10 mg sorbent SPE cartridges were obtained from Waters (Dublin, Ireland). All bacterial growth media were purchased from Oxoid Sparks Lab Suppliers (Dublin, Ireland). Protein content in the samples was determined using Pierce Bicinchoninic Acid Assay (BCA) protein concentration kit, ThermoFisher Scientific (Dublin, Ireland). Formic acid, Optima water and acetonitrile were all obtained from Fisher Chemical (Dublin, Ireland).

2.2.2 Bacterial Culture Preparation and Growth Conditions

E. coli NCTC 12900 (Serotype O157:H7, verocytotoxin/shigatoxin negative) was gifted by the Department of Food Science and Environmental Health, Dublin Institute of Technology, Ireland. *E. coli* cultures were maintained at -80ºC in 15% glycerol. *E. coli* was grown in Mueller Hinton broth (MHB) medium. Cultures were prepared by inoculating 10 mL of the selected media with bacteria and incubating overnight for 18 hours at 37ºC with agitation. A subculture was prepared from the overnight bacterial suspension by diluting it to an optical density at 600 nm (OD_{600}) of ~ 0.05 in fresh media and re-incubating under growth optimal conditions for 2-3 hours until logarithmic phase was reached (OD_{600} ~ 0.5). Bacterial cultures were then adjusted to the desired concentration for assay in phosphate buffer no salt (PBNS).

2.2.3 Lettuce Leaf Model Preparation

A fresh head of iceberg lettuce (*Lactuca sativa* var. *capitata*) grown in Spain was purchased at a local supermarket in Dublin, Ireland for each experiment. The lettuce was stored at 4 °C (degrees Celsius) and used within three hours of purchase. The outer leaves of the lettuce were removed and discarded. The remaining leaves were cut into 3 x 3 cm^2 pieces using a sterile knife. The leaves were dipped for 10 seconds in 70% ethanol (EtOH) before being submerged into sterile distilled water (dH_2O) for a further 10 seconds. Afterwards, the lettuce pieces were inoculated with 500 µL of *E. coli* at ~$1.1x10^4$ cells/mL. The leaves were dried in the biosafety cabinet for 15 minutes to facilitate bacterial adherence.

2.2.4 Hydrolysis of Pisum sativum Protein Powder and Preparation of PSH

Protein hydrolysis was carried out according to Aluko with modifications (Rotimi Aluko 2018). In brief, *P. sativum* protein powder was homogenised in a water bath under constant agitation. Enzymatic hydrolysis (enzyme selection was proprietary to Nuritas ltd.) was carried out under enzyme-specific conditions, after which, the soluble fraction was separated by centrifugation and further purified by SPE. Samples were then stored at 4˚C for antimicrobial activity testing.

2.2.5 Solid Phase Extraction of PSH

The PSH was prepared to a concentration of 10 mg/mL and acidified by directly adding formic acid (reagent grade, Fisher Chemical, Dublin, Ireland) to a final concentration of 0.1%. The extraction cartridges were then placed in the vacuum manifold and the vacuum switched on with the main tap off. 2 mL of acetonitrile (Optima grade, Fisher Chemical, Dublin, Ireland) was added to each cartridge; 1 mL at a time allowing the cartridge to empty 80% of the liquid before the next addition. 2 mL of formic acid dissolved in dH_2O was added in the same way. The acidified sample was loaded at gravity flow of 1 mL/min. The cartridges were washed with 4X the sample volume loaded using 0.1% formic acid, then with optima water, 1mL at a time, respectively. After the final wash, the volume was increased to 15 milligrams of mercury (mm-Hg) and the cartridge was dried out. The vacuum tap was closed and released using the emergency valve. The waste tubes were replaced with 1.5 mL tubes for sample collection. With the vacuum valve closed, the samples were eluted with 60% acetonitrile at half the sample volume loaded at gravity flow. Once dry, the vacuum was released with the emergency valve prior to removing the samples. After elution, the sample tubes were placed into a speed-vac and dried overnight. Each 1.5 mL sample tube was resuspended in 70 µL of Optima water before testing.

2.2.6 Bicinchoninic Acid Protein Determination Assay

A bovine serum albumin (BSA) stock solution of 2 mg/mL was prepared by adding 10 mg of BSA into 5 mL of dH_2O. Following this, protein standards of 0 – 2000 µg/mL were prepared using dH_2O according to manufacturer's instructions. Protein hydrolysates samples were diluted in dH_2O to a dilution factor of 10 and 20 to ensure the values would fall within the linear range of the standard curve. Copper II solution and bicinchoninic acid were mixed in a ratio of 1:50, respectively. Following this, 25 µL of the standards and diluted samples were added in triplicate onto a 96 well plate. Once completed, 200 µL of the pre-prepared BCA working reagent was added to the standards and samples and the plate was incubated at 37°C for 30 minutes. After incubation, the OD was read at 562 nm in a microplate

spectrophotometer (Synergy H1 Biotek). The protein/peptide concentration was determined by intrapolation of the readings of the samples into the equation of the standard curve using the Synergy H1 software.

2.2.7 Mass Spectrometry Analysis of PSH Samples

Firstly, PSH samples were filtered through 10 kDa MWCO filters to remove the enzymes used during hydrolysis and any insoluble fractions (V. K. Pasupuleki and Braun 2010). The filtered samples were then further cleaned by running them through SPE cartridges (previously described above; section 2.5), before being spray dried. Samples were reconstituted in Optima water to assess peptide concentration *via* the BCA assay (detailed in section 2.7). LC was performed using a 60-minute gradient from 5 to 75% acetonitrile on an EasySpray reverse-phase column (ES804, ThermoFisher Scientific, Ireland) coupled to a Q-Exactive mass spectrometer (Thermo Fisher, Ireland). Peptides were loaded on a trapping column and eluted over a 15 cm analytical column with a 1-hour gradient at a flow rate of 300 nL min^{-1}. The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 Full width at half maximum (FWHM) and 17,500 FWHM resolution, respectively. From the MS scan, the fifteen most intense ions were selected for MS/MS. Fragmentation spectra from putative peptides collected by data-dependent acquisition were used for peptide identification. Relative quantification was performed by integrating precursor signal intensities using the PEAKS software (Ma *et al*. 2003). MS sample preparation was performed by Dr. Sanja Trajkovic and data analysis was performed by Dr. John Savage and Dr. Gael Jalowicki, Nurtias Ltd.

2.2.8 Antibacterial activity of PSH against E. coli O157:H7

The complete elimination (CE) method was primarily used as the antimicrobial susceptibility testing method. This was performed to avoid any interference of cations within the test media on the bioactivity of the protein hydrolysate. The assay was performed according to Chen *et al.* (2003) with minor modifications. Briefly, *E. coli* O157:H7 was grown in liquid broth for 18 hours at 37°C before being sub-cultured and allowed to grow to mid exponential phase. PBNS was used to make serial dilutions of bacteria at a density of 1.0 x 10^4 and 1.0 x 10^5 cells/mL in a 96 well plate. PSH was added to the wells at the desired concentrations and made up to a final volume of 100 µL. PBNS alone was added to the bacterial suspension as an untreated control. The 96 well plate was incubated for 24 hours at 37°C. Post challenge, 3 x 10 µL from each well was spotted onto MHA. This was conducted in triplicate on three separate days. The spots were left to dry, and the plates were

incubated inverted. The first concentration to inhibit bacterial growth was determined as the CE concentration. Surviving colonies at sub-CE concentrations were counted after 18 hours incubation at 37°C. The reduction in colony forming units (CFU) was assessed relative to the untreated control and the log reduction induced by the hydrolysate treatment was calculated.

2.2.9 The Effects of PSH at Inhibiting the Growth of E. coli O157:H7 in an Infected Lettuce Leaf Model – Stamp on Agar Method

The lettuce was prepared according to section 2.3 The lettuce was inoculated with 500 µL of *E.* coli at ~1.1 x 10^4 cells/mL and the leaves were dried in the biosafety cabinet for 15 minutes. After this time, 100 µl of PSH was spotted onto the underside of a single leaf piece and spread onto the surface with a sterile cotton swab. PSH was applied to three separate leaves at the concentration previously determined to induce bactericidal effects in vitro. PBNS was used as a negative control, as it was the buffer solution for the PSH samples. Leucidal liquid was used as a positive control. The antimicrobial activity of Leucidal Liquid against various Gram-negative and -positive pathogens is available online and so this was used in the infected leaf model as a comparative bench mark for PSH (Active Micro Technologies 2014). The concentrations of Leucidal Liquid used in experiments was expressed as a percentage, as recommended by the manufacturer. While attempts to determine the protein content in Leucidal Liquid were performed via the BCA method in order to make the concentrations comparable to PSH, the levels of protein detected were insufficient. It is likely that as Leucidal Liquid is a fermented product, that it may contain other ingredients beyond hydrolysed proteins, such as metabolites which may contribute to its activity. Leucidal Liquid is included as a commercial control, however, its limitations regarding comparable units of PSH concentrations were considered. The treated leaves were placed in sterile petri dishes and refrigerated at 4°C for 2 hours. After this time, the leaves were pressed gently onto agar using sterile forceps. The plates were then inverted and incubated at 37°C for 24 hours. The colonies growing over the 3x3 cm2 surface stamped area were counted the following day. PSH treated samples were compared to the untreated control.

2.2.10 Toxicity Assay

The toxicity of the PSH was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor method (MTT, Sigma) in a cellular viability with human THP-1 macrophages (Silva *et al*. 2016). Macrophages were grown in Roswell Park

Memorial Institute (RPMI) medium supplemented with 10% of heat inactivated foetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin. When the cells reached a confluency of 90%, the cells were pelleted *via* centrifugation and diluted to a 1:5 ratio with complete RPMI. Differentiation of the monocytes was performed by adding 20 ng/mL phorbol 12-myristate 13-acetate (PMA) to cells before seeding in a 96 well plate at a density of 1.0 x 10^4 cells/well. The plate was incubated with 5% CO_2 for 72 hours at 37ºC. Following this, cells were treated with increasing concentrations (10, 100, 1000, 10000 µg/mL) of PSH before re-incubation for 24 hours. The highest concentration tested, 10000 µg/mL, was limited by the protein content of the PSH samples. A SPE method, capable of larger sample volumes, would allow for greater yields, concentration of the sample and therefore higher protein content values available for testing in toxicity assays.

After PSH treatment, the media was removed and replaced with 110 µL of MTT solution was added to each well at a final concentration of 500 µg/mL and incubated at 5% CO_2 for 2 hours at 37ºC. Post-incubation, the media was removed and replaced with of 110 µL of 100% dimethyl sulfoxide (DMSO) in each well at room temperature. The place was covered in tin foil and gently agitated on a plate shaker to 5 min encourage the dissolution of formazan crystals. The OD was measured at 570 nm in a microplate spectrophotometer (Synergy H1, Biotek). Maximum toxicity was determined by cells incubated with 100% DMSO. Cell viability was calculated as a percentage of the untreated control cells. Three technical replicates were performed each day on three independent days.

2.2.11 Statistical Analysis

All data are presented as mean ± SD. Replicate numbers for each experiment are indicated in figure legends. Results of *in vitro* experiments were analysed by one-way ANOVA. Statistical significance was defined as * $p < 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

2.3 Results

2.3.1 Size Exclusion - High Performance Liquid Chromatography Chromatograms of Hydrolysed and Unhydrolysed PSH

SEC was used to highlight the different molecular weight profile of hydrolysed and unhydrolysed PSH. The unhydrolysed material (Fig. 2.1 - green chromatogram) elutes 90% of its material between 9-18 minutes whereas the hydrolysed material (Fig. 2.1 – blue chromatogram) elutes mostly between 14-21 minutes. Based on the chromatogram it is clear

that the unhydrolysed PSH elutes faster based on the content of the higher molecular weight molecules. The unhydrolysed PSH contains smaller molecular weight peptides and amino acids which weave through the porous beads in the column and therefore elute up to 5 minutes after the unhydrolysed sample. As the only difference between the two samples was the addition (or not) of enzymes, the SEC chromatogram is indicative of successful hydrolysis whereby proteins have been digested into smaller molecular weight fragments.

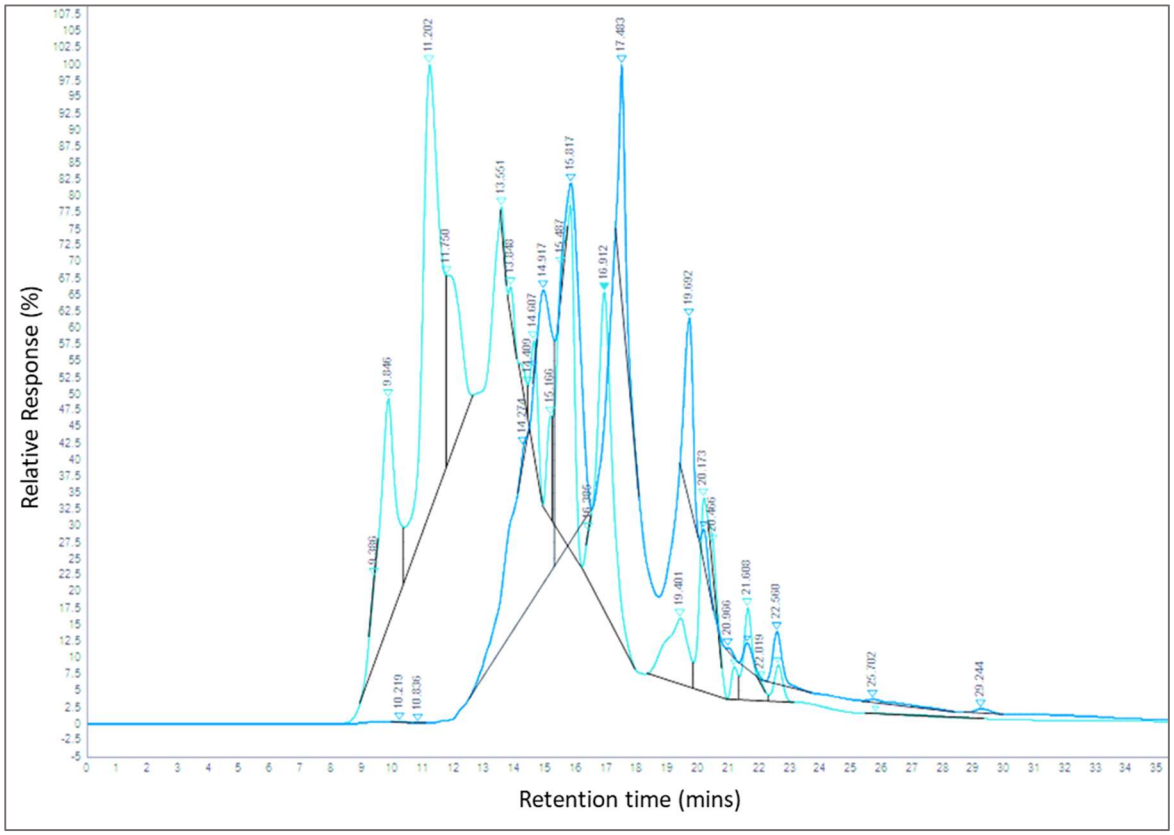


Figure 2.1 Size Exclusion-High Performance Liquid Chromatography (SE-HPLC) of unhydrolysed *P. sativum* overlaid on PSH. Molecular weight chromatograms of both *P. sativum* materials pre- and post-enzymatic digestion. The unhydrolysed sample has a shorter retention time (9-18 minutes) while the hydrolysed PSH eludes later (14-21 minutes). Unhydrolysed *P. sativum* (Green) and PSH (Blue).

2.3.2 Mass Spectrometry Analysis Indicating the % Hydrophobicity, Charge and Length Distribution of Peptides within Replicate PSH Samples

MS allowed for peptides released by the enzymatic treatment of *P. sativum* proteins to be studied. The variability in peptide content in three hydrolysate samples; PSH 1, PSH 2 and PSH 3 which were hydrolysed on three separate days was also determined. Studying the overlapping area of the Venn diagram in Fig. 2.2A, allowed us to investigate further only the peptides which were cleaved in a consistent manner, *i.e*. the samples releasing identical peptides in every hydrolysis. In general, there was a good overlap between the three hydrolysates. On average ~3520 peptides were released post-hydrolysis and ~ 55% (1949 peptides) of these were common across the samples. The peptides present in the PSH samples have a net charge distribution from -2 to + 2 with the majority having a -1 or neutral charge (Fig. 2.2B). It has been well documented that the percentage of hydrophobic residues in AMPs is around 50%, respectively (Y. Huang, Huang, and Chen 2010). On average, the hydrophobic percentage range between 40 and 60% was where the highest frequency was observed among the three samples (Fig. 2.2C). PSH 2 and PSH 3 were almost identical and overlapping. Regarding PSH 1, it appears to have less hydrophobic percentage in its peptidic content. Good consistency was observed among all samples. Finally, the length of the peptides in the three samples showed good consistency (Fig 2.2D). This indicates a robust hydrolysis procedure and efficacious enzymes as the proteins were being cleaved to release peptides of almost the same length every independent time the experiments were performed.

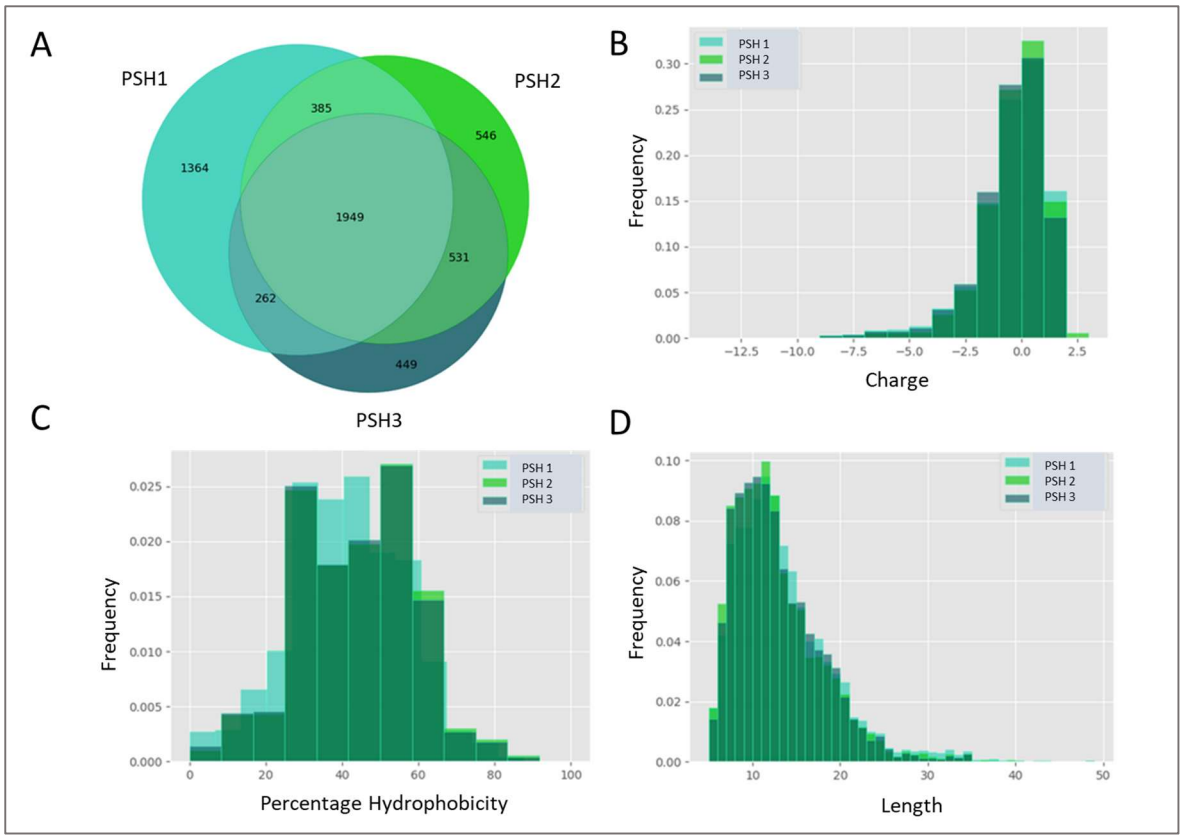


Figure 2.2 Analysis of the peptide content in PSH 1, PSH 2 and PSH 3 hydrolysed replicates of *P. sativum*. Histograms represent the peptides in each individual sample according to **A:** a Venn diagram of the number of overlapping peptides in each replicate. In **B:** Charge, **C:** Percentage of Hydrophobicity and **D:** Length of the three samples are shown. MS sample preparation was performed by Dr. Sanja Trajkovic and data analysis was performed by Dr. John Savage and Dr. Gael Jalowicki, Nurtias Ltd.

2.3.3 The effects of PSH against E. coli in vitro

The antimicrobial activity of PSH against *E. coli* was evaluated using the CE method in PBNS, thus avoiding any possible interference between ions in the buffer and the cationic peptides in the PSH. The results are shown in Table 2.1. All of the PSH biological triplicates exhibited a CE concentration of 8 mg/mL against *E. coli.* This was considered equivalent to the minimum bactericidal concentration (MBC) usually determined as per European Committee on Antimicrobial Susceptibility Testing (EUCAST) or Clinical Laboratory Standards Institute (CLSI) guidelines, as microbial growth was eliminated at this concentration. The unhydrolysed material did not exhibit any bactericidal action, indicating that the whole protein material did not possess any antimicrobial activities, and these only arose after enzymatic hydrolysis, and subsequent peptide release. Leucidal liquid was included as a positive control and was active within the concentration ranges (2-4%) previously reported by Active Micro Technologies (Active Micro Technologies 2014). While *E. coli* O157:H7 was the primary pathogen of interest in the study*, Salmonella* Typhimurium 14028S was also tested and equivalent results were found (CE concentration of 8 mg/mL) which is interesting as this is another important pathogen involved in food borne disease.

Table 2.1 Complete Elimination values of PSH samples against *E. coli* 0157:H7 *in vitro*

Hydrolysate samples	*E. coli* O157:H7
	(mg/mL)
Unhydrolysed	>20
PSH 1	8
PSH 2	8
PSH 3	8
Leucidal Liquid (control)	2%

Legend: Values of PSH replicates 1, 2, and 3 required to induce bacterial clearance *via* the CE method against *E. coli* O157:H7. Unhydrolysed *P. sativum* protein powder was used as a negative control. Values shown represent the average of three independent experiments on three independent days. PSH 1-3 represent biological triplicates.

2.3.4 Antibacterial activity of PSH at inhibiting the growth of E. coli O157:H7 in a lettuce leaf model

The stamp on agar method was performed to evaluate surviving colonies of *E. coli* O157:H7 after treatment with PSH, Leucidal liquid or buffer alone. While many studies describe making broths from food products for testing *via* the broth microdilution method, this is not representative of the final application of the PSH bio-preservative wash or surface treatment of the lettuce leaves (Papagianni and Papamichael 2007; Charalampopoulos *et al*. 2002; Gutierrez, Barry-Ryan, and Bourke 2009). As shown in Fig. 2.3, when the three biological replicates of PSH were applied as a surface treatment spread on *E. coli* infected leaves, significant reductions (** $p \leq 0.01$, *** $p \leq 0.001$) in bacteria were observed after only 2 hours of treatment compared to the untreated control. In fact, the number of bacteria recovered was less than for the commercially available hydrolysate Leucidal Liquid.

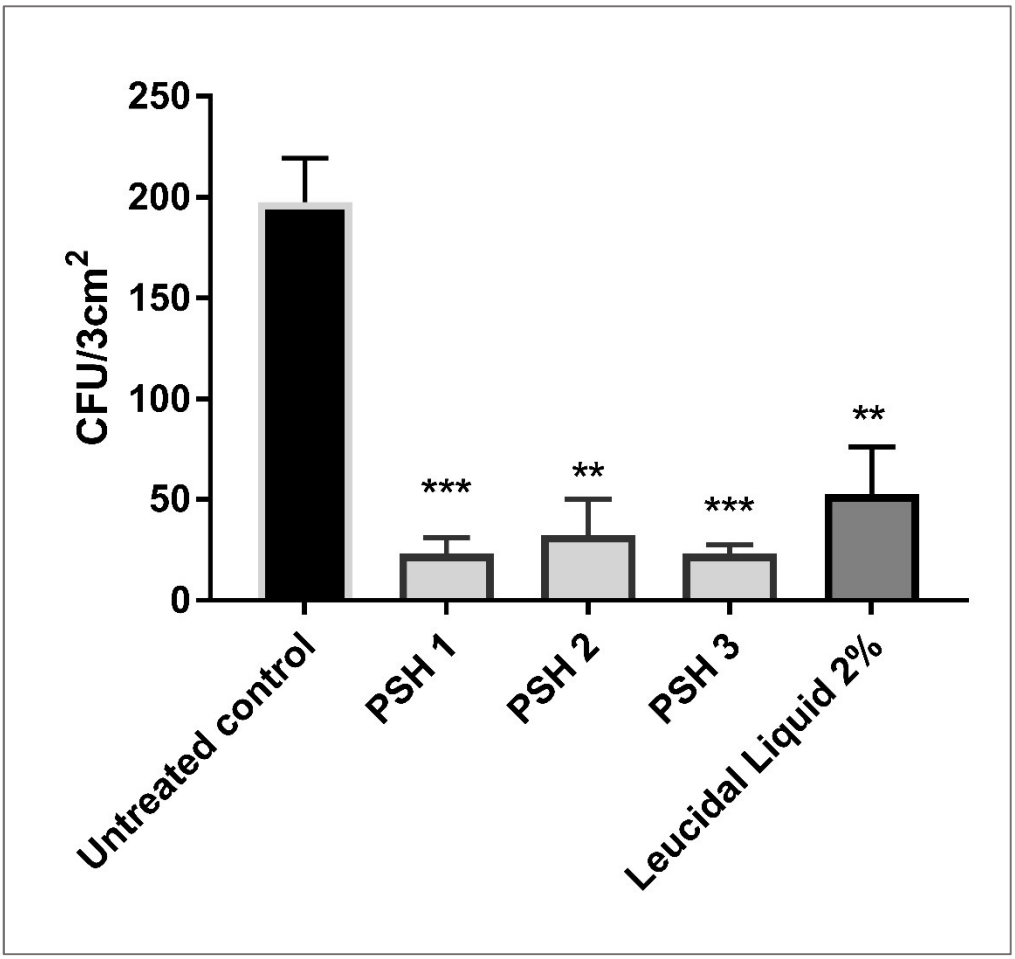


Figure 2.3 Antibacterial activity of PSH samples at reducing populations of *E. coli* O175:H7 in an infected lettuce leaf model. Reductions in the CFU/3cm^2 of *E. coli* recovered from the surface of an inoculated lettuce leaf were enumerated after treatment for 2 hours with PBNS (untreated control), PSH 1, PSH 2, PSH 3- all at 8 mg/mL and Leucidal Liquid at 2%. Bacteria were recovered on MHA. Data represent the mean of three experiments performed in triplicate and is expressed as the mean ± standard deviation (SD). Statistical analysis was performed using a One-way ANOVA where ** $p \leq 0.01$ and *** $p \leq 0.001$ was considered statistically significant.

2.3.5 Peptides predicted to contribute to antimicrobial activity present in the overlap of the PSH samples

As each of the PSH biological triplicates was shown to have an equivalent CE concentration against *E. coli* O157:H7, we hypothesise that the activity could be due to peptides, which are common among the replicates. The 1949 overlapping peptides that are displayed in the Venn diagram in Fig. 2.2C were listed and ranked according to 4 main features; net charge, length, percentage of hydrophobic residues and their PI. The top 10 peptides that were obtained with these characteristics are shown in Table 2.2. These features were chosen as top classifiers for peptides which may exert antimicrobial functions based on our previous study on NuriPep 1653, which was released from the P54 protein of hydrolysis of *P. sativum* (Mohan *et al*. 2019). This was combined with the available information in the literature on the defining features of AMPs important in conferring antimicrobial activity in a peptide sequence (Tossi, Sandri, and Giangaspero 2000). While outside the scope of this paper, it would be interesting to synthesise these peptides and confirm if they had antimicrobial properties individually.

Table 2.2 Top 10 peptides ranked according to charge, length, percentage of hydrophobic residues and isoelectric point identified within the overlapping peptide regions of PSH 1, -2, and -3 identified by mass spectrometry

Peptide ID	Charge	Length	% Hydrophobicity	PI
NuriPep 1653	4	22	50	11.26
PSPep 1	2	20	60	8.64
PSPep 2	2	20	60	8.80
PSPep 3	2	20	50	8.50
PSPep 4	2	20	50	8.50
PSPep 5	1	20	60	6.79
PSPep 6	1	19	63.16	6.79
PSPep 7	1	19	63.16	6.79
PSPep 8	1	18	44.44	8.63
PSPep 9	1	19	36.84	8.59
PSPep 10	1	18	55.56	8.59

Legend: PSPep 1 – 10 represent the top 10 peptides identified within the overlapping section of the triplicate PSH samples which have a charge, length, percentage of hydrophobicity and PI similar to NuriPep 1653, a previously described potent AMP obtained through the hydrolysis of *P. sativum* (Mohan *et al*. 2019). The longer peptides were shown to have the highest charge, and hydrophobicity however, the PI did not follow this pattern.

2.3.6 Effects of PSH on the viability of differentiated human macrophages

Differentiated human macrophages were used to assess the toxicity profile of PSH and compare this to the bactericidal concentrations obtained *in vitro*. As shown in Fig. 2.4, no significant adverse effects were observed on the cellular viability of the macrophages at the highest concentration of PHS 1 tested, 10000 μg/mL with 85% of cells still viable. PSH 1 was chosen as a representative of all the hydrolysate samples as toxicity results observed were equivalent. DMSO performed as expected and was cytotoxic to cells. These results indicate that the concentrations of PSH required to induce bactericidal effects *in vitro* and to reduce the populations of *E. coli* O157:H7 on infected lettuce leaves is less than the toxic concentration to human cells, indicating a good safety profile.

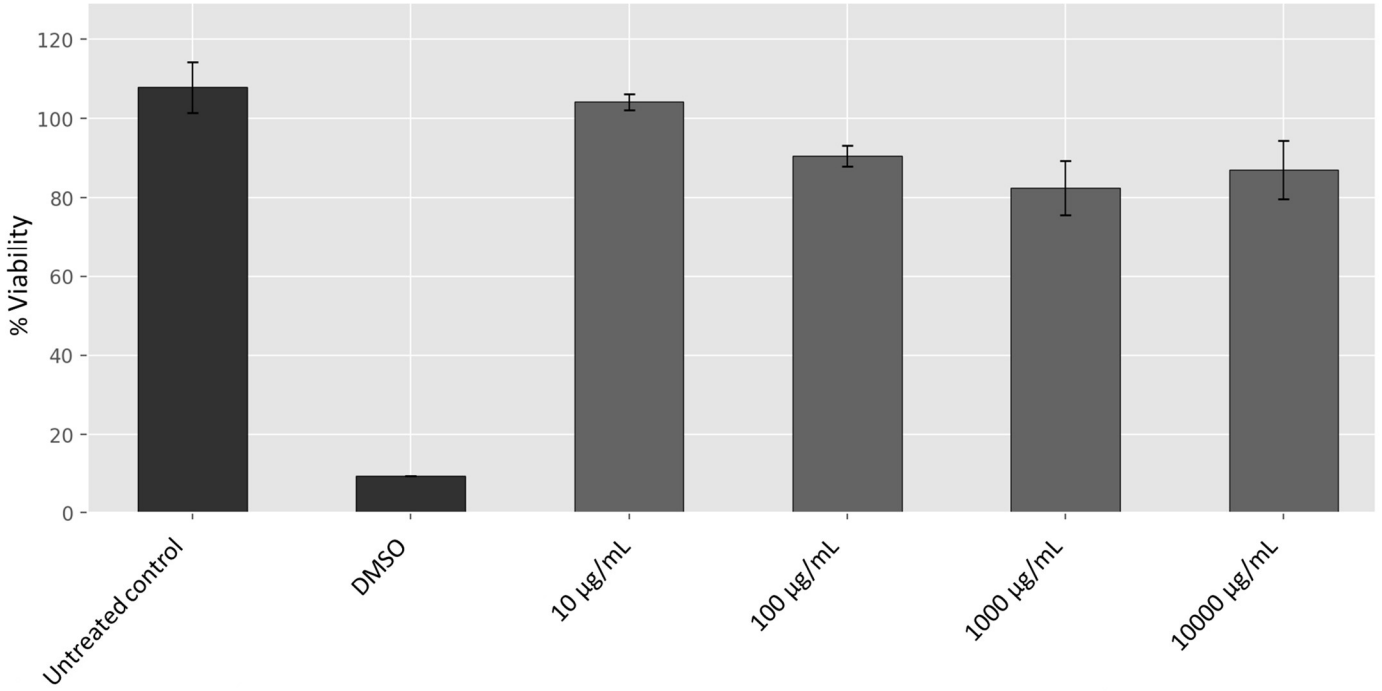


Figure 2.4 E**ffects of PSH on the viability of differentiated human macrophages.** Cells were differentiated with PMA 20 ng/mL for 72 hours into macrophages before treatment with the peptide for 24 hours. Cell viability was assessed by the MTT assay as per the manufacturer's guidelines. Values correspond to concentrations of PSH 1 tested at a range from 10 - 1000µg/mL. DMSO 100% and untreated cells served as controls. Data represent the mean of three experiments performed in triplicate and is expressed as the mean ± SD. Statistical analysis was performed using a Student's t test where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

2.4 Discussion

The focus of the present study was to produce a novel bio-preservative capable of inhibiting the growth of *E. coli* O157:H7 *in vitro*. Further, an infected leaf model was used to evaluate the potential activity of PSH as a bio-preservative against *E. coli* O157:H7. Here, we show that by conducting an enzymatic hydrolysis on *P. sativum*, a protein hydrolysate, PSH, could be produced which showed antimicrobial activity against this Gram-negative pathogen frequently implicated in foodborne disease. Other studies have described the potential preserving properties of PSH for example, as antifungals in the baking industry increasing the shelf life of wheat flour up to 21 days against certain strains (Rizzello *et al*. 2015).

Protein hydrolysis was conducted using food grade enzyme/s as has been commonly reported previously for plant ingredients such as wheat, rice and pea in order to release AMPs (Barac *et al*. 2012; Mccarthy, Callaghan, and Brien 2013; Hou *et al*. 2017). This is a contrast to keratinous proteins such as those from feathers and horns which are usually hydrolysed using acids (V. K. Pasupuleki and Braun 2010; Hou et al. 2017). PSH induced bactericidal effects *in vitro* against *E. coli* O157:H7 at 8 mg/mL. Furthermore, activity was observed with PSH against *S.* Typhimurium (data not shown), another relevant food safety pathogen, expanding the potential of the hydrolysate to a range of other food sources such as meat, poultry and seafood. Antimicrobial activity was absent in the unhydrolysed sample, indicating that it was the release of the peptide fragments from larger proteins which generated the bactericidal effects (Chalamaiah, Yu, and Wu 2018). The size exclusion – high performance liquid chromatography (SE-HPLC) chromatograms illustrate the difference in the hydrolysed and unhydrolysed samples where the latter eludes faster from the column based on its high molecular weight components, which avoided passage through the smaller pores.

NuriPep 1653, outlined in a previous paper (Mohan *et al*. 2019), was also identified from *P. sativum* and was shown to exhibit potent activity against multidrug resistant strains such as *A. baumannii*. The study also sheds light on the mechanism of action of the cationic peptide, which involves electrostatic attraction to the bacterial membrane followed by irreversible permeabilisation and lysis. This membrane active mode of action is common among many AMPs therefore it is likely that some of the peptides within the PSH work in this way to induce antimicrobial activity (Yeaman and Yount 2003a). Furthermore, protein hydrolysates contain a mixture of peptides which may function synergistically or individually conferring a wide range of bioactivities (Yoo *et al*. 2014). The MS output post-hydrolysis was screened

for peptides similar to NuriPep 1653 which may be responsible for the antimicrobial action of the PSH. While no peptides were identified bearing as high a charge (+4) or PI (11.26) as NuriPep 1653, peptides of similar length and percentage of hydrophobic residues were identified as shown and ranked in Table 2.2. We hypothesise that these peptides may indeed contribute to the functions of the PSH and could be investigated for their individual antimicrobial activities and potential use as antibiotic alternatives. This is suggested as isolated peptides are better suited to pharmaceutical applications given that they can be produced to a high quality, consistently, and their pharmacokinetic profile studied more easily than for protein hydrolysates. Also, the high production cost of pure synthetic peptides is prohibitive for application in food industry where profit margins are very tight.

Protein hydrolysates are cheap to produce, natural and may be able to replace some of the artificial ingredients or chemical washes currently employed in the food industry to control microbial spoilage. We have shown that PSH significantly reduced survival of *E. coli* O157:H7 on the surface of a lettuce leaf after just two hours of exposure to the protein hydrolysate. Furthermore, it outperformed the commercially available hydrolysate Leucidal liquid when used at 2%. One of the constraints of using hydrolysates in food products sometimes resides in the changes in the appearance and colour of the products. Some studies have described ways and means to decolourise and reduce haze in hydrolysates such as by adding charcoal powder (Hou *et al*. 2017). Based on our results, this would not be necessary for PSH since the visual appearance and odour of the treated lettuce compared to the untreated control sample did not change during this time (data not shown). Bitterness can be encountered with protein hydrolysates, in particular those with low molecular weight peptides, bulky hydrophobic amino acids at the C-terminus and basic amino acids at the N-terminus; all of these features have been implicated in the generation of adverse flavours (Lafarga and Hayes 2016; Maehashi and Huang 2009a; Hou *et al*. 2017). Sensory properties by means of tasting the PSH were not performed in the present study, however, protein hydrolysates are likely to be better tolerated and present less adverse impacts on the organoleptic profile when applied to food when compared to other natural preservative such as essential oils (Sultanbawa 2011). Natural oils have also been implicated with potentially altering the gut microbiome (Dorman and Deans 2000). This is not a concern with peptides and protein hydrolysates as they are subjected to natural digestion and absorption in the GI limiting antimicrobial activity to the actions on food prior to consumption (H Korhonen 2009).

The safety of various artificial preservatives and washing treatments has come into question in recent years. This is a paradox, as the aim of preservation is to ensure the safety of the ingested food from a microbiological perspective but may in fact cause biological harm in another way. PSH did not cause any cytotoxic effects at concentrations required to kill bacteria *in vitro* or on the lettuce. Protein hydrolysates in general have been reported as well tolerated and non-toxic, similar to the intake of intact proteins which have a history of safe consumption over hundreds of years and are subject to natural breakdown in the body, as described above (Schaafsma 2009). Here, the MTT assay was conducted in order to assess toxicity, however other methods such as the tryphan blue exclusion assay, resazurin blue assay, ATP assay and real time viability assay employing flow cytometry could also be used as a better measure of viability.

While limited toxicity is an advantage of protein hydrolysates, variability can sometimes occur based on seasonal, biological and chemical changes in the natural source material, which can limit their use. Despite this, little batch to batch variation was observed as all three independent PSH samples 1, 2 and 3 gave equivalent CE concentrations, performed well in the infected lettuce, and showed the same cytotoxicity profile in human cells. MS analysis of the peptides in each sample showed more than a 50% overlap in constitutively released peptides after hydrolysis, therefore we hypothesise, based on our findings that it is these commonly occurring peptides which are responsible for activity.

Most of the currently employed decontamination methods for fresh produce are not favourable for a number of reasons, but most importantly their safety to humans and the environment. Protein hydrolysates, such as PSH, represent an environmentally friendly, plant-based means to preserve food using the naturally occurring bioactive components found within food proteins. This cost effective, natural and novel bio-preservative may be applied to protect against various other pathogens in different foods and its full potential will be explored in future studies. This work opens the door to a “from food – for food” preservation approach for food products which will hopefully lead to the discovery of many more bioactive hydrolysates with applications in the food industry and beyond.

Chapter 3

Unlocking NuriPep 1653 from common Pea Protein: A Potent Antimicrobial Peptide to Tackle a Pan-Drug Resistant *Acinetobacter baumannii*

Note: This chapter is an accepted peer reviewed published article under the following citation;

Niamh M Mohan, Amine Zorgani, Gael Jalowicki, Alish Kerr, Nora Khaldi and Marta Martins (2019) Unlocking NuriPep 1653 from common Pea Protein: A Potent Antimicrobial Peptide to Tackle a Pan-Drug Resistant *Acinetobacter baumannii. Frontiers in Microbiology*. **10**, e2086. doi: 10.3389/fmicb.2019.02086.

3.1 Introduction

As we descend deeper into a post antibiotic era, the incidence of multidrug resistance extends to epidemic proportions. A significant threat, as recognised by the World Health Organisation (WHO) as a primary critical priority concern, is carbapenem-resistant *A. baumannii* (Tacconelli *et al*. 2018; World Health Organisation 2017). *A. baumannii* is an opportunistic, coccobacillus, Gram-negative bacterium. The importance of this nosocomial pathogen is escalating based on its ability to persist on artificial surfaces for extended periods as well as its propensity to acquire resistance to antibiotics (Ahmed *et al*. 2016). It is often life-threatening for critically ill, immunocompromised patients and is a major factor in complicating the treatment and rehabilitation of injured soldiers (Ahmed *et al*. 2016). For many years, carbapenems were considered as the standard therapeutic agents for MDR *A. baumannii* infections, however, their effectiveness is waning as the incidence of extensively drug resistant (XDR) pathogens continues to rise (Qureshi *et al*. 2015; T. Kim *et al*. 2014). Despite being abandoned for use in the 1970's, due to significant renal and neurological toxicity, bacterially derived lipopeptides, such as colistin, are now frequently used as a salvage treatment for XDR *A. baumannii* infections (Spapen *et al*. 2011; Pogue, Cohen, and Marchaim 2015). Unfortunately, increased reliance on this last-line therapy has also spurred resistance and has fuelled the surging progression from PDR to XDR *A. baumannii* posing major complications for modern healthcare (Subramani, Narayanasamy, and Feussner 2017; Tacconelli *et al*. 2018; Qureshi *et al*. 2015).

A recent revival in the exploration of AMPs as antibiotic alternatives has come as a consequence of the dwindling armoury of effective antimicrobial drugs and subsequent rise in resistance. The revival has transpired based on two critical attributes, their low propensity for inducing resistance and their broad spectrum activity based on a general mechanism of action (P. Meher *et al*. 2017). These attributes come as a result of the two major mechanisms associated to cationic peptides including direct bacterial killing, which is either membrane

or non-membrane targeting, and immune modulation. It is well described in the literature that the majority of AMPs display the former, where the peptide is attracted to the cell membrane by electrostatic interactions between the positively charged residues of the peptide and the negatively charged components in the bacterial cell membrane (Yeaman and Yount 2003b; Kumar, Kizhakkedathu, and Straus 2018). The general mechanisms of action described for AMPs means that they are often active against both antibiotic susceptible and resistant bacteria, which places them competitively among new antimicrobial solutions for the pharmaceutical sector.

The majority of AMPs discovered to date are non-ribosomal secondary metabolites assembled by enzymes in mammals, insects and bacteria. Until recently, very few had been exploited commercially as antimicrobials based on their toxicity, complex structures, and high production costs. A largely unexplored class of peptides, which may have an improved safety profile based on their source, are those found within regions of proteins (Schaafsma 2009). While the reason for the presence of these sequences, entrenched in proteins, is largely undetermined, it provides a new avenue of discovery for anti-infectives and peptides with other bioactivities. Recently, human pepsinogen A3-derived peptides were shown to exhibit potent antimicrobial functions in skin infection models whilst showing no toxicity to human cells (Pane *et al*. 2018). In another study, lactoferricin, a non-toxic AMP released from the milk glycoprotein, lactoferrin, post digestion is described (Bruni *et al*. 2016). In the case of milk, the release of functional peptides from proteins occurs in the mammary gland, were complex proteases pre-digest the proteins into bioactive peptide fragments for neonatal health (Dallas and German 2017). While further investigation is needed, bioactive peptides within phyto-proteins may confer specific health benefits or protection once digested in a similar way to milk, or else may have a role in the life cycle of the plant. In this study, we explore if we can apply the same rationale for AMP discovery in milk proteins, to plants, by finding peptides within edible plant proteins which exhibit potent antimicrobial effects against PDR *A. baumannii*.

Here, we describe NuriPep 1653, a novel, 22-mer peptide with the sequence VRGLAPKKSLWPFGGPFKSPFN, which was discovered in the region between residues 271 – 292 of the P54 nutrient reservoir protein of *P. sativum*. NuriPep 1653 was among one of the peptides in the library which were released after hydrolysis and was chosen based on a combination of features most associated with conferring antimicrobial activity. These 20 features are listed in Supplementary Table 3.1S. We provide an insight into NuriPep 1653's

potent bactericidal activity against *A. baumannii*. We also show its limited toxicity, adjuvant properties with colistin and its reduced likelihood to induce resistance. This study highlights the discovery of a novel AMP sequence from within a plant protein which may have important therapeutic significance against MDR, XDR and PDR strains.

3.2 Materials and Methods

3.2.1 Deciphering Features Conferring Antimicrobial Activity in Peptides

The discovery pipeline is shown in Fig 3.1. AMPs were collected from the Collection of Anti-Microbial Peptides (CAMP)[6] database (Waghu *et al*. 2016). To focus on relatively short peptides, active sequences were filtered by a length threshold of 40 for a total of 1957 sequences. A set of 5000 non-AMPs whose length does not exceed 40 amino-acids was also extracted from Uniprot (https://www.uniprot.org/). Only sequences containing standard amino acids were considered. Individual physicochemical properties of amino acids were pre-selected from curated cationic AMPs. For each physicochemical property, values were summed across each peptide sequence and then averaged by sequence length. To evaluate which properties were relevant to antimicrobial activity, property distributions from both active and negative datasets were compared using the non-parametric Kolmogorov-Smirnov test. Sequence scores were then ranked, and the top 20 properties kept as the most significant to antimicrobial activity. This approach was designed in conjunction with Dr. Gael Jalowicki, Nuritas, Ltd.

[6] CAMP: (http://www.camp.bicnirrh.res.in/)

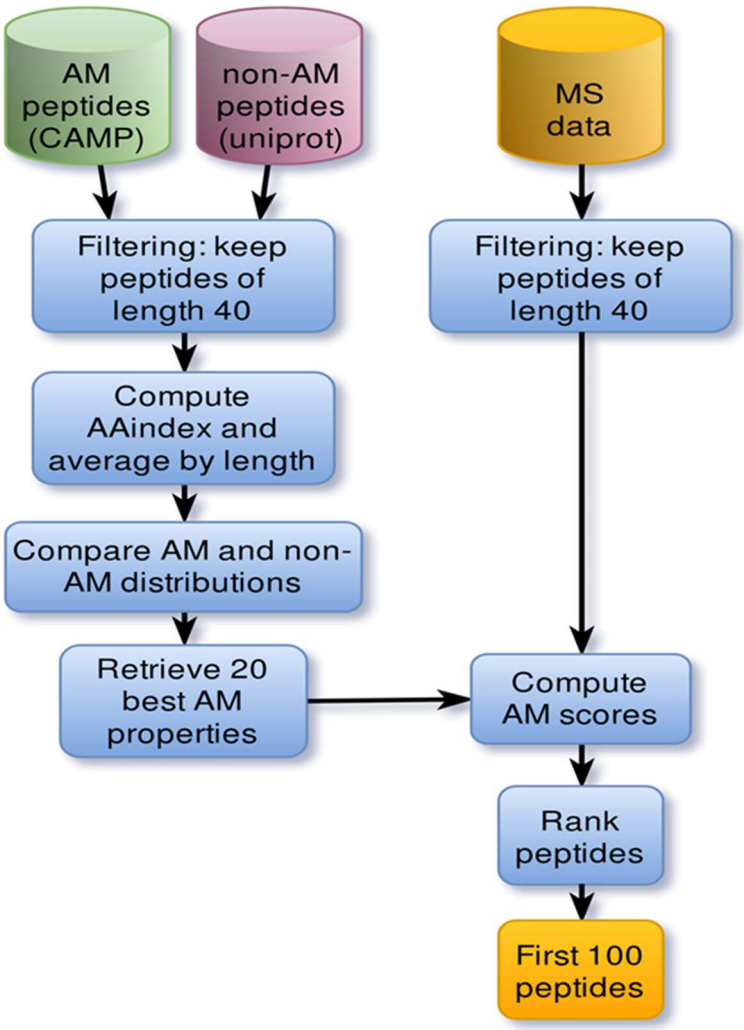


Figure 3.1 Main steps of the pipeline that led to the discovery of NuriPep 1653. Computational steps involved in the discovery of antimicrobial related properties and AMPs. Cylinders correspond to datasets. First positive (green) and negative (red) datasets were extracted from CAMP and Uniprot databases, respectively. Sequences whose length exceed 40 amino acids were discarded based on the cut-off length for peptides. Sequence physicochemical properties were computed based on individual amino acid scores retrieved from AAindex. Those scores were then normalised by sequence length. Comparison of property distributions yielded 20 main antimicrobial properties which were subsequently used to rank peptides identified with MS. The resulting set of 100 peptides were then manually curated. This approach was designed in conjunction with Dr. Gael Jalowicki, Nuritas, Ltd.

3.2.2 Peptides, Antibiotics and Reagents

The peptide sequence was synthesised by GenScript (Piscataway, NJ) using solid-phase 9-fluorenylmethoxy carbonyl (Fmoc) chemistry and purified to a purity of 95-99% using reverse-phase HPLC. Peptide mass was confirmed by MS. MHB and MHA. THP-1 cells were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA). Oxoid brand were purchased from Sparks Lab Suppliers, Ireland. All antibiotics powders and discs were purchased from Sparks Lab suppliers. The LIVE/DEAD *Bac*Light kit was supplied by Molecular Probes (Invitrogen, Ireland).

3.2.3 Bacterial Culture Preparation and Growth Conditions

A. baumannii resistant (ColRAB) and susceptible (colSAB) (ATCC 19606) to colistin were kindly donated from the Institute of Hygiene and Tropical Medicine, Universidade Nova de Lisboa, Lisbon, Portugal. ColRAB was isolated from a bronchial secretion of a patient presenting with pneumonia and a urinary tract infection post bladder reconstruction (Machado *et al*. 2018). *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *S.* Typhimurium ATCC 14028, *Listeria monocytogenes* ATCC 11994, *Enterobacter aerogenes* ATCC 13048 and *Staphylococcus aureus* ATCC 25923 were gifted from the School of Food Science and Environmental health, Dublin Institute of Technology, Dublin. *Burkholderia cepacia* NCTC 10744 and *Stenotrophomonas maltophilia* NCTC 10257 were purchased from Public Health England. *Klebsiella pneumoniae* isolate 50183 was kindly donated by St. James' hospital, Dublin. Cultures were maintained at -80ºC in 15% glycerol. All bacteria were grown in MHB. Cultures were prepared by inoculating 10 mL of the selected media with bacteria and incubating overnight for 18 hours at 37ºC. A subculture was prepared from the overnight bacterial suspension by diluting it to an OD at 600 nm (OD_{600}) of ~ 0.05 in fresh media and re-incubating under optimal conditions for each bacterium for 2-3 hours until logarithmic phase was reached (OD_{600} ~ 0.5). Bacterial cultures were then adjusted to the desired concentration for assay in PBNS.

3.2.4 Disk Diffusion Method

The antibiotic susceptibility profile of the clinical isolate of *A. baumannii* and colSAB was rechecked against the values previously reported by Machado *et al*. (2018). This was conducted using the Kirby Bauer disk diffusion method as per CLSI guidelines (CLSI 2013). Briefly, agar plates were lawned with bacteria diluted to OD_{600} ~ 0.1. Antibiotic disks were placed onto the agar surface and allowed to incubate overnight at 37°C. The zone of inhibition (ZOI) produced by the antibiotic was then used as a measure of susceptibility or

resistance of the bacteria to the antibiotic according to the breakpoints outlined in the EUCAST guidelines (The European Committee on Antimicrobial Susceptibility Testing 2019).

3.2.5 Antimicrobial Activity Assays

3.2.5.1 Broth Microdilution Method

The MIC and MBC were determined aerobically in a 96 well plate as previously described (CLSI 2013). This method was used to determine the antibiotic susceptibility profile of the bacteria to antibiotics only, as an alternative method, described below, was used in the case of the peptides. Briefly, bacterial strains were grown aerobically shaking to mid exponential phase at 37°C in 5 mL of appropriate broth, depending on the strain. The compounds were then serially diluted to the desired concentrations in the microtiter plate to a final volume of 100 µL. Cultures were diluted in PBNS to a final concentration of 2.0×10^4 and 4.5×10^5 cells/mL and added to each well of the plate, except the negative control containing only media. The MIC was determined as the lowest concentration inhibiting bacterial growth after incubation at 37°C for 18 hours and the MBC was defined as the lowest concentration to completely kill the bacteria. The MBC is performed using a pin replicator to transfer 1 µL from every well of the MIC plate into the corresponding wells of a compound free plate containing only 100 µL of media. The plate is then incubated at 37°C for 18 hours. The MBC was determined as the concentration showing no growth at all (determined either by eye or by reading in a spectrophotometer at 600 nm) indicating complete cell death.

3.2.5.2 Complete Elimination Drop Plate Method

The CE method with PBNS buffer was primarily used as the antimicrobial susceptibility testing method for peptides used in this study. It should be mentioned that the CE assay was performed in various media including cation-adjusted MBH, Nutrient Broth (NB), Brain Heart Infusion Broth (BHI) and RPMI, however the ion content in all the above-mentioned media, interfered with the activity of the peptide. Hence, PBNS was used in order to perform the experiments included in this paper and to solely study the action of the peptide on the bacteria and avoid any interference of cations within the test media or buffer on the bioactivity of the peptides. The CE concentrations in the above-mentioned media are highlighted in Supplementary Table 3.2S. Farkas *et al.* (2018) and Travis *et al.* (2000)

explain the modified buffers they used in order to avoid ion interference. The CE assay was performed by the method outlined by Chen *et al* (2003) with minor modifications. Briefly, bacteria were grown overnight and sub-cultured to mid exponential phase. PBNS was used to make serial dilutions of bacteria to a concentration of between 1.0 x 10^4 and 1.0 x 10^5 cells/mL. The appropriate volume of peptide was added to the wells at a final volume of 100 µL. As a control, PBNS was added to the bacterial suspensions without any peptide. The microtiter plate was incubated for 90 minutes at 37°C. Post challenge, 10 µL from each well was spotted onto appropriate media, depending on the strain. This was conducted in triplicate on three separate days. The spots were left to dry, and the plates were incubated inverted. The first concentration to inhibit growth across all bacterial dilutions was determined as the CE concentration. Surviving colonies at sub-CE concentrations were counted after 18 hours incubation at 37°C. The reduction in CFU was assessed relative to the untreated control and the log reduction induced by peptide treatment was calculated. All antimicrobial activity assays were performed in triplicate and on independent days.

3.2.6 Synergy Studies and Measurement of Fractional Inhibitory Concentration

Synergy studies were conducted using the above described CE drop plate method, however two peptides were mixed in a series of concentration combinations using PBNS as a diluent before bacterial inoculation and subsequent incubation. The highest concentration combination tested was the individual CE concentrations of NuriPep 1653 and colistin, which were 12 µg/mL and 8 µg/mL, respectively. The amounts of each compound were then reduced sequentially in various proportions until the lowest possible active combination was identified.

Traditionally, the fractional inhibitory concentration (FIC) in a drug mixture is defined as the sum of the FICs (ΣFIC) and is calculated with the equation;

$$\Sigma FIC = FIC_A + FIC_B = (CE^{comb}{}_A/CE_A) + (CE^{comb}{}_B/CE_B)$$

Where CE_A and CE_B are the killing concentrations of individual peptides A and B, respectively, and $CE^{comb}{}_A$ and $CE^{comb}{}_B$ are the killing concentrations of the peptides in combination. In this work we used a similar concept and analysed the ratios of FIC_A and FIC_B. Synergy was interpreted as a FIC ≤ 0.5, antagonism as FIC > 4.0 and no interaction as FIC > 0.5–4.0 (Odds 2003).

3.2.7 Valence Sensitivity Study

A variation of the CE drop plate method was used to determine valence sensitivity of the peptide. The concentration of NuriPep 1653 required to kill bacteria diluted in sodium chloride (NaCl) or calcium chloride ($CaCl_2$) at concentrations ranging from 0 to 150 mM was determined (Menzel *et al*. 2016).

3.2.8 Kinetics of Antimicrobial Activity

The killing kinetics, or time kill assay was performed according to a previously published standard protocol (Lorian 2005). The experiment was performed in duplicate. Bacterial cells from mid exponential growth were collected, washed and adjusted to a final density of 1.0 x 10^7 to 1.0 x 10^8 cells/mL in PBNS buffer. The cells were incubated at 37°C with different concentrations (12, 48 and 192µg/mL) of NuriPep 1653 to a final volume of 2 mL. At T10, 20, 30, 60 and 120 mins, 0.1 mL of the culture was removed, diluted appropriately in PBNS and spread plated on MHA. The CFU/mL at each timepoint was calculated after overnight incubation at 37°C. Cells treated with PBNS alone were used as the control.

3.2.9 Membrane Damage of Bacterial Cells

3.2.9.1 Cell Viability and Membrane Permeabilization Assessed by Flow Cytometry

The Live/Dead *Bac*Light viability kit was used as described by Joshi *et al.* (2010) with minor modifications [26]. *A. baumannii* cells were collected in mid exponential phase, washed three times with PBNS, and resuspended at a final concentration of 1 × 10^6 cells/mL in the same buffer. This was followed by addition of NuriPep 1653 at the CE concentrations (12µg/mL) and incubation at 37°C for 90 minutes. Cells treated with PBNS were used as the live control and cells heat treated for 60 minutes at 90°C served as the "dead" control (maximum of fluorescence). These samples were used to establish the initial gate between live and dead populations (data not shown). All samples were prepared to a final volume of 1 mL before the addition of 6 µL of a premixed solution containing a 1:1 ratio of SYTO9 (3.34 mM), a green-fluorescent nucleic acid stain and propidium iodide (Pi) (20 mM), a red-fluorescent nucleic acid stain. Once the dyes were added, samples were incubated for 15 minutes at room temperature in the dark. Flow cytometry analysis was conducted using the Accuri C6 Flow Cytometer. For each sample 50,000 cells were analysed. The height of the forward scatter (FSC) and side scatter (SSC) of stained cells was analysed using 488/530

emission and excitation filters. The experiment was conducted in duplicate and data was analysed by C Flow Plus software (Becton Dickinson, San Jose, CA, USA).

3.2.9.2 Scanning Electron Microscopy

Suspensions of *A. baumannii* were grown in MHB until mid-exponential phase before being washed three times with PBNS and resuspended at an OD_{600} of ~ 0.5. The CE concentrations of NuriPep 1653 against this bacterial density was determined (32 µg/mL) and used to treat the cells for 90 minutes. After incubation, the cells were pelleted *via* centrifugation at 14,000 RPM for 10 minutes and then washed with PBNS. This process was repeated three times with the final re-suspension done using glutaraldehyde 2.5% (weight/volume) (w/v) diluted in PBNS to fix the cells. The fixed cells were dehydrated in a hexamethyldisilasane (HDMS) gradient at 10, 30, 50, 70, 90, 100% before a final overnight drying *via* evaporation. Finally, the samples were mounted onto stubs and sputter coated with gold. Scanning Electron Microscopy (SEM) images were obtained in the Core Imaging facilities in the Conway Institute, University College Dublin (Dublin, Ireland) using a Hitachi Scanning Electron Microscope (Hitachi High Technologies Europe) at a voltage of 5.0 kilovolts (kV).

3.2.10 In vitro Resistance Study

This assay was conducted as outlined by Ge *et al*. (1999) with some modifications. Briefly, the *in vitro* passage study involved exposing bacteria diluted to 1 x 10^4 cells/mL in PBNS to NuriPep 1653 at one-half of the previously established bactericidal concentration. After 90 minutes of incubation at 37°C, 10 µL was spotted in triplicate on MHB and incubated again overnight. Post-incubation, surviving colonies were collected, diluted in PBNS and adjusted to 1 x 10^4 cells/mL before repeated treatment with the sub-killing concentration of NuriPep 1653. The CE concentration was determined prior to the initiation of the study, after the 4^{th}, 7^{th}, 11^{th} and 14^{th} passages. If the CE concentration after the fourth passage was greater than the original killing concentration, then for passages 5, 6, and 7, the amount of peptide used during the bacterial challenge was increased to one-half of that new CE concentration. This was continued over a 14-day course. Magainin and colistin were used as controls for the *in vitro* resistance study. This assay was performed in independent duplicates.

3.2.11 Toxicity Assay

3.2.11.1 Ex-vivo viability assay

Cellular viability was assessed in human THP-1 macrophages by the MTT colorimetric assay (MTT, Sigma) (Silva *et al*. 2016). Cells were grown in RPMI medium supplemented with 10% of heat inactivated FBS, 1% L-glutamine and 1% penicillin-streptomycin. At 90% confluence, cells were centrifuged and diluted to a 1:5 ratio with complete RPMI. The monocytes were differentiated into macrophages using PMA 20 ng/mL before being seeded in a 96 well plate at a density of 1.0 x 10^4 cells/well and incubated in the presence of 5% CO_2 for 72 hours at 37ºC. Following this, cells were treated with increasing concentrations (0.05, 0.5, 5, 50, 500 µg/mL) of NuriPep 1653, magainin 2 or colistin before re-incubation for a further 24 hours. Post-incubation, 110 µL of MTT was added to each well at a final concentration of 500 µg/mL and incubated at 5% CO_2 for 2 hours at 37ºC. Formazan crystals were dissolved by the addition of 100 µL 100% DMSO and gentle shaking for 5 minutes at room temperature in the dark. The absorbance was measured at 570nm in a microplate spectrophotometer (Synergy H1, Biotek). Maximum toxicity was determined by cells incubated with 100 % DMSO. Cell viability was calculated as a per cent of a control treated with PBNS. Three technical replicates were performed each day on three independent days.

3.2.11.2 Haemolytic Assay

The effects of NuriPep 1653 on sheep red blood cells (RBCs) was evaluated by a haemolysis assay (Lu *et al*. 2016). In brief, 100 µL of fresh peripheral blood from a healthy sheep was added with 4 µL of heparin and centrifuged at 25ºC for 15 minutes at 2000 rpm. The RBCs were washed and resuspended three times in phosphate buffer (PBS) and before being prepared as a 2% suspension. The RBCs were seeded in a 96 well plate along with increasing concentrations of the peptide to a final volume of 100 µL. Triton X-100 was used as a positive control (for lysis) and PBS used as the negative control (no lysis). Post-incubation at 37ºC for 90 minutes, the samples were centrifuged at 2000 rpm for 10 minutes and the absorbance was measured at 540 nm in a microplate spectrophotometer (Synergy H1, Biotek). The degree of haemolysis was calculated according to the following: % of haemolysis = [(Sample absorbance – negative control) / (positive control – negative control)] × 100%.

3.3 Results

3.3.1 Antibiotic Susceptibility Profile of A. baumannii Strains

A. baumannii ATCC 19606 was susceptible to 7 of the 8 antibiotic classes tested (Table 3.1). Based on its susceptibility to colistin, it will be denoted as colSAB. Conversely, the clinical isolate showed resistance to all the antibiotics tested, including the last line lipopeptide, colistin, defining it as a PDR isolate which will be referred in this study as colRAB. Our results were in line with previously reported findings (Machado *et al*. 2018).

Table 3.1 Antibiotic resistance profile of colRAB and colSAB

	Penicillin		Cephams		Carbapenems			Aminoglycosides	Tetracycline	Fluoroquinolone	Folate pathway inhibitors	Lipopeptides
Bacterial Strain	**AMP**	**TIC**	**CN**	**CTX**	**MEM**	**ETP**	**IMP**	**AK**	**TE**	**CIP**	**W**	**Colistin***
***A. baumannii* ATCC 19606**	20	22	29	23	20	21	31	20	30	29	0	0.25
***A. baumannii* colRAB**	0	0	0	14	0	0	10	0	0	0	0	128
***E. coli* ATCC 25922**	24	22	32	24	23	24	30	17	25	26	18	0.25

Legend: The values refer to the diameter of a ZOI produced by the antibiotic, measured in millimetres. Based on this value, the susceptibility profile to the antibiotics are grouped as defined by the CLSI guidelines (CLSI 2013) as light grey = susceptible and dark grey = resistant. AMP; Ampicillin, TIC; Ticarcillin, CN; Cefotetan. CXT; Cefotaxime, MEM; Meropenem, ETP; Ertapenem, IMP, Imipenem, AK; Amikacin, TE; Tetracycline, CIP; Ciprofloxacin, W; Trimethoprim, and Colistin. *The antibiotic susceptibility profile of colistin was determined *via* broth microdilution method. *E. coli* ATCC 25922 was used as a reference quality control strain as per CLSI recommendations.

3.3.2 Unlocking and Identifying Potent Peptide Sequences from Vegetable/Legume Proteins

The peptide discovery approach used in this study is outlined in Fig. 3.1. By using the top 20 features identified as important in conferring antimicrobial activity to rank the sequences identified from the MS data of hydrolysed *P. sativum* protein material, NuriPep 1653 was identified as the top candidate for further investigation. Fig. 3.2 is a t-distributed Stochastic Neighbour Embedding (t-SNE) plot representing all the peptides used in the study; positive and negative data sets obtained from CAMP and Uniprot[7] databases, respectively; MS data obtained by hydrolysis of *P. sativum*; and top scoring peptides from the feature ranking. Clusters in the data can be observed which may spear based on similar structures and characteristics *e.g.* negative peptides in co-ordinates (Grey -65, -15), and positive peptides in co-ordinates (Yellow -25, 45). The ~4000 peptides identified by MS were wide spread across the plot (Blue) as were the 100 top ranked peptides (Red). NuriPep 1653 is represented by a pink star at (-12, -38). Dr. Gael Jalowicki, Nuritas Ltd., was involved in the design and generation of the t-SNE plots, data-mining and aided in designing the discovery approach.

[7] Uniprot: https://www.uniprot.org/

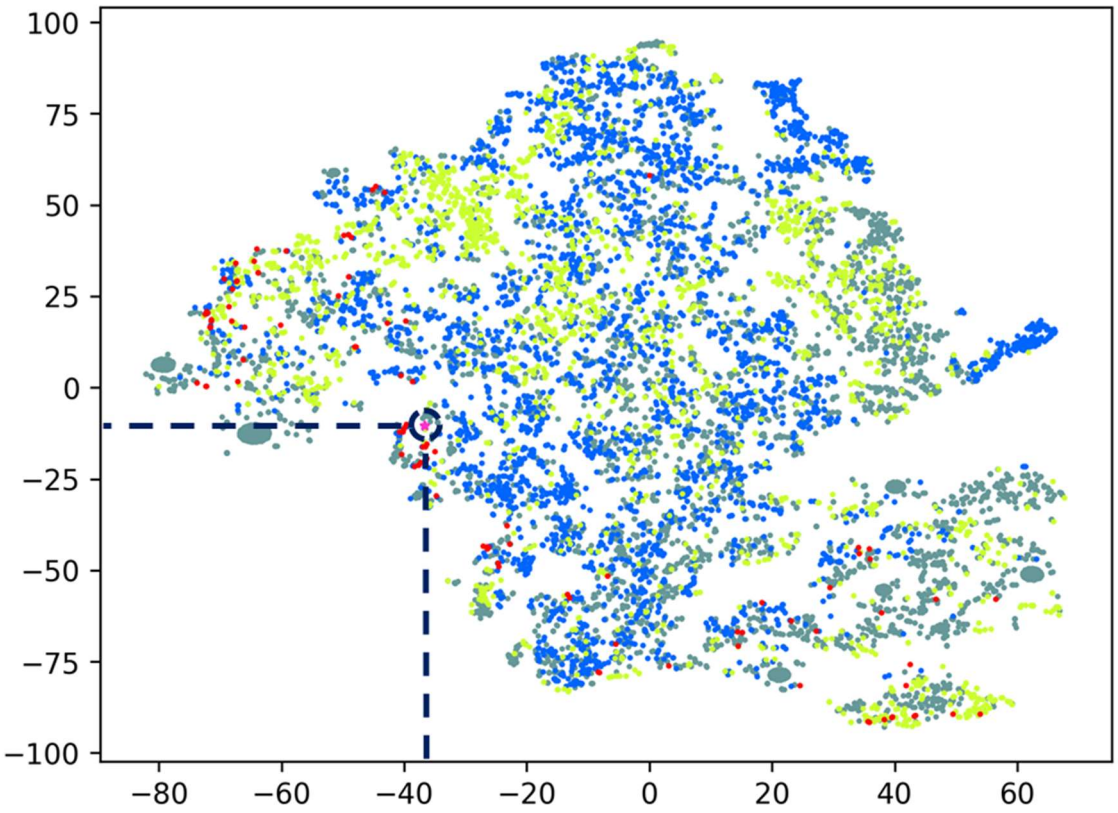


Figure 3.2 t-distributed Stochastic Neighbour Embedding plot comprising all peptide data used in this study. Peptide sequences were transformed using all physicochemical features, averaged by peptide length. Grey: non-active peptides; yellow: active peptides; blue: peptides from MS analysis; red: top active peptides; Pink star: NuriPep 1653 (identified with a navy-blue broken circle). Generated by Dr. Gael Jalowicki, Nuritas Ltd.

NuriPep 1653 (VRGLAPKKSLWPFGGPFKSPFN) is a linear 22-mer peptide with a global positive charge (+4) as a result of 1 arginine and 3 lysine residues in the sequence. Based on the cationic and amphipathic characteristics, we can assume a membranolytic step being involved in the killing of *A. baumannii*. The structure of the protein and isolated peptide was predicted by Iterative Threading ASSEmbly Refinement (I-TASSER)[8] (Fig. 3.3A). It identifies structural templates from the PDB by multiple threading approach, with full-length atomic models constructed by iterative template-based fragment assembly simulations. The bioactive peptide is located inside the non-antimicrobial, storage protein P54 from *P. sativum* (Fig. 3.3B) and is predicted to have a mostly linear structure with a single turn and a bend at residue 13 and 14, respectively (Fig. 3.3C). The lack of cysteine residues contributes to the linearity of the sequence. Finally, motifs identified as common from a set of 3496 known AMPs are outlined in Fig. 3.3D. The 2-amino acid residues KK located in position 7 and 8 of NuriPep 1653 appear in 33% of the curated AMPs. Glycine paired with leucine, glycine and arginine also features highly as commonly conserved motifs and therefore might be important in antimicrobial function.

[8] I-TASSER: (https://zhanglab.ccmb.med.umich.edu/I-TASSER/).

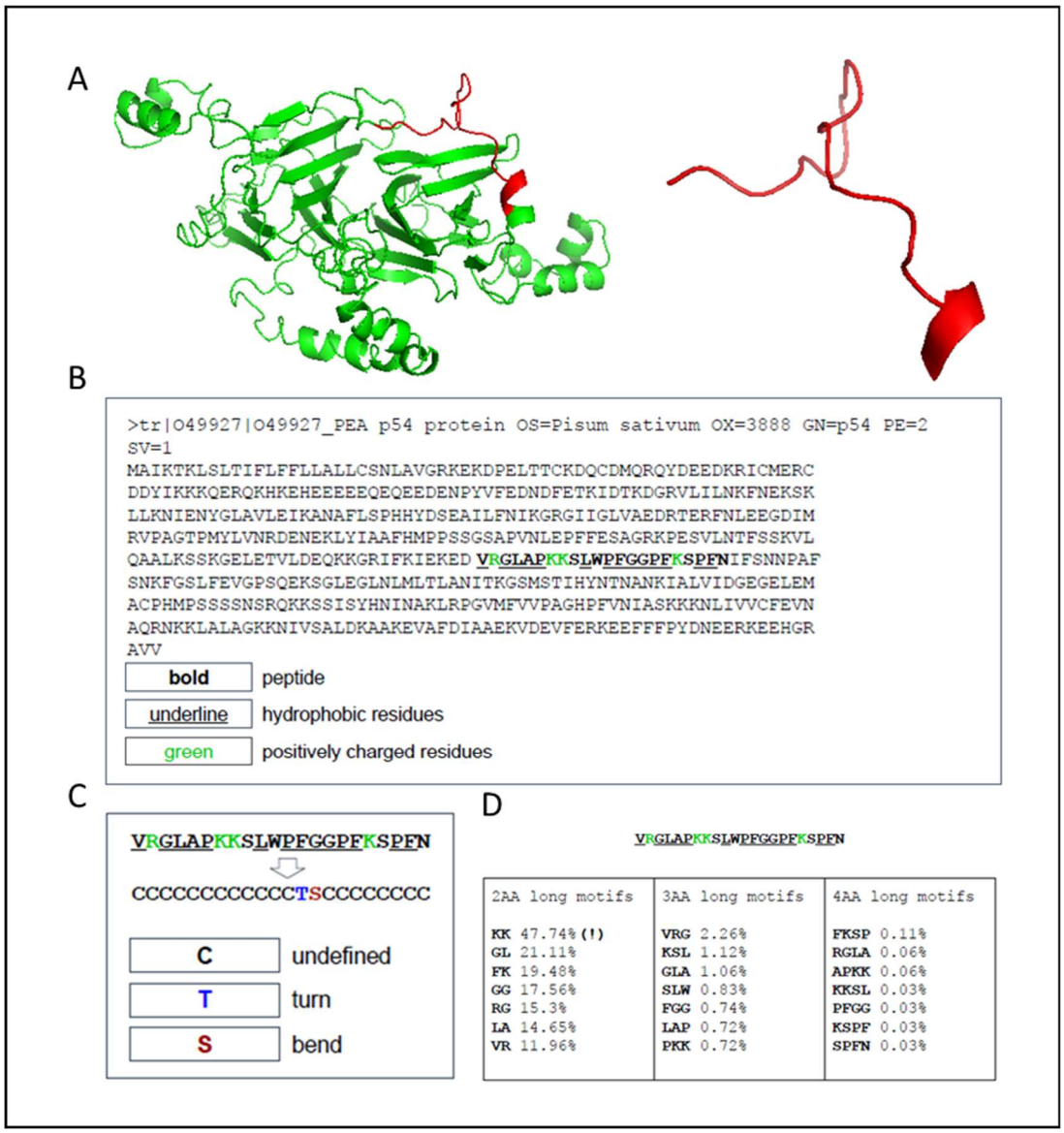


Figure 3.3 Sequence Location, structure and antimicrobial motifs of NuriPep 1653 derived from P54 *P. sativum* protein. The sequence structure and folding pattern as predicted by I-TASSER is shown in (**A**). The sequence of the storage reservoir P54 reservoir protein with the location of NuriPep 1653 highlighted in bold, hydrophobic residues underlined and positive resides in green (**B**). The peptide conformation is shown in (**C**) as determined by SCRATCH-1D. The most recurrent exact motifs of length 2, 3 and 4 amino acids within NuriPep 1653 and occurring in 3496 curated AMPs are shown in (**D**). Recurrent motif analysis was performed in conjunction with Dr. Alessandro Adelfio, Nuritas Ltd.

3.3.3 Antimicrobial activity of NuriPep 1653 Against colRAB

The antimicrobial activity of NuriPep 1653, magainin 2 and colistin against colSAB and colRAB are summarised in Table 3.2. The activity of NuriPep 1653 against a range of Gram-negative and -positive are also shown. Interestingly, the concentrations of NuriPep 1653 required to eliminate both colSAB and colRAB, were identical at 12 μg/mL indicating that the mechanism of action of the peptides is not hindered by any of the genes associated which confer resistance or enhanced virulence in the clinical isolate. Similar results were observed with magainin 2, however at concentrations 4 times higher than NuriPep 1653. The CE concentration of 8 μg/mL confirmed resistance to colistin according to the EUCAST guidelines (MIC ≤ 2 μg/mL) (The European Committee on Antimicrobial Susceptibility Testing 2019). While the peptides likely share an initial membranolytic mechanism of action based on their cationic nature, the difference in activity observed between NuriPep 1653 and colistin against colSAB and colRAB suggests that a difference may exist in a subsequent bactericidal action inside the cell. In addition to its activity against *A. baumannii*, NuriPep 1653 displays activity against three other ESKAPE pathogens (*P. aeruginosa, K. pneumoniae,* and *E. aerogenes*) with CE concentrations ranging from 8 μg/mL – 400 μg/mL. None of the peptides shown here display activity against either of the Gram–positive bacteria. This may be due to the difference in the cell wall composition and thickness compared to Gram-negative strains, however testing in a wider range of strains would be necessary to validate this.

Table 3.2 Complete Elimination values of NuriPep 1653 against colSAB and colRAB as well as a range of Gram-negative and -positive pathogens

Strain	NuriPep 1653	Magainin 2	Colistin
		μg/mL	
colSAB	12	50	0.25
colRAB	12	50	8 (R)
S. maltophilia	64	NT	NT
B. cepacia	NI	NT	NT
P. aeruginosa	8	NT	NT
K. pneumoniae	400	NT	NT
E. coli	100	NT	NT
***S.* Typhimurium**	100	NT	NT
S. aureus	NI	NT	NT
L. monocytogenes	NI	NT	NT
E. aerogenes	200	NT	NT

Legend: Values of NuriPep 1653 required to induce bacterial clearance *via* the CE method against colSAB, colRAB and a range of Gram-negative and -positive pathogens. Magainin 2 and colistin were used as controls for the *A. baumannii* strains used in this study. (R) = resistant as defined by EUCAST breakpoint guidelines as (MIC ≤ 2 μg/mL). NI = No inhibition at concentrations < 800 μg/mL. NT = Not tested. Values shown represent the average of three independent experiments on three independent days.

3.3.4 Kinetics of Antimicrobial Action Induced by NuriPep 1653 Against colSAB and colRAB

As shown in Fig. 3.4, the rate of bacterial killing was concentration dependent in both colSAB and colRAB. At the CE concentration (12 μg/mL), between 10^4 and 10^5 CFU/mL were eliminated after 120 minutes for colSAB and colRAB, respectively. However, when the peptide concentration was increased to 48 μg/mL, total bacterial clearance (10^8 CFU/mL) was observed after just 20 minutes in colRAB as opposed to 60 minutes in colSAB. At 16x the CE (192 μg/mL) rapid killing kinetics was achieved at 10 and 20 minutes for colSAB and colRAB, respectively. No regrowth was observed after 24 hours indicating bactericidal activity as opposed to inhibitory action.

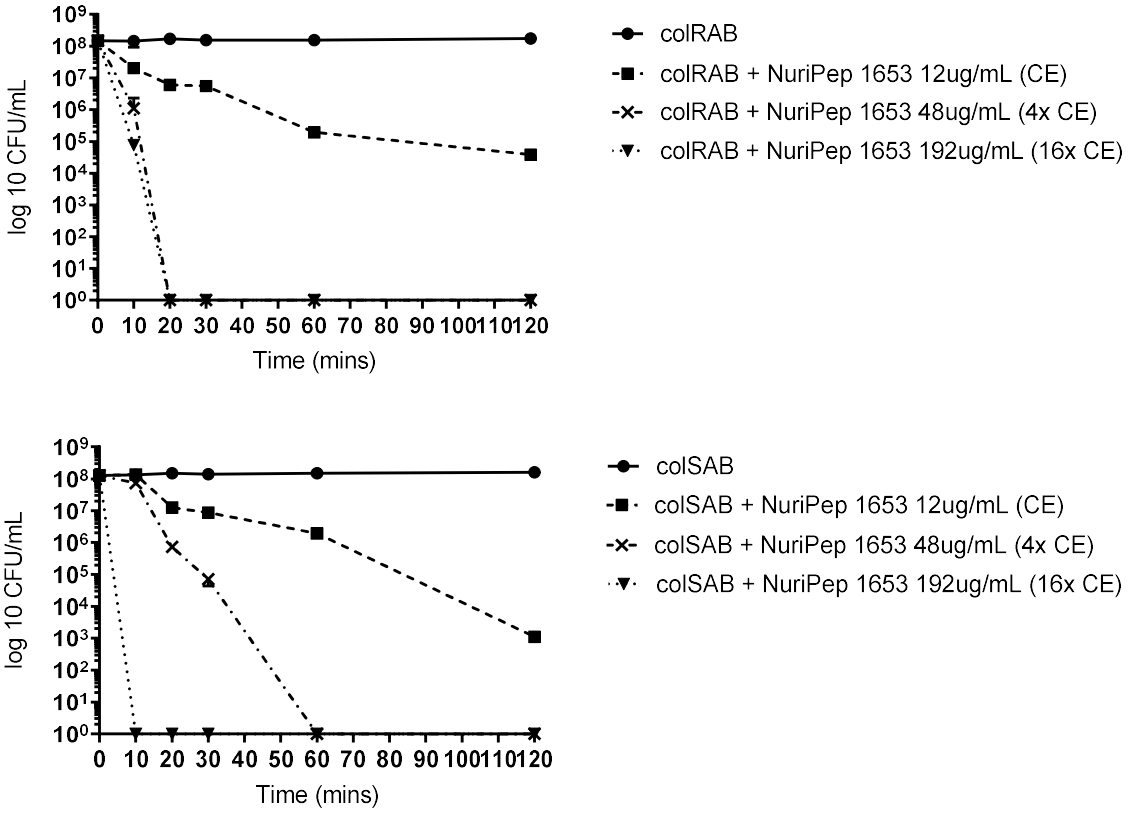
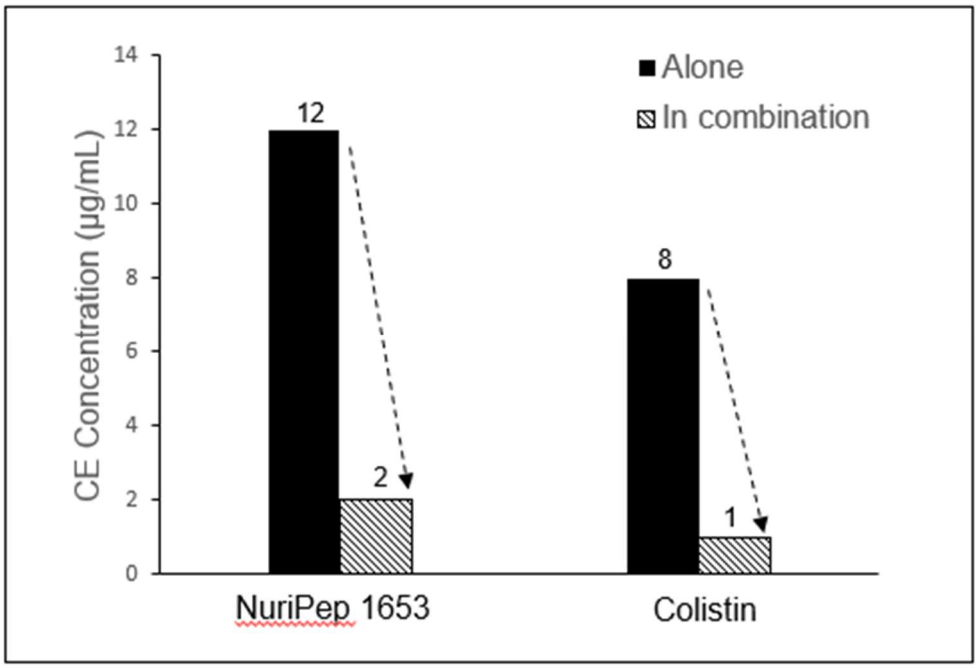


Figure 3.4 Killing kinetics of NuriPep 1653 against colRAB and colSAB. The killing activity of NuriPep 1653 against (**A**) colSAB and (**B**) colRAB was monitored for 120 minutes. Peptide treatments at 1x CE (12 µg/mL), 4x CE (48 µg/mL) and 16x CE (192 µg/mL) concentrations were tested. The CFU/mL was calculated at the timepoints 10, 20, 30, 60 and 120 minutes and was compared to the untreated control. Data represent the mean of three experiments performed in triplicate and is expressed as the mean ± SD.

3.3.5 Synergy Assessment of NuriPep 1653 and Colistin Against colRAB

In order to assess potential synergistic interactions between NuriPep 1653 and other FDA approved AMPs, we combined the use of NuriPep 1653 with colistin, a last resort cationic lipopeptide. When NuriPep 1653 was combined with colistin, the CE concentrations could be reduced from 12 µg/mL and 8 µg/mL, the concentrations previously determined to induce killing alone respectively, to 2 µg/mL and 1 µg/mL (Fig. 3.5). Using the FIC index, this provides a score of 0.291, thus indicating a synergy between the two peptides. This is indicative that both peptides may kill *A. baumannii via* different mechanisms post initial disruption of the membrane based on the improved combined action.



Compound	CE ug/mL	FIC score
NuriPep 1653	12	N/A
Colistin	8	N/A
NuriPep 1653 + Colistin	2 + 1	0.291

Figure 3.5 Assessment of synergistic interactions between NuriPep 1653 and colistin against colRAB. Changes in CE concentration observed with NuriPep 1653 and colistin against colRAB when tested alone *versus* in combination together to determine synergistic effects. A FIC index score of 0.291 for both peptides combined indicates synergy.

3.3.6 Significance of Electrostatic Interactions in the Initial Mechanism of Action of NuriPep 1653

To validate the importance of electrostatic interactions in the mechanism of action of the cationic peptide NuriPep 1653, we assessed the activity of the peptide when pre-exposed to mono- and di-valent cations in the form of NaCl and $CaCl_2$. Valence sensitivity was observed with the CE concentration increasing from 8 to 100 μg/mL in the presence of 12.5 mM of NaCl (Fig. 3.6). Activity was completely lost when the peptide was exposed to either of the cationic buffers above 50 mM. These results confirm that disruption of electrostatic attractions induced a concentration and ion dependent inhibition, which impacted peptide primary attachment and subsequent cellular disruption in colSAB.

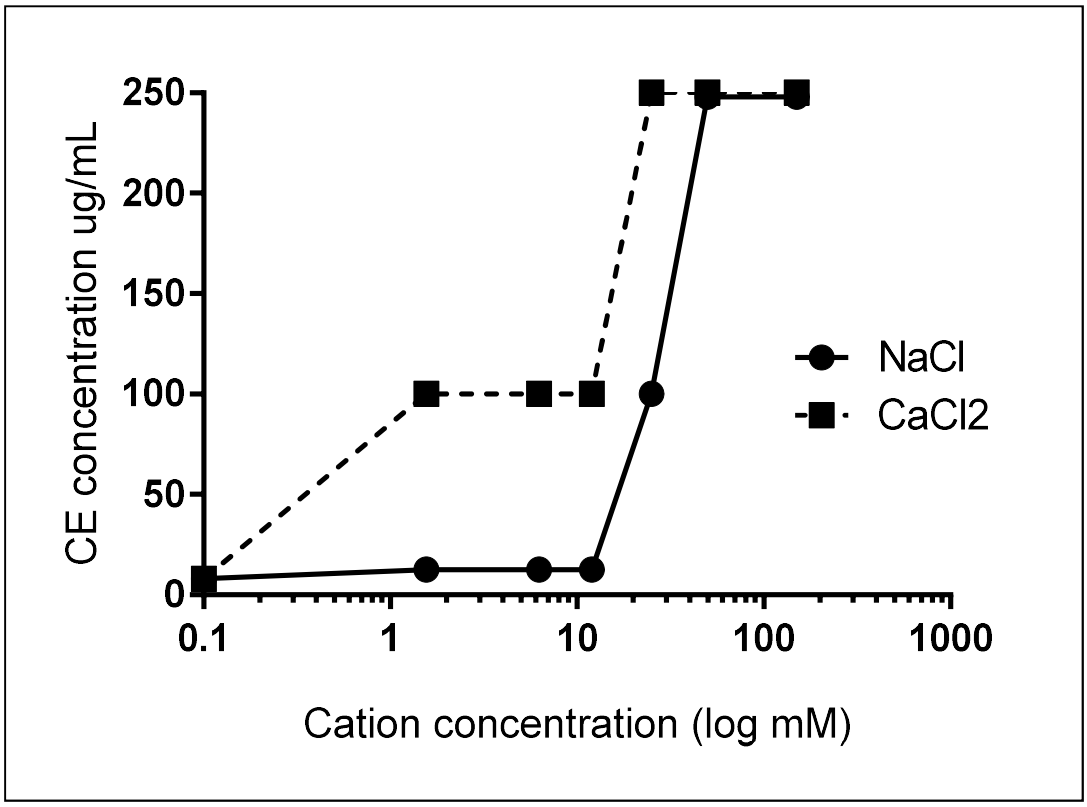
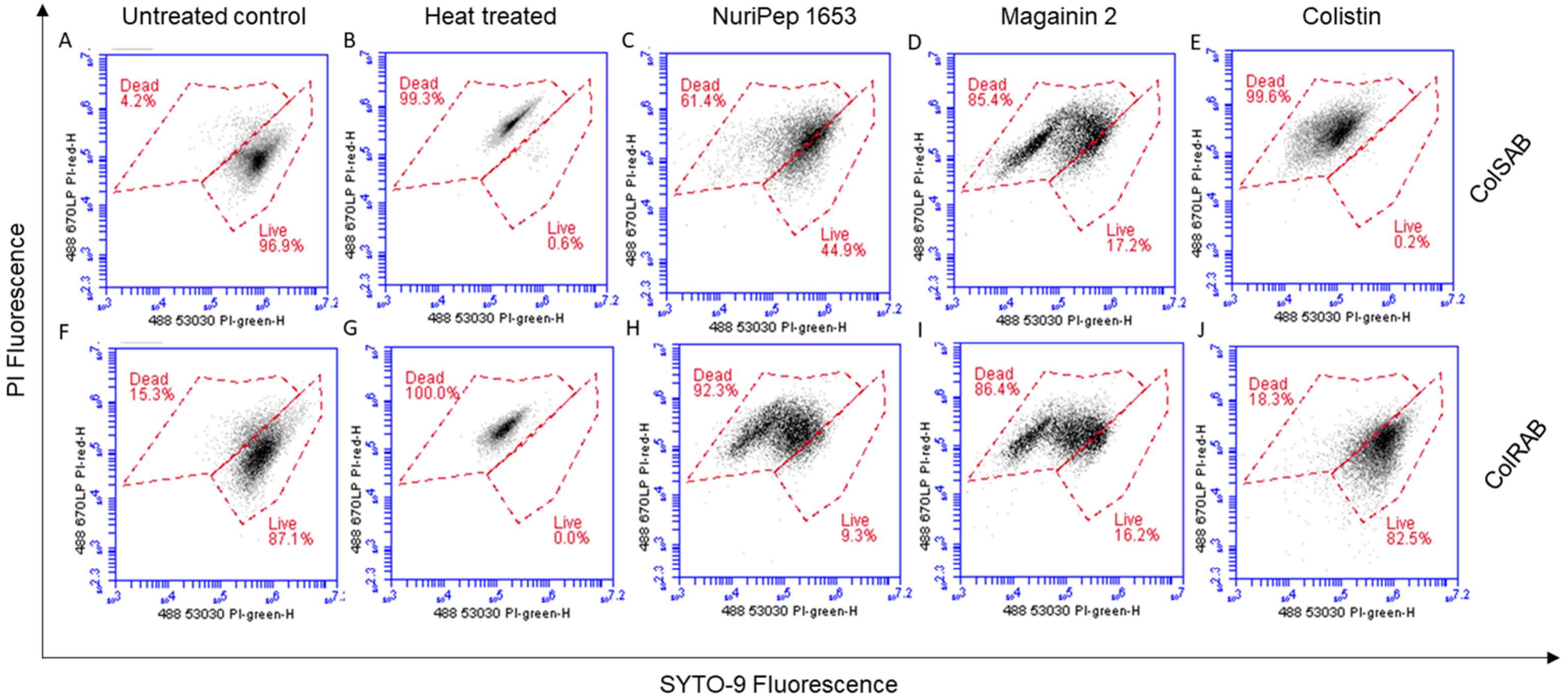


Figure 3.6 Electrostatic interactions and the impact of cations on the antimicrobial activity of NuriPep 1653. CE concentrations against colSAB were determined for NuriPep 1653 in the presence of increasing concentrations of NaCl or $CaCl_2$. The initial CE under standard conditions was 12 μg/mL.

3.3.7 Cell Viability and Membrane Permeabilisation of A. baumannii Post-Treatment with NuriPep 1653

Cellular viability and membrane integrity of colSAB (Fig. 3.7A-E) and colRAB (Fig 3.7F-J) pre- and post-treatment with NuriPep 1653 was analysed by co-staining of the bacterial cells with two nucleic acid dyes (PI and SYTO-9) and measuring fluorescence. Untreated cells and heat-treated cells were used as measures of viability as observed by minimal and maximal PI fluorescence, respectively. ColSAB and colRAB cells exposed to NuriPep 1653 at 12 µg/mL are observed in Fig. 3.7C & 7H respectively, where 61% and 92% of cells are labelled as dead i.e. stained fluorescent red from PI penetration and reduced SYTO9. Magainin 2 treated cells are shown in Fig. 3.7D and 7I for the susceptible and resistant strains, where only ~17% and 16% of cells were viable post-treatment, respectively. In Fig. 3.7E, ~99% of the colSAB cells treated with colistin showed PI uptake, indicating complete bacterial clearance as a consequence of membrane permeabilisation, damage and death. Conversely, ~82% of the colRAB cells remained viable further confirming the resistance profile and ineffectiveness of the lipopeptide treatment (Fig. 3.7J). Interestingly, the percentage populations of live and dead bacteria treated with NuriPep 1653 are different depending on the presence of colistin resistance. From our results, the peptide appears to work more efficiently in colRAB where 92.3% of cells are labelled as dead versus 61.4% in colSAB. The improved action of the peptide in colRAB may be as a result of collateral sensitivity based its antibiotic drug resistant profile (Lázár *et al*. 2018). These results clearly indicate an increased permeability of *A. baumannii*, in both susceptible and resistant phenotypes, in response to NuriPep 1653 treatment.


Untreated control
Heat treated
NuriPep 1653
Magainin 2
Colistin
A
B
C
D
E
F
G
H
I
J
Dead 4.2%
Live 96.9%
Dead 99.3%
Live 0.6%
Dead 61.4%
Live 44.9%
Dead 85.4%
Live 17.2%
Dead 99.6%
Live 0.2%
Dead 15.3%
Live 87.1%
Dead 100.0%
Live 0.0%
Dead 92.3%
Live 9.3%
Dead 86.4%
Live 16.2%
Dead 18.3%
Live 82.5%
488 670LP PI-red-H
488 53030 PI-green-H
PI Fluorescence
SYTO-9 Fluorescence
ColSAB
ColRAB

	Untreated		Heat Inactivated		NuriPep 1653 at 12µg/mL		Magainin 2 at 12µg/mL		Colistin at 4µg/mL	
	Live %	Dead %	Live %	Dead %	Live %	Dead %	Live %	Dead %	Live %	Dead %
ColSAB rep 1	96.9	4.2	0.6	99.3	44.9	61.4	17.2	85.4	0.2	99.6
ColSAB rep 2	97.2	2.8	0	100	58.3	41.5	20.9	79.1	1.1	98.9
ColRAB rep 1	87.1	15.3	0	100	9.3	92.3	16.2	86.4	82.5	18.3
ColRAB rep 2	90.3	9.7	1.1	98.9	5.2	94.8	30.4	69.6	90.0	8.8

Figure 3.7 Analysis of the effects of NuriPep 1653 on the membrane integrity and viability of colRAB and colSAB. Quantification of viable *versus* non-viable cells represented by the uptake of fluorescent PI in (**A**) untreated colSAB; (**B**) heat inactivated colSAB at 95°C for 60 minutes; (**C**) colSAB treated with NuriPep 1653 at 12µg/mL; (**D**) colSAB treated with magainin 2 at 12µg/mL; (**E**) colSAB treated with colistin at 4µg/mL; (**F**) untreated colRAB; (**G**) heat inactivated colRAB at 95°C for 60 minutes; (**H**) colRAB treated with NuriPep 1653 at 12µg/mL; (**I**) colRAB treated with magainin 2 at 12µg/mL; (**J**) colSAB treated with colistin at 4µg/mL. A minimum of 50,000 single events were collected per sample. Data is displayed as scatterplots of PI fluorescence (y-axis) and SYTO-9 fluorescence (x-axis). When used alone, the SYTO9 stain generally labels all bacteria in a population. In contrast, PI penetrates only bacteria with damaged membranes, causing a reduction in the SYTO9 stain fluorescence when both dyes are present. Therefore, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. Bacterial OD were optimised for cell events. Experiment was conducted in duplicate on independent days with repeat (rep) 1 and 2 for populations stained as live or dead shown above.

3.3.8 In vitro Development of Bacterial Resistance in Response to NuriPep 1653

ColSAB and colRAB were both passaged repeatedly in the presence of either NuriPep 1653, magainin 2 and colistin at 50% of the CE value to explore the development of resistance. This concentration was insufficient to induce killing but the continual exposure allows for the selection of mutants that may exist or develop in a population, which can eventually persist as a resistant population. A schematic overview of the experimental procedure is shown in Fig. 3.8A and the fold change observed in CE concentration over 14 days in Fig. 3.8B. When NuriPep 1653 and colistin treatment in colSAB are compared, a 2-fold *versus* a 6-fold increase in the CE concentrations were observed, respectively, over 14 days. Interestingly, the greatest increase (16-fold) was seen in colistin treated colSAB. Here, the cells developed resistance after 8 days, where resistance was defined as >10-fold the original CE concentration (Deslouches *et al*. 2015). ColRAB cells remained susceptible to NuriPep 1653 over 14 days. The trend observed in magainin 2 treated cells suggests a slow tolerisation and adaptation of cells over time. As colRAB was already identified as resistant to colistin by the EUCAST breakpoints (The European Committee on Antimicrobial Susceptibility Testing 2019), the development of resistance through a fold change in CE was not determined and was represented as resistant from day 0 in Fig. 3.8. NuriPep 1653 and colistin treated cells behave differently over the 14 days. The initially susceptible cells develop resistance to colistin just over half way through the time course. The CE concentration of NuriPep 1653 is identical in both colRAB and colSAB and neither develop resistance over 14 days exposure. These contrasting responses suggest different mechanisms of action however, further experiments are needed to explore the details of this.

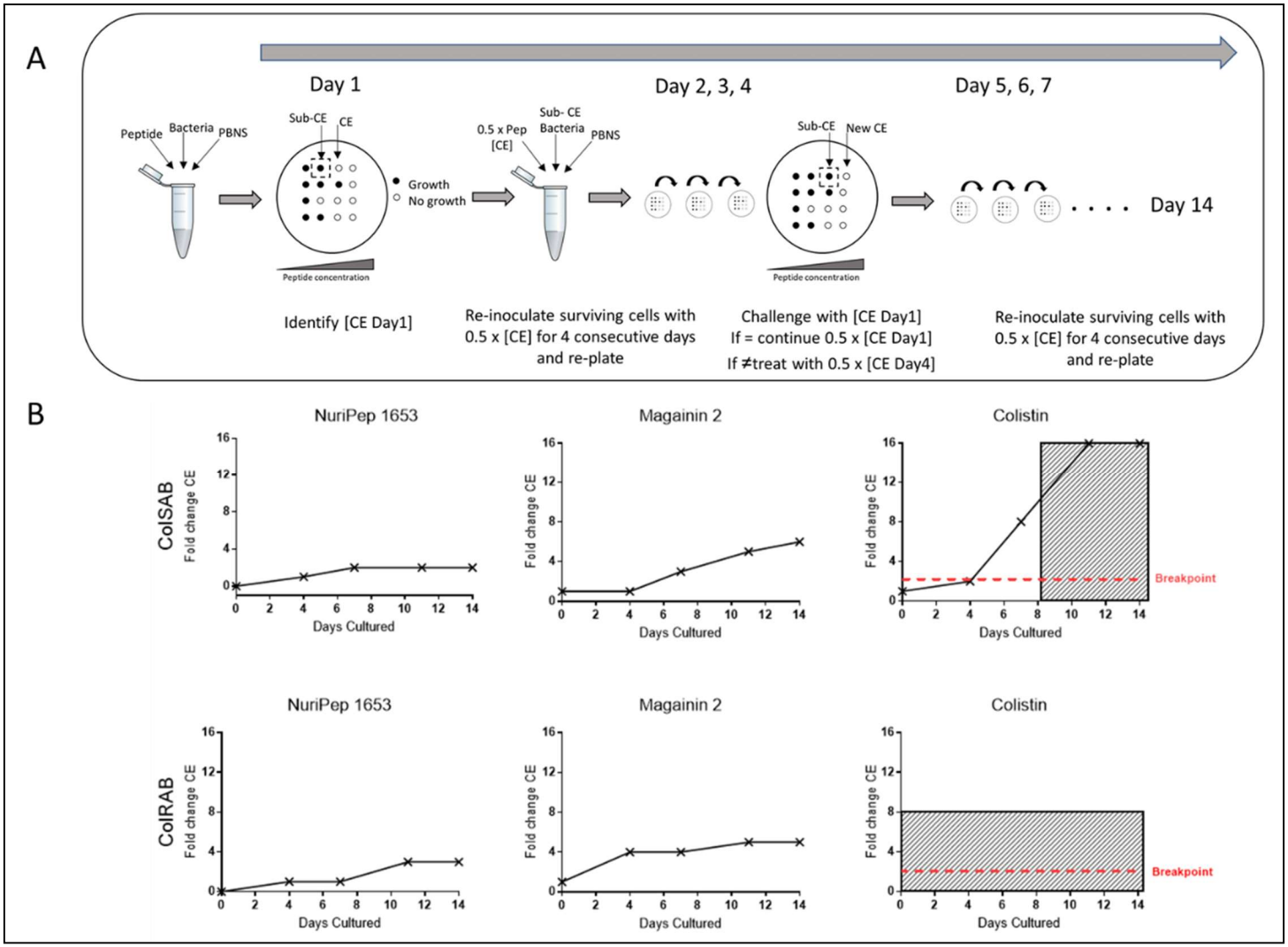

A
Day 1
Day 2, 3, 4
Day 5, 6, 7
Peptide
Bacteria
PBNS
Sub-CE
CE
Growth
No growth
Peptide concentration
0.5 x Pep [CE]
Sub- CE
Bacteria
PBNS
Sub-CE
New CE
Peptide concentration
Day 14
Identify [CE Day1]
Re-inoculate surviving cells with 0.5 x [CE] for 4 consecutive days and re-plate
Challenge with [CE Day1]
If = continue 0.5 x [CE Day1]
If ≠treat with 0.5 x [CE Day4]
Re-inoculate surviving cells with 0.5 x [CE] for 4 consecutive days and re-plate
B
NuriPep 1653
Magainin 2
Colistin
ColSAB
Fold change CE
Days Cultured
Breakpoint
NuriPep 1653
Magainin 2
Colistin
ColRAB
Fold change CE
Days Cultured
Breakpoint

Figure 3.8 Assessment of the development of resistance to NuriPep 1653, magainin 2 and colistin in colRAB and colSAB over 14 sequential passages. A schematic representation of the resistance assay is shown in (**A**). On day 0, bacterial cells were diluted in PBNS to 1 x 10^4 cells/mL and were treated with either colistin, magainin 2 or NuriPep 1653 at a range of concentrations for 90 minutes. The CE concentration was determined on Day 1 after 18 hours incubation at 37°C. Surviving cells exposed to a sub-killing concentration were collected with an inoculating loop and re-diluted to the same bacterial density in PBNS and exposed to 50% of their CE concentration of the peptide again and incubated for 90 minutes before plating. This was repeated throughout the experiment. On days 4, 7, 11 and 14, the bacteria were challenged with the previously determined CE concentration Where the killing concentration was higher than that on day 0, the treatment for the following 3 days was increased to one half of the new CE concentration. The CE concentrations represented as a fold change over 14 days during repeated exposure of colSAB with sub inhibitory doses of NuriPep 1653, magainin 2 and colistin from left to right are shown in the top row (**B**) and again against colRAB in the bottom row. The development of resistance was defined as >10 fold the CE and is shown as shaded areas in the graphs (Deslouches *et al*. 2015). Colistin resistance in colRAB was previously determined by the EUCAST breakpoints (The European Committee on Antimicrobial Susceptibility Testing 2019) and is represented as resistant from day 0. The experiment was conducted in duplicate on independent days.

3.3.9 Effects of NuriPep 1653 on the Viability of Differentiated Human Macrophages

Toxicity studies were carried out to determine the concentration dependent toxicity profile of the peptide in differentiated human macrophages and compare this to the bactericidal concentrations *in vitro*. The assay was performed in RPMI media and the CE concentration against colRAB was increased to >200 μg/mL. This increase may be due to disrupted conformation in this complex media, or interference with some of the components of the media and should be considered in the context of these results. As shown in Fig. 3.9, no significant adverse impact on cellular viability was observed with concentrations of NuriPep 1653 as high as 50 μg/mL however, a 24% decrease in cell viability was observed at 500 μg/mL ($p \leq 0.05$). In the case of magainin 2, exposure of the cells to 50 μg/mL caused a decrease of 22% in viability ($p \leq 0.01$). Contrastingly, at just 0.05 μg/mL of colistin, 12% of cells were killed ($p \leq 0.05$). Furthermore, less than 50% of cells survived the highest treatment of 500 μg/mL ($p \leq 0.001$). The results revealed that the toxic concentration of NuriPep 1653 is over 40 times higher than what is required for antimicrobial action, indicating a good therapeutic index or ratio of the toxic concentration compared to the therapeutic dose. Contrastingly, the toxic concentration for colistin is just 6.8 times higher than the active concentration against colRAB. NuriPep 1653 differs from the other two peptides in that it was identified as a naturally occurring sequence within a region of a *P. sativum* protein. It may display a better safety profile compared to the other two peptides as it is not produced as a secondary metabolite and due to its natural, edible source material. As mentioned above, these assays are conducted in RPMI media to give an initial idea of the toxic effects of the peptide however, a more accurate evaluation of this would be to perform toxicity studies in a murine *in vivo* model over a time course. When applied to sheep red RBCs, neither NuriPep 1653, magainin 2, nor colistin were shown to induce significant haemolysis at concentrations as high as 500 μg/mL (data not shown).

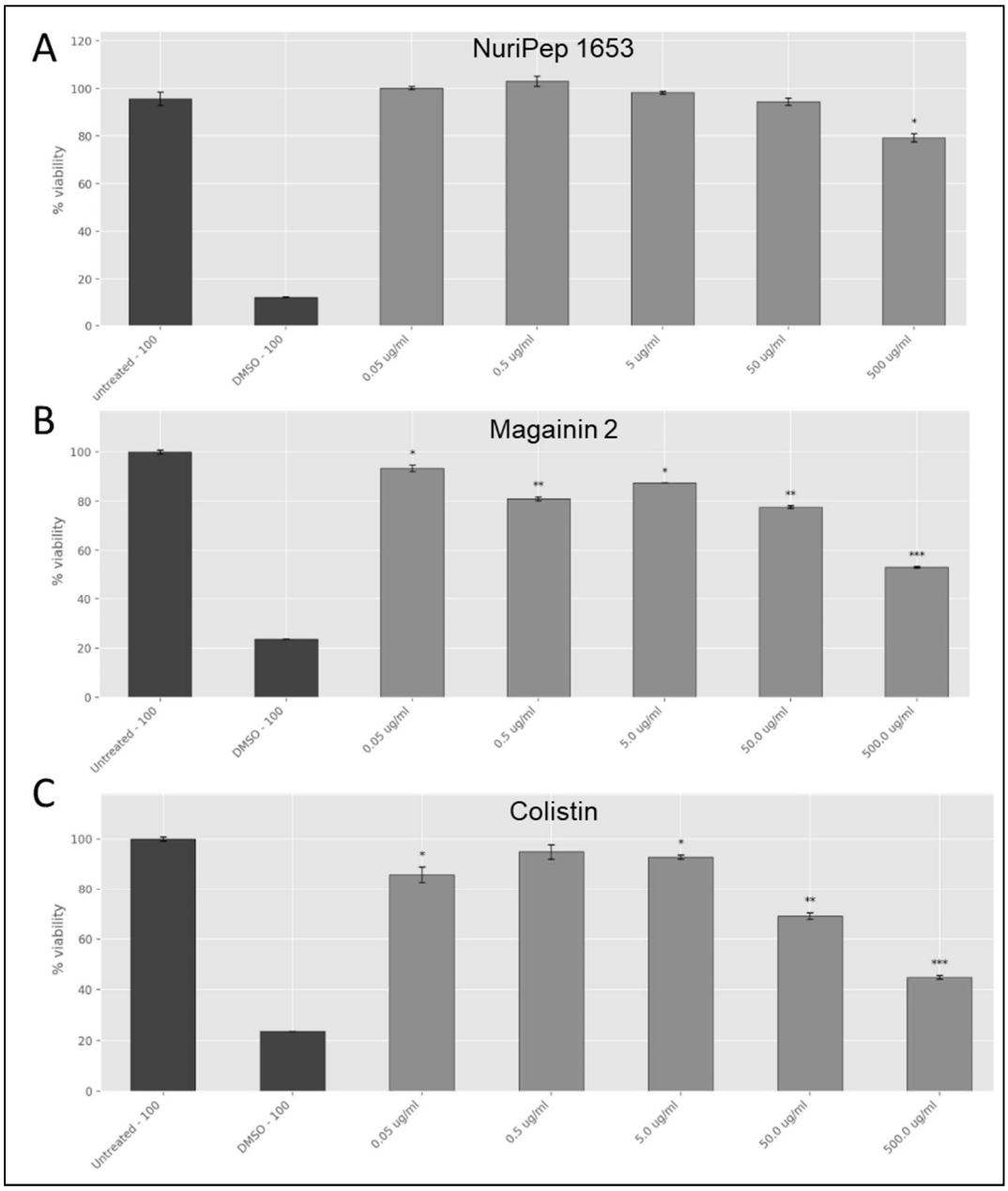


Figure 3.9 Effects of NuriPep 1653 on the viability of differentiated human macrophages. Cells were differentiated with PMA 20 ng/mL for 72 hours into macrophages before treatment with the peptide for 24 hours. Cell viability was assessed by MTT assay as per the manufacturer's guidelines. Values correspond to concentrations of (**A**) NuriPep 1653, (**B**) magainin 2 and (**C**) colistin tested at a range from 0.5 - 500µg/mL. DMSO 100% and untreated cells served as controls. Data represent the mean of three experiments performed in triplicate and is expressed as the mean ± SD. Statistical analysis was performed using a Student's t test where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.4 Discussion

The disbalance between the rate of bacterial resistance and discovery of anti-infective countermeasures is spiralling out of control (O'Neill 2015). While carbapenem resistant *A. baumannii* is listed by the WHO as a "critical priority" pathogen, Qureshi *et al.* report the complex resistance mechanisms and surging prevalence of colistin resistance emerging in *A. baumannii* strains isolated from hospital settings (Ahmed *et al*. 2016; Qureshi *et al*. 2015). ColRAB was included in this study on this basis where remaining treatment options have been completely depleted. While cationic linear peptides have been proposed by many researchers as having significant potential as new anti-infective drugs, few have been successful based on toxicity, poor commercial viability and inferior activity when compared to antibiotics (Deslouches *et al*. 2015; Ge *et al*. 1999; Baltzar and Brown 2011). In this work, we aimed to go beyond the realms of secondary metabolites and explore if AMPs can be identified within regions of non-antimicrobial proteins.

Embedded peptides are usually inactive in the parent protein but once proteolytically released, exert potent bioactivities (Mine, Ma, and Lauriau 2004; Hannu Korhonen and Pihlanto 2006). The reasons behind the existence of these sequences within larger proteins remain unknown. It may be that the peptides exist to confer health benefits once ingested. Since the nineteenth century, it was understood that milk, once consumed, played a vital role in neonatal protection and that the beneficial components were potentiated through digestion (Ehrlich 1892; Fokker 1890; Fadaei 2012). Interestingly, this concept and the evolutionary importance of these sub-peptide sequences is virtually unexplored in plants. It is conceivable to think, that peptides get released by natural cleavage at a given stage in the life cycle of the plant before being further broken down into individual amino acid building blocks. The functionalities of many of the intermediate breakdown products remain undetermined. We explore legumes and their bioactive breakdown protein products, expanding knowledge beyond the well-studied scope of milk.

NuriPep 1653, a novel natural non-toxic peptide was identified from within a region of the P54 protein in *P. sativum* and was released post enzymatic digestion of the protein. To the best of our knowledge, the sequence of the peptide has not been previously reported. NuriPep 1653 features highly in pre-established antimicrobial characteristics and displayed potent bactericidal action against PDR *A. baumannii*, at 12 µg/mL *in vitro*. This peptide was also shown to possess activity against three other ESKAPE pathogens (*P. aeruginosa, S.* Typhimurium and *E. aerogenes*) as well as *S. maltophilia,* which is prevalent in cystic

fibrosis and respiratory disease. It should be noted that the majority of peptides with antimicrobial activity in line with that observed for NuriPep 1653 against a PDR strain, are either completely synthetic creations, or engineered versions of natural compounds (Deslouches *et al*. 2015). NuriPep 1653 is completely unmodified, linear, shorter than the majority of known plant peptides and naturally occurring in a phyto-protein. While in this work we highlight just one peptide with a focus on its potential pharmaceutical application, the mere existence of NuriPep 1653 highlights the possible presence of many other AMPs of the same nature. This approach vastly extends the potential of peptide discovery from plants beyond those which are ribosomally derived or produced as metabolites.

Common to other AMPs, the activity of NuriPep 1653 was disrupted by salt sensitivity (Holthaus, Spisni, and Alibardi 2016; Klüver, Adermann, and Schulz 2006), however the peptide showed thermostability when heated at 95°C for 1 hour prior to bacterial challenge (Supplementary Fig. 3.2S). Further details of the assay are outlined in supplemental methods and results. Cationic, amphipathic peptides rely on electrostatic interactions for bacterial attachment or insertion as an initial step in their mechanism (Menzel *et al*. 2016). We confirmed this by observing a concentration- and valence- dependent inhibition of the bioactivity of NuriPep 1653 once solubilised in buffers with varying anionic strength. Considering this, a general lack of toxicity of AMPs to eukaryotic cells is assumed based on their zwitterionic phospholipids membrane, which have minimal electrostatic potential (Yeaman and Yount 2003a). We determined the cytotoxic concentrations of NuriPep 1653 in THP-1 cells which was 40 times higher than that required to eliminate bacterial growth. Other methods for determining toxicity are mentioned above in section 2.4. The toxic concentration of colistin was in line with that reported in literature where a significant reduction in cell viability occurred at 0.05µg/mL (Uhlig *et al*. 2014; Marr, Gooderham, and Hancock 2006). The improved safety profile of NuriPep 1653 over other AMPs may be based on its natural occurrence, natural edible source and lack of non-natural amino acids.

Following membrane binding, PI was used as an indicator of membrane permeability to assess if NuriPep 1653 exerts its action through a membrane active mechanism, commonly observed among AMPs (G. Wang *et al*. 2015; Bahar and Ren 2013). The cellular integrity of both colRAB and colSAB were affected with 92 and 61% of cells labelled as non-viable post peptide treatment. Mirroring the findings from the killing kinetics, NuriPep 1653 showed improved activity in the PDR isolate over colSAB. Lázár *et al*. highlighted that increased collateral sensitivity to AMPs in MDR strains was stimulated by regulatory

changes affecting the LPS composition of the outer membrane, which strengthened attachment and slowed down *de novo* evolution of resistance. From this we can assume that NuriPep 1653 may kill colRAB faster as developing resistance to antibiotics might have induced a high frequency collateral sensitivity to AMPs (Lázár *et al*. 2018).

SEM images (Supplementary Fig. 3.1S) further highlight the effects of NuriPep 1653 on the permeabilisation of the bacterial outer membrane. Further details of the assay are outlined in Supplemental methods and results. Control cells are shown in Supplementary Fig. 3.1SA&C. Similar ultrastructural degenerative aspects were observed with colSAB (Supplementary Fig. 3.1SB) and colRAB (Supplementary Fig. 3.1SD), however in the latter, an elongated morphology was observed as the cells attempt to increase their surface area to dilute the potency of the peptide on the membrane. Complete lysis and cell debris were seen when cells were exposed to a higher concentration of the peptide.

Taken together, the above results indicate that NuriPep 1653 performs similarly to other cationic AMPs as regards its initial antimicrobial mechanism (Bahar and Ren 2013). However, the synergistic effects observed against colRAB when the peptide was combined with colistin are suggestive of different actions post membrane disruption. CE concentration reductions of 6 and 8-fold were observed for NuriPep 1653 and colistin, respectively indicating compound potentiation. This was reinforced in the induction of resistant phenotypes experiment. ColSAB remained sensitive to both NuriPep 1653 and magainin 2 throughout the 14 days as active concentrations remained below the threshold for resistance (fold change CE >10). These findings are in line with other literature where unsuccessful attempts to generate resistance in several bacterial species through 14 repeated passages of pexiganan were reported (Ge *et al*. 1999). Similarly, in another study, *P. aeruginosa* was shown to develop resistance to colistin almost twice as fast as to two *de novo* engineered peptides, WLBU2 and WR12 and 7 times faster to rifampicin (Deslouches *et al*. 2015). The differences in the generation of mutants by the eigth day between colistin and NuriPep 1653 treated cells eludes to a distinct mechanism of action between these two peptides post bacterial attachment. The limited propensity for inducing resistance adds to the attractiveness of NuriPep 1653 as a novel anti-infective or adjuvant compound capable of reverting resistant phenotypes.

The lack of observed development of resistance and action of NuriPep 1653 against clinically relevant pathogens positions NuriPep 1653 as an appealing candidate which

warrants consideration for development and further testing on a larger cohort of PDR and XDR isolates. Therapeutic areas of particular interest may include cystic fibrosis and lung/skin infections given the results against the strains highlighted in Table 3.2. Future perspectives for NuriPep 1653 include increasing the salt resistance profile of the peptide. This may be investigated in future work through replacing tryptophan or histidine residues with the bulky amino acids β-naphthylalanine and β-(4,4′-biphenyl)alanine, as described by Yu *et al*. for their novel peptide P-113 (Yu *et al*. 2011). Furthermore, a deeper exploration into activity, protease resistance, bioavailability and optimal delivery strategies in murine models will also be investigated in the future.

3.5 Supplementary Data

3.5.1 Scanning Electron Microscopy

The structural damage and morphological changes induced in colSAB and colRAB after treatment with NuriPep 1653 at a sub lethal concentration using SEM are shown in Supplementary Fig. 3.1S. Untreated colSAB (Fig. 3.1SA) and colRAB (Fig. 3.1SC) display smooth, intact surfaces and active proliferation. The cells are numerous, appear healthy and viable which was confirmed by plating onto MHA and determining the CFU/mL before fixation with glutaraldehyde (data not shown). Conversely, NuriPep 1653 treated cells, shown in Fig. 3.1SB and 3.1SD exhibit major morphological changes such as roughening of the cell surface and distorted cell surface formation. The peptide treated colSAB cells (Fig. 3.1SB) appear elongated compared to the control cells (Fig. 3.1SA). This is likely an axial stress response induced by the peptide. The cells attempt to increase their cell surface area to dilute the potency of NuriPep 1653 on a site in the membrane. When a higher concentration of the peptide was used, no intact cells were observed and instead, substantial debris was seen indicating complete cell disruption and a loss of cellular integrity, as shown by the flow cytometry results (Fig. 3.7C and 3.7H).

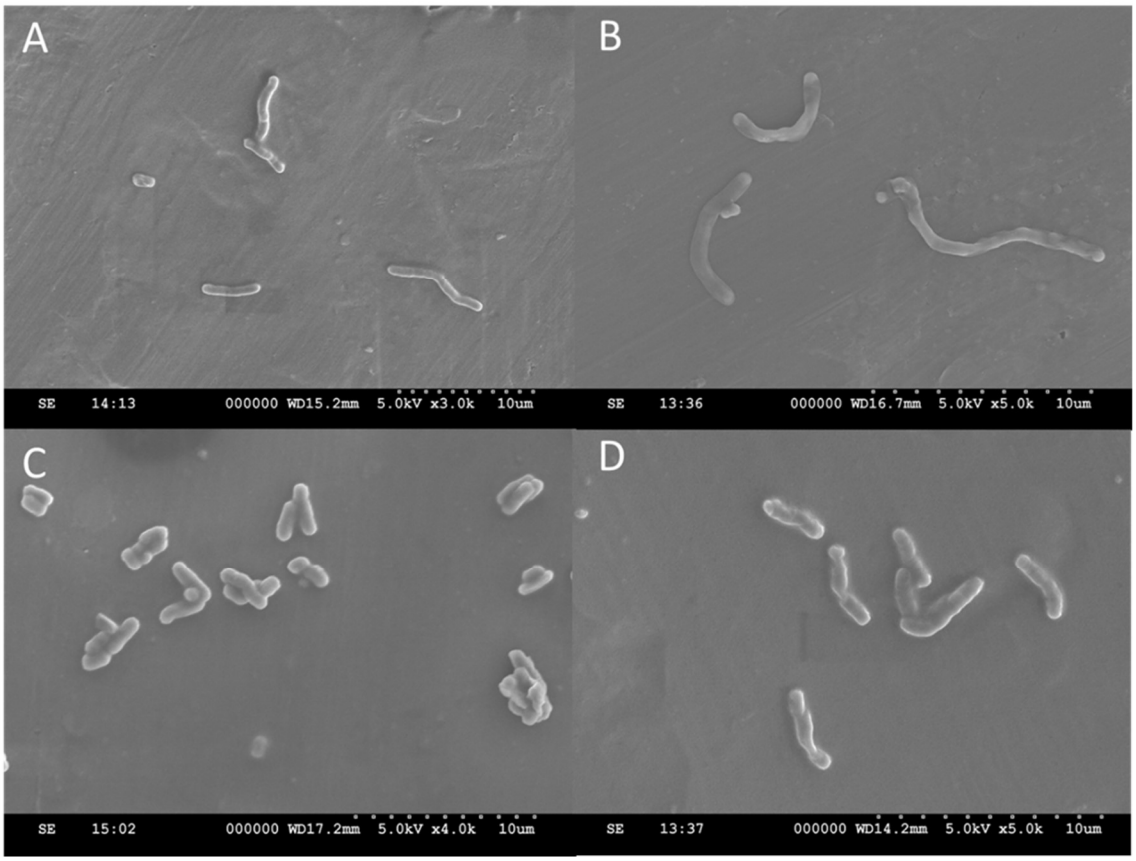


Figure 3.1S Scanning electron micrographs of colSAB and colRAB post treatment with NuriPep 1653. The images highlight the morphology of (**A**) Untreated colSAB; (**B**) NuriPep 1653 treated colSAB; (**C**) Untreated colRAB; (**D**) NuriPep 1653 treated colRAB. Cells were exposed to the peptide for 90 minutes before fixation. The treatment of *A. baumannii* with the peptide induced roughening of cell surface, elongation, disruption and debris while smooth cell surfaces were observed in cells without treatment.

3.5.2 Thermostability Study

The thermostability of NuriPep 1653 was assessed as per the CE method described above, however, prior to the bacterial challenge, the peptide was incubated at temperatures of 37, 75, 95 and 121°C.

The thermostability of NuriPep 1653 was evaluated in colSAB (Supplementary Fig. 3.2S). The peptide was shown to resist high temperatures and retain the same CE concentration (12 μg/mL) under both standard conditions at 37°C and after exposure to 95°C for 60 minutes. Only after incubation at 121°C was the peptide rendered inactive likely due to complete denaturation and breakdown of the sequence.

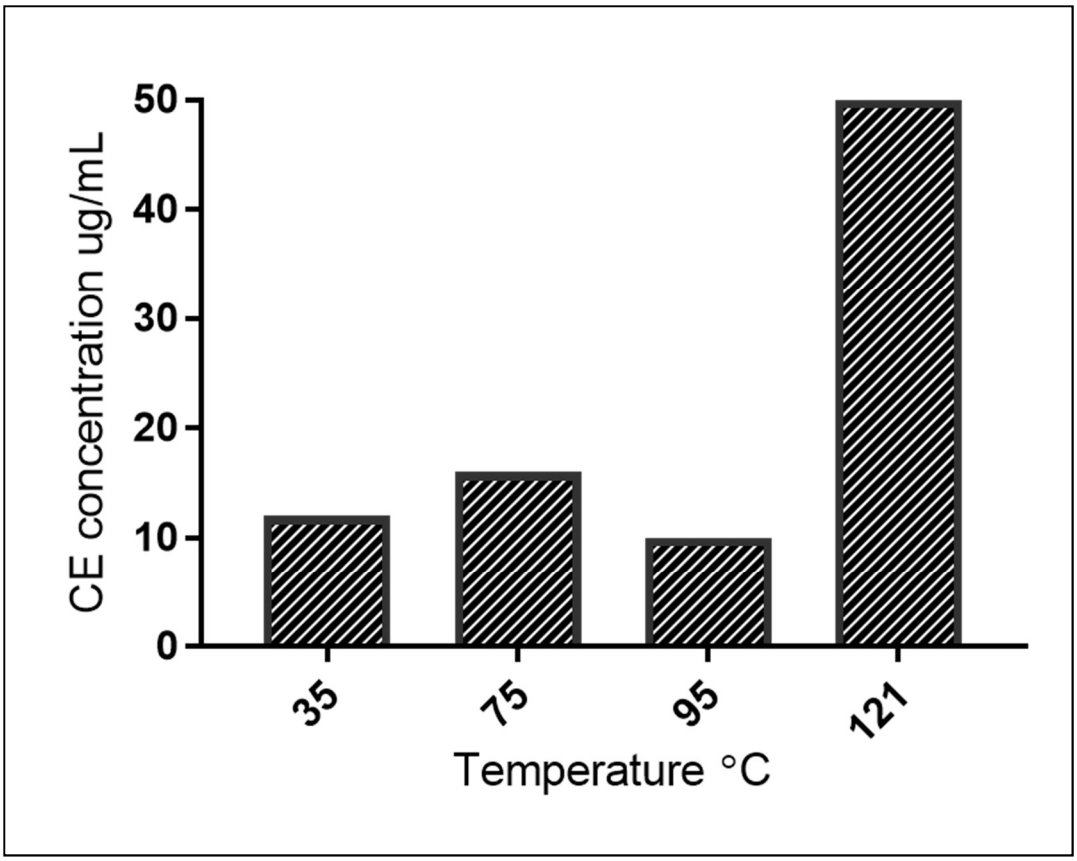


Figure 3.2S Thermostability of NuriPep 1653: The CE concentrations of NuriPep 1653 against colSAB were determined when incubated at 35, 75, 95 or 121°C for 60 minutes prior to the bacterial challenge. Under standard conditions, assays were performed at room temperature ~ 21 ± 2°C.

3.5.3 Sequence Physicochemical Properties Ranked as Important in Conferring Antimicrobial Activity to Peptides in the Discovery Pipeline

Supplementary Table 3.1S defines the AAIndex identifier string and description of the top 20 ranked features employed in the discovery pipeline (section 2.1) to sort the peptides identified by MS according to predicted activity. The full list of 566 amino acid indices can be searched on the AAIndex[9]. The 20 features can be grouped broadly into four groups: charge, structure, hydrophobicity and amino acid composition. Membrane interaction is critical to antimicrobial activity along with positive charge and hydrophobicity, which allow a sequence to adopt a helical structure from a cytoplasmic coil structure.

[9] https://www.genome.jp/aaindex/

Supplemental Table 3.1S Ranked list of top 20 sequence physicochemical properties in conferring antimicrobial activity to peptides

Feature ranking	AAIndex identifier	AAIndex identifier feature	Feature group
1	KLEP840101	net charge	Charge
2	QIAN880113	Weights for alpha-helix, pos 6	Structure
3	QIAN880127	Weights for coil at the window position of -6	Structure
4	QIAN880101	Weights for alpha-helix, pos -6	Structure
5	FAUJ880111	positive charge	Charge
6	SUEM840102	Zimm-Bragg parameter sigma - helix-coil transition	Structure
7	WILM950101	Hydrophobicity coefficient in RP-HPLC, C18 with 0.1%TFA/MeCN/H2O	Hydrophobicity
8	VENT840101	bitterness	
9	ARGP820103	Membrane-buried preference parameters	Hydrophobicity
10	BROC820102	Retention coefficient in HFBA	Hydrophobicity
11	WILM950102	Hydrophobicity coefficient in RP-HPLC, C8 with 0.1%TFA/MeCN/H2O	Hydrophobicity
12	NAKH920108	AA composition of MEM of multi-spanning proteins	Cytoplasmic vs extracellular AA composition of the membrane
13	NAKH900110	Normalized composition of membrane proteins	Cytoplasmic vs extracellular AA composition of the membrane
14	QIAN880130	Weights for coil at the window position of -3	Structure
15	MEEJ800101	Retention coefficient in HPLC, pH7.4	Hydrophobicity
16	ROBB760104	Information measure for C-terminal helix	Structure
17	NAKH900112	Transmembrane regions of mt-proteins	Cytoplasmic vs extracellular AA composition of the membrane
18	ROBB760107	Information measure for extended without H-bond	Structure
19	GARJ730101	Partition coefficient	Hydrophobicity
20	PONP800107	Accessibility reduction ratio	Hydrophobicity

Legend: Sequence physicochemical properties obtained from the AAIndex and ranked according to the discovery pipeline in Figure 3.1. Scores are normalised by peptide length and ranked backwards from 1 – 20 with number 1 being the top ranked. These features we used in the ranking of the MS peptide output according to most likely to possess antimicrobial activity. The features can be grouped broadly into 4 categories: charge, structure, hydrophobicity and amino acid composition. Generated in conjunction with Dr. Gael Jalowicki, Nuritas Ltd.

3.6 Data Supporting Chapter 3 but excluded from the Publication

3.6.1 Antimicrobial Activity of Randomised Versions of NuriPep1653

In order to determine if the order of amino acids in the sequence of NuriPep 1653 was important in conferring activity, three unordered versions were synthesised and tested. Two of these sequences were generated randomly by Nuritas software (NuriPep -2206 and -2207). NuriPep 2208 was designed by hand with the aim of splitting up hydrophobic and hydrophilic residues across the length of the sequence as much as possible. Hydrophobic segments of the peptides may be important in bacterial killing as these parts of the sequence can interact with fatty acyl groups of phospholipids in the membrane and encourage anchorage. The hydrophobic residues of the sequence, Alanine (A), Valine (V), Leucine (L), Isoleucine (I), Proline (P), Phenylalanine (F), Tryptophan (W), and Methionine (M) are highlighted in red in Fig. 3.3S below.

The concentrations of peptide required for the elimination of bacteria are shown in Table 3.2. The CE method was performed as described above in PBNS buffer to test the activity of the randomised peptide versions. NuriPep 1653 demonstrated a higher antimicrobial activity against the strains tested. The hydrophobic and hydrophilic residues in this sequence are grouped mostly in segments of two and three (1, 2, 3, 3, 4, 2, 2, 2, 2, 1 - hydrophobic in red and hydrophilic in green). NuriPep -2206 and -2207 display similar antimicrobial action when compared to -1653. The slight reductions in activity may result from the differing proportions of hydrophobic and hydrophilic segment splits or due to certain amino acids being split up such as GG, which we previously determined to be a motif common in AMPs (Mohan *et al*. 2019). NuriPep 2208 demonstrated lower inhibitory activity in the antimicrobial assay. There are hardly any hydrophobic segments in this sequence with individual alternating hydrophobic residues and hydrophilic residues featuring most. While authors have described the importance of overall hydrophobicity in AMPs (Yin *et al.* 2012), we hypothesise that the distribution of the hydrophobic charges may also be an important factor in conferring activity to a sequence. A two- to three- fold increase in CE concentrations were observed here with a complete loss of activity against *P. aeruginosa* and *S.* Typhimurium. No activity was observed against *S. aureus*, similar to the results obtained with NuriPep 1653.

ID	Sequence																						Basis of Choice
NuriPep1653	V	R	G	L	A	P	K	K	S	L	W	P	F	G	G	P	F	K	S	P	F	N	Original
2206	G	S	V	P	P	P	R	N	K	A	S	F	G	K	L	P	G	K	F	W	F	L	Random
2207	P	A	V	P	S	G	L	S	R	F	N	K	P	W	F	F	K	L	G	P	K	G	Random
2208	S	V	R	W	K	P	A	L	N	P	G	F	G	F	P	K	F	G	K	P	S	L	Hydrophobic split

Figure 3.3S Unordered versions of NuriPep 1653. Three versions of NuriPep 1653 are shown with their amino acid order altered. NuriPep -2206 and -2207 are randomly generated mixed up versions. NuriPep 2208 was chosen on the basis that hydrophobic and hydrophilic alternating segments of three/four residues may be important in conferring activity. In this peptide, the alternations are more frequent, and the polar segments are shorter when compared to the original sequence.

Table 3.2S Complete Elimination values of NuriPep 1653 and its randomised versions against a range of Gram -negative and -positive bacteria

	NuriPep ID number			
	1653	**2206**	**2207**	**2208**
	CE (μg/mL)			
P. aeruginosa ATCC 27453	8	50	25	NI
E. coli ATCC 25922	100	100	100	200
S. Typhimurium ATCC 14028	100	NI	100	NI
A. baumannii ATCC 19606 (colSAB)	12	12	25	50
A. baumannii (colRAB)	12	12	25	50
S. aureus ATCC 25923	NI	NI	NI	NI

Legend: Values of NuriPep -1653, -2206, -2207 and -2208 required to induce bacterial clearance *via* the CE method against a range of Gram -negative and -positive strains. NI = No inhibition at concentrations < 800 μg/mL. Values shown represent the average of three independent experiments on three independent days

3.6.2 Structural Prediction of the P54 Storage Pea Protein and the Location of NuriPep 1653 using I-TASSER Platform.

The 3D structure of the P54 *P. sativum* protein has not yet been solved experimentally and so, we used the I-TASSER[10] platform to predict its structure. I-TASSER is among the top-ranked, most widely used online prediction server for protein structure models (Zheng *et al*. 2019). The structural modelling strategy, which was developed by Wu et al, has four major steps: template threading, structural fragment assembly, model refinement and structure-based function annotation, which are shown in Fig 3.4S. (Zheng *et al.* 2019; Wu, Skolnick, and Zhang 2007). Template threading involves locating templates of known proteins from the Protein Data Bank (PDB)[11] which share homology with the protein of interest (Wu, Skolnick, and Zhang 2007). The second step involves removing continuous sections from the threading alignments from the template models. The unaligned portions are then constructed by I-TASSER. The aligned and unaligned parts are incorporated into Replica-Exchange Monte Carlo simulation (REMC), which creates thousands of conformations based on pair-wise structural similarity (Wu, Skolnick, and Zhang 2007). In the third step, the conformations are clustered by tertiary structure and narrowed down to the best five corresponding to low-free energy, and geometry occurring in nature. The best five best models generated by I-TASSER of NuriPep 1653 are shown in Fig. 3.5S. The samples were ranked by their C-score, or the confidence of each model calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations (Zheng *et al*. 2019). In the case of NuriPep 1653, model A had the lowest C-score, and therefore was chosen as the best structural conformation for the peptide. Functional annotation is the final step, however prior to the generation of the structural model of NuriPep 1653, the activity was predicted and validated according to the methods outlined in Figure 3.1, so this final step was not relevant.

[10] https://zhanglab.ccmb.med.umich.edu/I-TASSER/

[11] www.rcsb.org

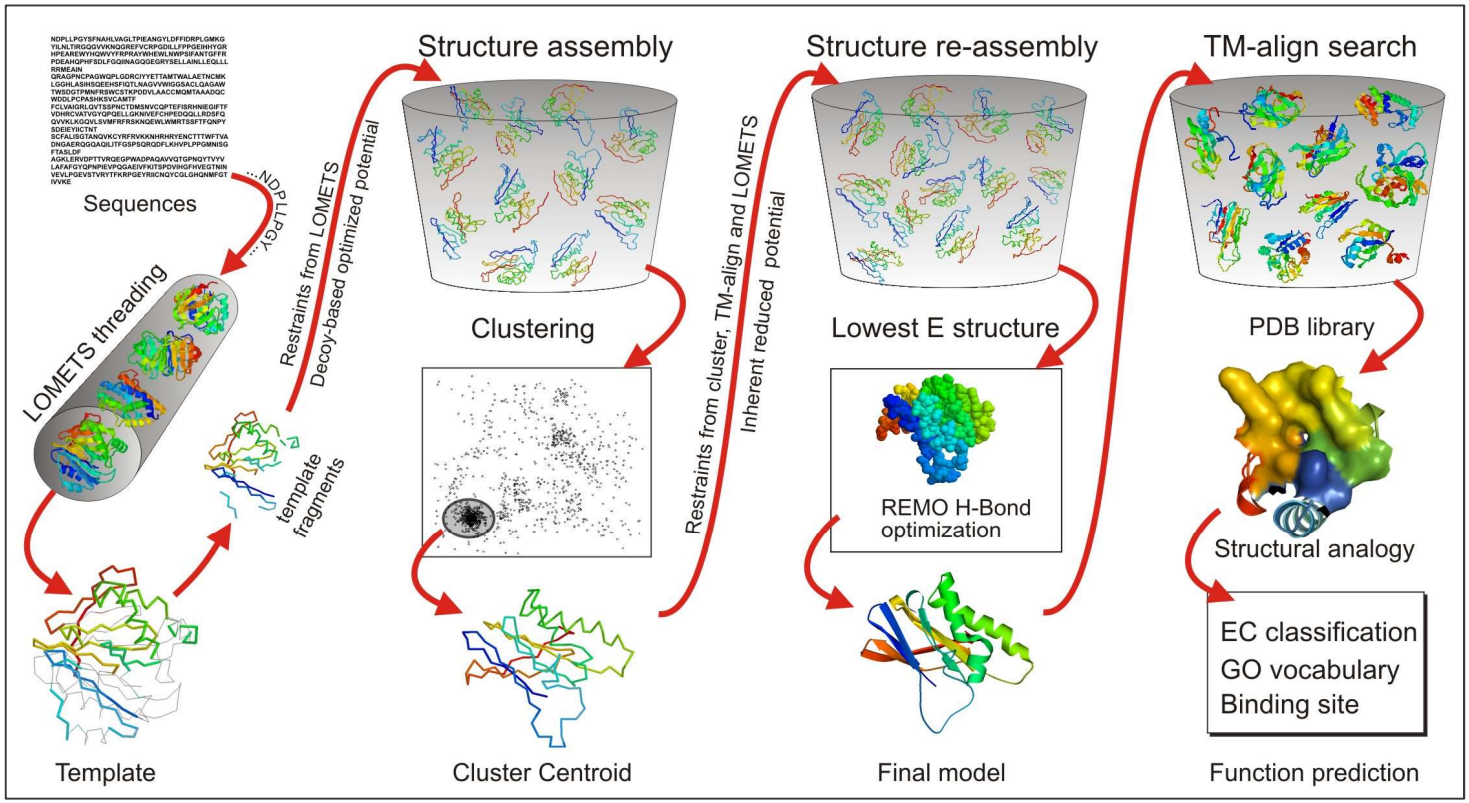


Fig. 3.4S. Schematic of the I-TASSER structure and function prediction protocol as designed : The four main stages in creating a protein model are shown here; 1 = structural template threading, 2 = structural fragment assembly, 3 = model refinement and 4 = structure-based protein function annotation similarity (Wu, Skolnick, and Zhang 2007).

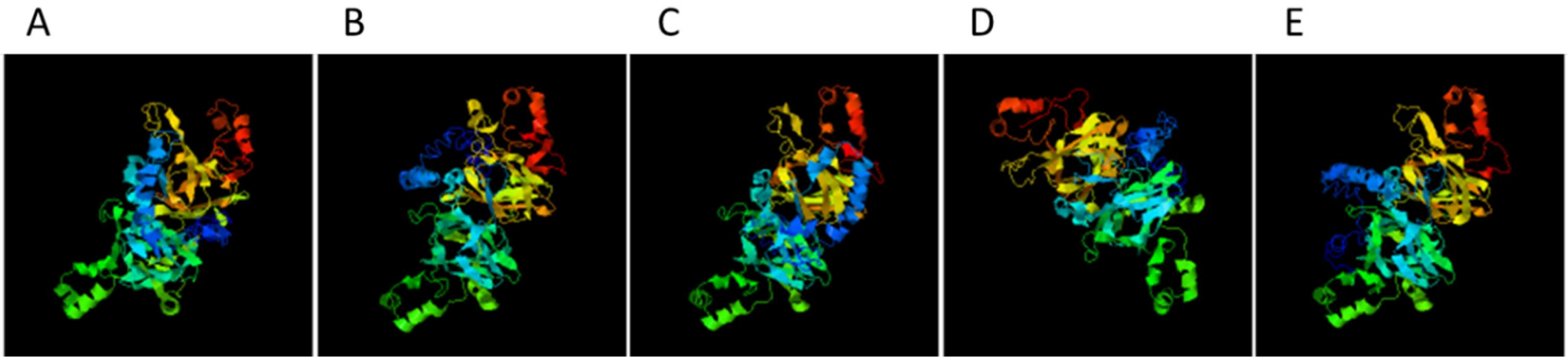


Figure 3.5S Structural models of NuriPep 1653 as predicted by I-TASSER algorithm: The C score is typically in the range of -5, 2, where a higher score indicates a higher confidence. The C score for the models are as follows; **A**: -2.06, **B**: -2.14, **C**: -2.14, **D**: -2.47, **E**: -2.59 therefore, in this case model A was the best.

Chapter 4
Immunomodulatory Activity of NuriPep 1653 - An Antimicrobial Peptide Derived from *Pisum sativum* Protein

4.1 Introduction

The immune system plays a crucial role in maintaining homeostasis in the body by identifying and eliminating pathogens or detecting "sterile inflammation" and cancerous cells. Bacterial infection can activate TLRs and trigger inflammatory signalling pathways of the immune system, most commonly the NF-κB pathway as well as trigger various inflammatory cells (L. Chen *et al*. 2018). Macrophages are important primary responder cells in the innate immune system which must be carefully regulated in the body. Macrophage activation results in the remove harmful cells and bacteria through phagocytosis and the release of cytokines and chemokines (Y. Wang *et al*. 2010). However, excessive activation has damaging effects for the host such as septic shock and organ dysfunction (Valledor *et al*. 2010). Proinflammatory cytokines, such as TNF-α, IL-1, IL-6, and IL-12 as well as anti-inflammatory cytokines, such as IL-10 and IL-13 are critical components in immune regulation (Kang *et al*. 2019).

In this study, we focus on TNF-α, which plays a particularly important role in infection. It is an endogenous alarm signal that stimulates gene expression and inflammatory response to infection, which promotes bacterial killing and tissue damage (Mizgerd 2003). However, careful modulation of TNF-α is essential, as when produced in excessive amounts, it has been shown to exacerbate bacterial infection, compromise respiratory, hepatic and circular physiology as well as cause tissue damage and haemodynamic changes (Meduri *et al*. 1999; Cohen 2002; Czaja 2014). Extensive research has been conducted on the ability of TNF-α antagonists to have a therapeutic benefit in inflammatory disorders such as rheumatoid arthritis (Johnston and Conly 2006). However, given the putative role of TNF-alpha in host defense, anti-TNF-α compounds have also been associated with an increased risk of serious infection. TNF-α acts alongside other cytokines such as interferon-delta, to generate competent cell-mediated immune response to intracellular pathogens such as *S.* Typhimurium (Rychly and DiPiro 2005). Gastroenteritis is an example of pathogen induced inflammation in the GI tract where modulation of the host cytokine response is vital to promote cells to the site of infection but limit excessive inflammation which can lead to further cell damage and stress. Recently, the therapeutic potential of AMPs has been magnified by the myriad of other activities attributed to them (Bowdish *et al*. 2005; Neshani *et al*. 2019). Among these, immunomodulatory properties are among the most appreciated.

Various publications highlight potent antimicrobial activities of AMPs in non-complex, low-salt media, as their sensitivity to anions is well known (Menzel *et al*. 2016). These peptides

often lose their apparent antimicrobial functions in physiologically relevant media, however, they may still be capable of boosting or dampening cells of the immune system by inducing or suppressing the production of pro- and anti-inflammatory, stimulating B-cells to produce antibodies, lymphocyte proliferation and increasing phagocytic abilities (Chalamaiah, Yu, and Wu 2018; Duarte, Vinderola, Ritz, Perdigón, and Matara 2006; Marr, Gooderham, and Hancock 2006; Bowdish, Davidson, and Hancock 2005). Certain immunomodulators, such as those which inhibit the action of TNF-α, can be expensive and toxic. In certain cases, anti-TNF-α medicines have been shown to induce serious side effects such as hepatotoxicity to patients, which restricts their use (French *et al*. 2016). This differs drastically from natural peptides which have limited side effects and are far less cytotoxic, adding to their attractiveness as immunomodulators (Kang *et al*. 2019).

In this study, we also address the allergenic immune response as it represent a very prevalent health issue affecting up to one quarter of the population in developed countries, allergies (Marth *et al*. 2014). Allergies are hypersensitive IgE mediated immune responses to triggers, usually proteins in the case of food-related allergies. Th-1 and Th-2 lymphocytes are involved in cytokine production and regulate inflammation. Furthermore, Th-17 cells are a subset of pro-inflammatory T helper cells defined by their production of IL-17 and have also been implicated in autoimmune and inflammatory disorders and play a vital role in adaptive immunity protecting the body against pathogens. During an allergic response the levels of Th-2 and Th-17 cytokines produced heavily outweigh the Th-1 type as IgE is produced (Asthma and Allergy Foundation of America 2004). IgE cross-links granulocytes such as basophil and stimulates the release of histamine and proinflammatory mediators from mast cells (Marth *et al*. 2014; Asthma and Allergy Foundation of America 2004). The allergic response can be sudden or late phase, with that latter being important in chronic inflammation associated allergy (Marth *et al*. 2014). While allergy testing is required before a compound can be developed as a drug, online predictors are an excellent resource which provide valuable information on the allergenic potential of a compound.

NuriPep 1653 (VRGLAPKKSLWPFGGPFKSPFN) is a novel, 22-mer peptide contained within the P54 storage reservoir protein in common garden pea, *P. sativum*. This cationic, amphipathic peptide was previously shown to possess potent antimicrobial activity against MDR pathogens including *A. baumannii* and *P. aeruginosa* through electrostatic and membranolytic mechanisms (Mohan *et al*. 2019). In this study, we highlight activity beyond antimicrobial functions and present our findings on how the peptide can modulate aspects of

the immune system. NuriPep 1653 was shown to either inhibit intracellular uptake or enhance phagocytosis of *S.* Typhimurium ATCC 14028 in THP-1 differentiated macrophages pre-treated with the peptide. Moreover, without impacting cellular viability, the peptide could significantly inhibit TNF-α in LPS stimulated human macrophages and modulate NF-κB levels, which is indicative of inflammation regulated immune functions. NuriPep 1653 was also shown to aid in the migration and proliferation of keratinocytes in a wound-healing model. Furthermore, we highlight our findings using the AlgPred[12] tool which suggest that NuriPep 1653 is a non-allergenic sequence.

The food-protein derived peptide, NuriPep 1653, with its combined antimicrobial and immunomodulatory activities, delivers a promising dual pronged approach to decelerate the surging prevalence of multidrug resistance -directly through bacterial killing, and -indirectly by modulating the host's defence responses and reducing inflammation.

4.2 Materials and Methods

4.2.1 Materials and Reagents

NuriPep 1653, previously described by Mohan *et al*. 2019, was synthesised by GenScript (Piscataway, NJ) using solid-phase Fmoc chemistry and purified to 95-99% using RP-HPLC). Peptide mass was confirmed by MS. THP-1, HaCaT, HepG2 and Caco-2 cells were acquired from ATCC (Manassas, VA, USA). All above cells except the HaCaT cells were grown in RPMI supplemented with 10% FBS and antibiotics (streptomycin 100 μg/mL and penicillin 100 U/mL) when required. HaCaT cells were cultured and maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented as above. All cells were grown in a humidified 5% CO_2 incubator at 37°C. THP-1 Blue™ NF-κB cells, QUANTI-Blue™ as well as normocin, and the selective antibiotic blasticidin were purchased from InvivoGen (California, USA). Heat inactivated FBS, penicillin and streptomycin were all obtained from Thermo Fisher Scientific (Dublin, Ireland). RPMI media was used to culture the cells and it was obtained from InvivoGen (California, USA). PMA was purchased from Sigma-Aldrich (United Kingdom).

[12] http://crdd.osdd.net/raghava/algpred/

4.2.2 Viability Assay in Human Cell Lines

Cellular viability was assessed in human THP-1 macrophages, Caco-2 human epithelial colorectal adenocarcinoma cells and HepG2 human liver cancer cells by the MTT colorimetric assay as outlined above in section 3.2.11.1 (Silva *et al.* 2016). The concentrations of NuriPep 1653 used in THP-1 cells was as follows: (0.05, 0.5, 5, 50, 500 µg/mL). In Caco-2 and HepG2 cells, NuriPep 1653 was tested at 100, 250 and 500 µg/mL. Cell viability was calculated as a per cent of a control treated with PBNS. Three technical replicates were performed each day on three independent days.

4.2.3 Predicted Allergenicity of NuriPep 1653

The allergenicity of NuriPep 1653 was predicted using the using the AlgPred[13] online predictor (Saha and Raghava 2006). Multiple prediction approaches were selected from the available tools including: mapping of the IgE epitopes; support vector machine (SVM) based on amino acid composition; Blast searching on allergen representative peptides; and Motif Alignment Search Tool (MAST).

4.2.4 THP-1 Macrophage Infection with Salmonella Typhimurium

The assay was performed according to Starr *et al.* (2018) with some modifications. THP-1 monocytes were grown in suspension in RPMI medium supplemented with 10% volume/volume (v/v) FBS at 37°C in an incubator with 5% CO_2. The cells were seeded at a density of 1 x 10^5 cells/mL in 24-well tissue culture treated plates in the presence of 20 ng/mL PMA for 3 days to differentiate the cells into macrophages. The experiment was performed in two different ways. Firstly, the THP-1 cells were treated with NuriPep 1653 at 0.5 µg/mL and 500 µg/mL for 2 hours or 20 hours before being infected with *S.* Typhimurium ATCC 14028, which was used as a model of an intracellular bacterial infection. In the second case, the macrophages were infected and then treated with the same concentrations of the peptide for the same length of time. For a multiplicity of infection (MOI) ~ 20 (1 x 10^5 THP-1 cells to 2 x 10^6 bacteria or 1 macrophage to 20 bacteria). The cells were washed with PBS twice and finally diluted in RPMI before being added to the

[13] http://crdd.osdd.net/raghava/algpred/

plate and spun at 1000xg for 5 minutes at room temperature to aid internalisation of the bacterial cells. In the case of cells pre-treated with the peptide, which represents a healthy state, (pre-treatments of 2 and 20 hours, respectively), the bacteria were added, and incubated for 30 minutes. After this time, cells were washed, gentamycin (100 µg/mL) added and the plate re-incubated for 60 minutes to kill any extracellular bacteria, which were not internalised into the macrophages. In the case of the cells which were firstly infected to represent a diseased state, and then treated with peptide, after the cells were exposed to the bacteria for 30 mins, the cells were washed and treated with 100 µg/mL gentamicin for 60 minutes at 37°C, 5% CO2, followed by incubation in 10 µg/mL gentamicin and 0.5 and 500 µg/mL of NuriPep 1653 for the remainder of the experiment. For enumeration of bacteria, monolayers with 2- or 20-hours peptide pre-treatment were solubilised with lysis buffer (0.2% sodium deoxycholate in PBS) and serially diluted in PBS before plating on LB agar plates. The assays were performed in triplicate on independent days.

4.2.5 Enzyme-Linked Immunosorbent Assay

Cells were pre-treated with NuriPep 1653 and incubated for 24 hours in a 37°C, 5% CO_2 humidified incubator. The inflammatory response was induced by exposing the cells to 100 ng/mL LPS followed by incubation for 24 hours in a 37°C, 5% CO_2 humidified incubator, prior to cell sample collection. Enzyme-Linked Immunosorbent Assay (ELISA) was used to quantify the TNF-α secretion in cell culture supernatants from immortalised THP-1 cells. Measurements were performed using BioLegend's LEGEND Max™ Human TNF-α ELISA kit, which was pre-coated with a monoclonal antibody specific to human TNF-α on a 96 well plate. The protocol was carried out according to the manufacturer's specifications. This colorimetric assay determined the concentration of TNF-α in the sample by measuring the OD values. The OD of each of the samples and standards were measured at 450 nm on a CLARIO star microplate spectrophotometer (BMG LABTECH, CLARIO star, UK). The CLARIO star software was used to plot the standard curve by measuring the OD values of the standards at 450 nm and plotting them against their known concentrations. The OD data from the sample was subsequently interpolated on a 4-parameter curve, within the OD dataset range of the standard curve to determine the TNF- α concentration in the cell supernatants.

4.2.6 Measurement of NF-κB modulation using THP-1 Blue Cells

THP-1 Blue cells are transfected with a construct which contains a secreted embryonic alkaline phosphatase (SEAP) reported induced by the NF-κB transcription factor (Zuliani-Alvarez, Piccinini, and Midwood 2017). Post-stimulation, NF-κB activation leads to the secretion of SEAP, which is quantifiable using QUANTI-Blue colometric solution. Therefore, the amount of the SEAP reporter detected in the media correlates with the triggering of this pathway. THP-1-Blue™ NF-κB cells are highly responsive to Pattern Recognition Receptors (PRR) agonists that activate the NF-kB pathway. Various TLRs can be used as stimulants. The assay was performed according to Zuliani-Alvarez, Piccinini, and Midwood (2017) and modified accordingly to include the peptide and ligands of interest in this study. 25 ng/mL Pam3CsK4 (TLR1/2), $2.5E^7$ ng/mL HKLM (TLR2), 30 ng/mL LPS (TLR4), 20 ng/mL FLA-ST (TLR5), 3 ng/mL FSL-1 (TLR2/6) and 3.13 ng/mL rTNF-α were used as ligands which gave a strong stimulatory response in the cells. TLR3, TLR7 and TLR9 were included in an initial screen, however as indicated by the manufacturers, the response to these ligands is hardly detectable and so were excluded for further analysis. The optimal concentrations of these ligands for use in the assay were determined prior to the addition of the peptide.

Firstly, the THP-1 Blue NF-κB cells were cultured for two passages in THP-1 NF-κB medium without blasticidin (RPMI supplemented with 10% FBS, 1% penicillin/streptomycin and 100 µg/ml Normocin) to allow recovery of the cells after defrosting. Post-recovery, the cells were cultured in the same media however, 10 µg/ml of blasticidin was added to maintain selection pressure for the reporter. In order to assess the effects of NuriPep 1653 on the modulation of the NF-κB pathway, 25 µL of each ligand was incubated with 25 µL of the peptide at a concentration range (500, 250, 125, 10, 5, 0.5 µg/mL), in duplicate. For the untreated cells, media was added to the wells containing the ligands only. Media alone was included as a blank control. THP-1 Blue NF-κB cells were diluted to a density of 1×10^5 cells/mL and 50 µL was added to each well on the plate giving a final volume of 100 µL per well. The plate was incubated for 24 h at 37 °C in 5% CO2. Post-incubation, the amount of NF-κB-induced secreted alkaline phosphatase was determined. QUANTI-Blue was prepared by mixing 1 mL of QUANTI-Blue reagent with 1 mL of QUANTI-Blue buffer and preparing this to a final volume of 100 mL, using sterile dH_2O. Resuspending thoroughly, but avoiding bubbles, 40 µL was transferred from each

well of the incubated plate into a fresh microtiter plate. Following this, 160 µL of the QUANTI-Blue solution was added to the cells, the plate was covered with tin foil and re-incubated for 60 mins at 37 °C in 5% CO2. The OD was read at 620 nm in a microplate reader (BMG LABTECH, CLARIO star, UK). A colour change in the media from pink to blue indicated the presence of SEAP.

4.2.7 Scratch-Assay in HaCat cells in order to Determine the Wound-healing Properties of NuriPep 1653

The assay was performed according to Muniandy *et al*. with minor modifications (Muniandy *et al*. 2018). HaCat cells were seeded at a cell density of 2×10^5 cells in each well of a 24-well plate and incubated with complete medium at 37°C and 5% CO_2. After 24 hours the media was removed and exchanged with serum-free media in order to starve the cells for a further 24 hours. After incubation, the monolayer confluent cells were scraped horizontally with a sterile P200 pipette tip. The cells were rinsed with PBS in order to remove any cell debris. NuriPep 1653 was diluted to a concentration range (0.1 – 100 µg/mL) in serum-free media and 500 µL was added to each well of the plate. Cells treated with 10% FBS were used as a positive control, while those with DMEM media only were the untreated controls. The wounds in the cells were observed with a microscope (Keyence VHX-5000 Digital Microscope, UK) and images captured using a phase contrast microscope (mag x 40) at time 0 hours. The plate was then incubated for 24 hours and the wounds were re-photographed. The migration was determined using Image J software with the wound-healing plug in for analysis. The area between the wound was measured and compared over the time period and compared to the untreated control. Experiments were performed in triplicate on independent days and the data was evaluated statistically using one-way ANOVA analysis.

4.3 Results

4.3.1 Effects of NuriPep 1653 on the Viability of Human Macrophage, Epithelial and Liver cells

Toxicity studies were carried out to determine the concentration dependent toxicity profile of NuriPep 1653 in THP-1 differentiated human macrophages, $HepG_2$ human liver cancer cells and Caco-2 human epithelial colorectal adenocarcinoma cells and compare this to both the bioactive concentrations of the peptide. As shown in Fig. 4.1, no cytotoxicity was

observed at concentrations of NuriPep 1653 as high as 250 µg/mL. However, a reduction in cell viability of ~21%, 16% and 18% was observed in the THP-1, $HepG_2$ and Caco-2 cells, respectively at the highest concentration tested, 500 µg/mL. Considering the MIC of NuriPep 1653 (12 µg/mL), this indicates that the peptide is well-tolerated *ex vivo* and provided a therapeutic window in which the peptide was non-toxic to THP-1 cells for the immunological assays. Furthermore, the results obtained in $HepG_2$ and Caco-2 cells add to the attractiveness of the peptide, as it is unlikely to induce intestinal or liver damage at biologically relevant concentration. NuriPep 1653 may exhibit a good safety profile based on its plant based source material, and given the fact that it was identified from within the sequence of a naturally occurring protein, which has been part of the diet of humans for hundreds of years and undergoes biological breakdown into amino acids for absorption (Schaafsma 2009). This result gives an initial insight into the safety of the peptide for *in vitro* and *ex vivo* studies.

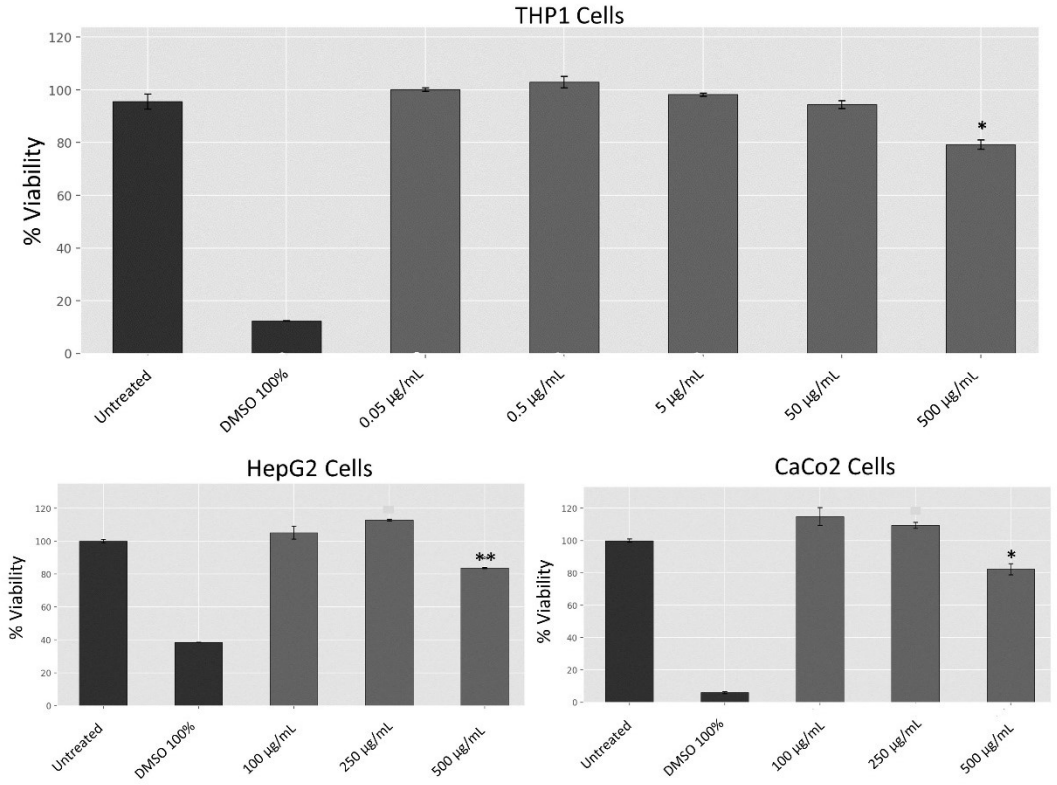


Figure 4.1 Effects of NuriPep 1653 on the viability of THP-1, HepG2 and Caco-2 cells. Cell viability was assessed by the MTT assay as per the manufacturer's guidelines. Values correspond to concentrations of NuriPep 1653 tested at a range from 0.5 – 500 µg/mL in THP-1 cells and 100, 250 and 500 µg/mL in HepG2 and Caco-2 cells. DMSO 100% and untreated cells served as controls. Data represent the mean of three experiments performed in triplicate and is expressed as the mean ± SD. Statistical analysis was performed using a one-way ANOVA where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4.3.2 Predicted Allergenicity of NuriPep 1653 as determined by AlgPred Online Tools

The results obtained from the online predictor confirmed that NuriPep 1653 is a non-allergenic peptide sequence and are summarised below. In the mapping of the IgE epitopes, a protein is a predicted allergen if it has one or more known IgE epitopes. As NuriPep 1653 does not contain any, it was deemed non-allergenic by this test. In the SVM model, the amino acid composition of all proteins (allergen and non-allergen) was computed. This composition was used as an input vector for training and testing of the SVM. The sequences were then compared against all of the sequences and their allergenicity predicted. NuriPep 1653 scored with a negative prediction value of 71.24% with this tool. The Blast searching uses a similar methodology to predicted allergenic proteins by performing alignment searches against representatives of allergenic peptides. Finally, the MAST tool uses motif searching of sections of proteins which known to be allergenic and compares this against the query sequence. NuriPep 1653 was not found to be allergenic using the Blast tools or to have an allergenic motif using the MAST predictor.

Mapping of the IgE epitopes:

Sequence does not contain experimentally proven IgE epitopes

SVM modules based on amino acid composition

- Non-Allergen – Score = 0.5374 (Threshold 0.4)
- Positive prediction value = 18.27%
- Negative prediction value = 71.24%

Blast searching on allergen representative peptides:

No hits found – Non allergen

MAST motifs

No hits found – Non allergen

4.3.3 Effects of NuriPep 1653 when used Pre- and Post-Infection on the Intracellular Fate of Salmonella Typhimurium in THP-1 Macrophages

THP-1 cells were exposed to NuriPep 1653 for a period of 2 or 20 hours either before or after infecting cells with *S.* Typhimurium. The macrophages were infected at a MOI of 20:1 and then spun to promote internalisation of the bacteria. In the case of cells that were pre-treated with NuriPep 1653 (Fig. 4.2A and 4.2B), the length of time of peptide exposure did not affect the cells, as almost identical results were obtained regardless of 2 hours or 20-hour macrophage pre-treatment. This was the only variation in the experiment, as in both cases, cells were lysed 1-hour post infection and the viable intracellular bacteria quantified by plating (CFU). Interestingly, at the lower concentrations tested, 0.5 and 50 μg/mL, there were no significant differences observed in the number of bacteria recovered when compared to the untreated control. However, when the cells were exposed to NuriPep 1653 at the highest concentration tested, 100 μg/mL (the MIC previously determined), no bacteria were recovered from the lysed macrophages (2-hour Fig.4. 2A and 20-hour Fig. 4.2B). This is a vast contrast to the results observed in the cells that were infected with *S.* Typhimurium before the addition of the peptide (Fig. 4.2C and Fig. 4.2D). Neither the length of time of NuriPep 1653 treatment post-infection nor the concentration resulted in any increase or decrease in the number of internalised bacteria compared to the control cells. In this assay, the activity of the peptide therefore appears to be specific for healthy cells and can potentially eliminate populations of internalised *S.* Typhimurium.

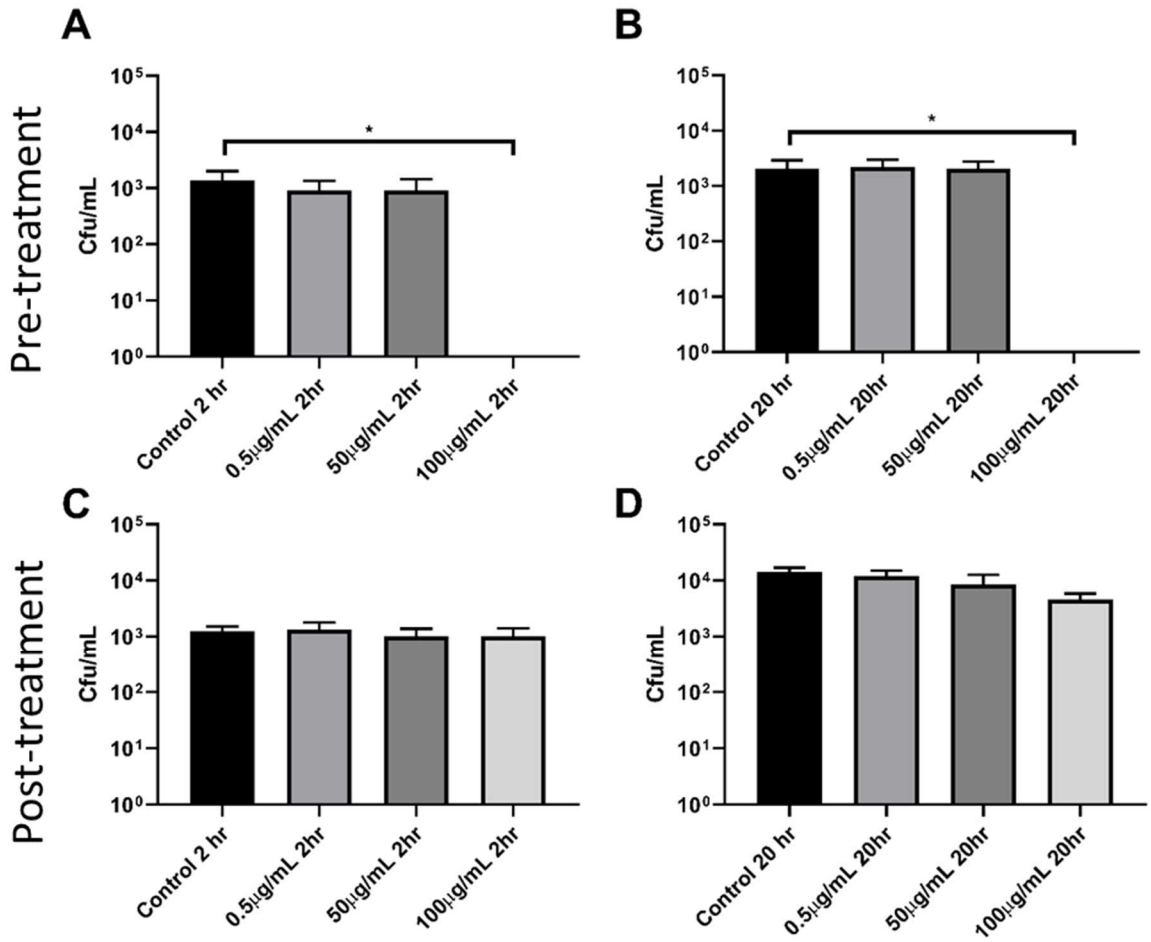


Figure 4.2 Effects of NuriPep 1653 when used pre- and post-infection in THP-1 macrophages infected with *S.* Typhimurium ATCC 14028. S. Typhimurium was used as a model for intracellular infection**. A**: 2 hours peptide exposure pre-infection, **B**: 20 hours peptide exposure pre-infection, **C**: 2 hours peptide exposure post-infection, **D**: 20 hours peptide exposure post-infection. Values represent the CFU/mL recovered from lysed macrophages after plating. Data represent the mean of three experiments performed in triplicate and is expressed as the mean ± SD. Individual treatments with the peptide at different concentrations were compared to the untreated, infected cells (* $p \leq 0.05$).

4.3.4 Effect of NuriPep 1653 on TNF-α Secretion from THP-1 Cells

The immunomodulatory effects of NuriPep 1653 was examined in differentiated THP-1 cells. Two concentrations were tested that were within the non-toxic concentration range of the peptide; 0.5 and 5 μg/mL. Differentiated THP-1 cells were pre-treated with NuriPep 1653 before stimulation with 100 ng/ml of LPS. Cell supernatants were subsequently collected and assayed for TNF-α by ELISA as per the manufacturer's instructions. The anti-inflammatory omega-3 fatty acid docosahexaenoic acid (DHA) was used as a positive control in the assay and successfully reduced TNF-α secretion from the THP-1 cells (Solanki *et al.* 2013). The reductions induced by NuriPep 1653 were presented as a reduction in TNF-α concentration relative to untreated, LPS stimulated cells. Considering that the cells treated with LPS alone represent the maximal TNF-α concentration, or 100%, DHA was shown to reduce TNF-α secretion by 61.7%. TNF- α percentage reductions from THP-1 cells treated with NuriPep 1653 compared to the LPS treated cells are shown in Fig. 4.3. Both concentrations of the peptide; 0.5 μg/mL and 5 μg/mL significantly reduced TNF-α secretion by 36.8 % and 33.7 %, respectively ($p \leq 0.001$). The peptide-induced reduction in this multifunctional cytokine suggests that NuriPep 1653 is indeed capable of triggering immunological functionalities associated with the stimulation of the immune system and regulation of inflammation.

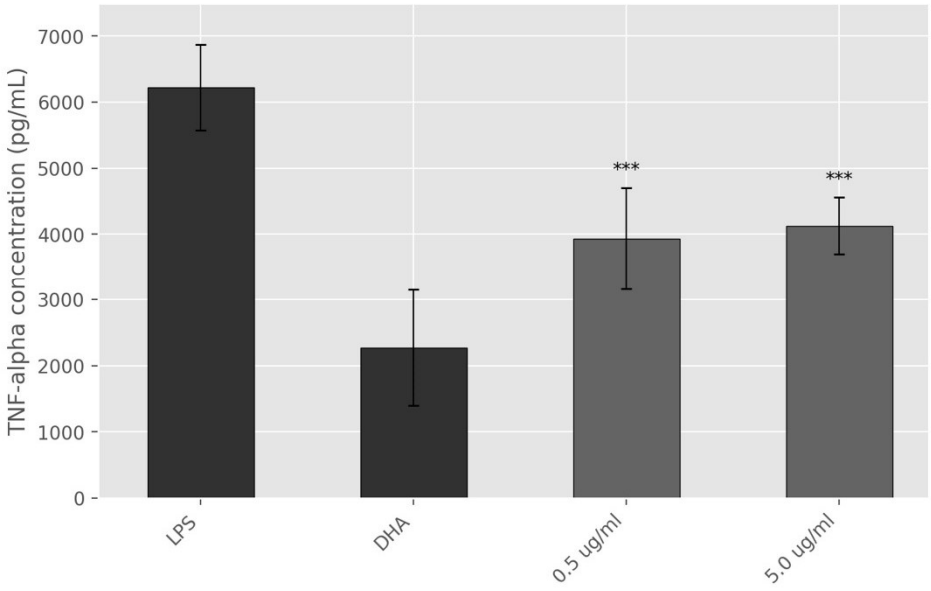


Figure 4.3 TNF-α reduction from THP-1 cells treated with NuriPep 1653 treatment. Cells were either treated with NuriPep 1653 at 0.5 and 5 μg/mL, DHA or media alone, which acted as the negative control. All cells were subject to LPS stimulation (100 ng/mL) before treatment. NuriPep 1653 significantly reduced the production of TNF-α, measured in pg/mL, in differentiated THP-1 cells. Unstimulated cells were included as an internal negative control in the assay but not incorporated in the graph as no TNF-α production was observed. Data presented as mean ± SD of three independent experiments; *p ≤0.05 between replicates, **p ≤0.01 between replicates, ***p ≤ 0.001 between replicates.

4.3.5 Measurement of NuriPep 1653 Induced NF-κB Modulation using THP-1 Blue Cells

TLRs are considered part of the first line of defence against pathogens and are responsible for detecting specific microbial motifs and stimulating the immune system (Caballero *et al*. 2017). They are also responsible for detecting danger signals within the body in the absence of microbial infection, hence they are critical mediators in inflammation (Matzinger 2012). The TLR ligands used in the assay had their optimal concentrations pre-determined so that signal saturation was avoided before the addition of the peptide to the cells. The ligand concentrations were as follows; FLA-ST 20 ng/mL, HKLM 2.7E7 ng/mL, FSL, 3ng/mL, LPS 30 ng/mL, rTNFα 3.13 ng/mL and Pam3CsK4 25 ng/mL. Each of the ligands corresponded to a different TLR, which produced a strong stimulatory response in the cells. In its inactive state, NF-κB complexes are bound to an inhibitor, inhibitor kappa-light-chain-enhancer of activated B-cells (IκB) (Gilmore 1999). However, when stimulated, IκB is phosphorylated and broken-down and allows NF-κB to translocate to the nucleus (Karin 1999). BAY 11-7082 (Sigma Aldrich, United Kingdom) selectively inhibits NF-κB activation by blocking TNF-α-induced phosphorylation of IκB and therefore, was included in the assay as an internal positive control (Pierce *et al*. 1997; Z. Zhang *et al*. 2019). When compared to the cells stimulated with FLA-ST (TLR5) alone, NuriPep 1653 was capable of significantly reducing the levels of SEAP detected in the supernatant in a dose dependant manner by 35.36%, 51.12% and 64.21% at 125 µg/mL, 250 µg/mL and 500 µg/mL, respectively ($p \leq 0.05$, $p \leq 0.001$, $p \leq 0.0001$), tested in triplicate on independent days (Fig. 4.4A). THP-1 Blue cells measure NF-κB production through the release of SEAP reporter, which is detected by measuring the OD. Activation of this pathway is usually associated stimulating the immune system and the production of proinflammatory cytokines, such as TNF-α. Our results revealed that NuriPep 1653 reduced the NF-κB production in cells stimulated specifically with TLR5, which recognise bacterial flagellin from invading mobile bacteria. This was measured by the lower detection of SEAP compared to the stimulated, but non-peptide treated cells. Importantly, the peptide reduces, but not inhibits, NF-κB production, indicating that the effects are modulatory and more specifically associated with inducing an anti-inflammatory immune response. NuriPep 1653 did not significantly modulate the levels of NF-κB when cells were co-stimulated with NuriPep 1653 and the remaining TLR ligands (Fig. 4.4B, C, D, and E). The levels of Pam3CsK4 were below detection levels and therefore not considered for further analysis (Fig. 4F)

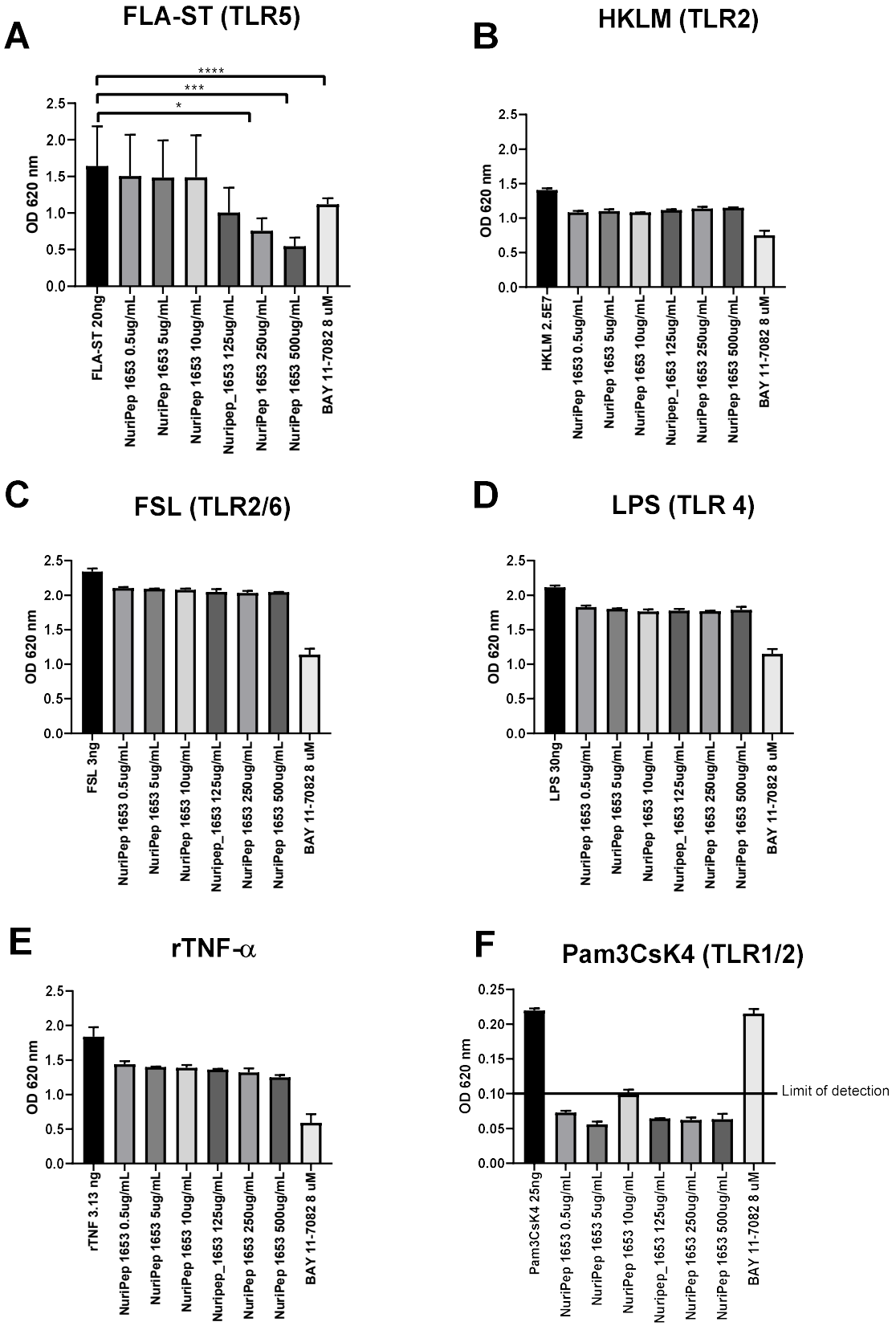

A
FLA-ST (TLR5)
OD 620 nm
FLA-ST 20ng
NuriPep 1653 0.5ug/mL
NuriPep 1653 5ug/mL
NuriPep 1653 10ug/mL
Nuripep_1653 125ug/mL
NuriPep 1653 250ug/mL
NuriPep 1653 500ug/mL
BAY 11-7082 8 uM
B
HKLM (TLR2)
HKLM 2.5E7
C
FSL (TLR2/6)
FSL 3ng
D
LPS (TLR 4)
LPS 30ng
E
rTNF-α
rTNF 3.13 ng
F
Pam3CsK4 (TLR1/2)
Pam3CsK4 25ng
Limit of detection

Figure 4.4 The effects of NuriPep 1653 on the modulation of the NF-κB pathway using THP-1 Blue cells. THP-1 Blue cells were stably transfected with SEAP before stimulation with the ligands and peptide exposure. Ligands for stimulation were used at the following concentrations; (A) 20 ng/mL FLA-ST (TLR5), (B) 2.5E7 ng/mL HKLM (TLR2), (C) 3 ng/mL FSL-1 (TLR2/6), (D) 30 ng/mL LPS (TLR4), (E) 3.13 ng/mL rTNF-α and (F) 25 ng/mL Pam3CsK4 (TLR1/2). Ligands that produced a weak response, TLR3, TLR7 and TLR9, were excluded from the analysis. NuriPep1653 was tested at 0.5, 5, 10, 125, 250 and 500 µg/mL. BAY 11-7082 was used at 8 µM as a positive control in the assay blocking NF-κB activation. No activation or reduction in the NF-κB cascade was observed in B, C, D, E and F. However, a significant reduction in the pathway was induced by the NuriPep 1653 in cells stimulated with FLA-ST (TLR5), A. Data represent the mean of three experiments performed in triplicate and is expressed as the mean ± SD. (*p ≤0.05, ***p ≤ 0.001, **** p ≤ 0.0001).

4.3.6 Effects of NuriPep 1653 in a Wound-healing HaCaT cell Model

We aimed to determine if NuriPep 1653 could stimulate proliferation as well as migration of keratinocytes in a scratch-wound assay using HaCat cells. As determined previously, this peptide displayed potent activity *in vitro* against *A. baumannii* (Mohan *et al*. 2019). Considering that this pathogen is frequently associated with skin infections, we wanted to investigate if NuriPep 1653 could be beneficial in healing a wound and promoting the migration of keratinocytes. Using Image J software analysis, the area between the wound edges could be determined after 24 hours of peptide treatment (Fig.4.6). Significant differences in the wound area were observed in all individual cases between time 0 and time 24 hours. However, no significant differences were observed between peptide treatment and the untreated control when the measure of wound closure, or when comparing healing at the final timepoint was considered, Fig. 4.5. Keratinocytes migrate naturally to heal wounds even in the absence of any growth factor (Bayram *et al*. 2005) and in this case, addition of the peptide did not induce a greater healing effect than that naturally observed in these cells.

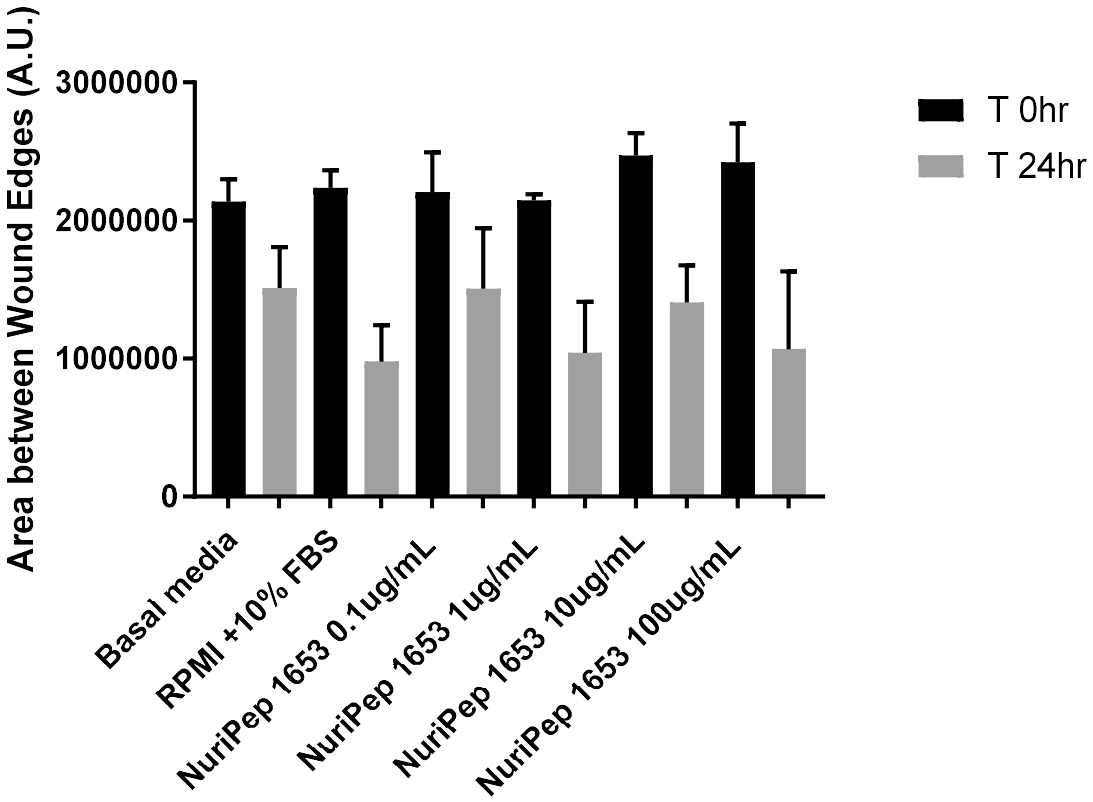


Figure 4.5 Wound-healing capabilities of NuriPep 1653 in HaCat cells *ex vivo* over 24 hours. Cells treated with media containing 10% FBS were included as a positive control as FBS encourages growth and migration of the cells. NuriPep 1653 was used at a concentration range of 0.1 µg/mL to 100 µg/mL. The area of the wound was measured at time 0 and time 24 hours and compared to cells grown in basal media with no growth factor. Wound-healing was measured as the migration of cells closing the area between wound edges using Image J software Version 1.52n and the wound-healing plug-in. Data represent the mean of three experiments performed in triplicate and is expressed as the mean ± SD. Statistical analysis was performed using a one-way ANOVA, however no significance was determined when 1: the difference between T0 and 24 hours was analysed across the samples or 2: when only the final area after 24 hours healing was considered.

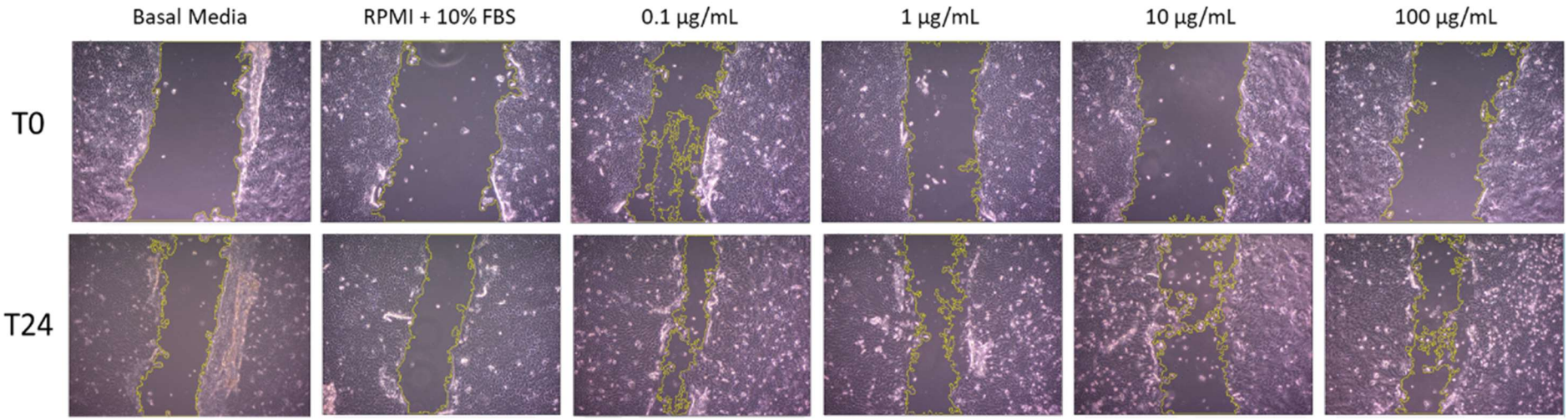


Figure 4.6 Image J analysis of wound-healing in HaCat cells post treatment with NuriPep 1653 over 24 hours. The wound-healing plug-in was used to measure the area between the wound edges in cells treated with basal media (untreated control), 10% FBS (growth control) and increasing concentrations of NuriPep 1653 (0.1 – 100 µg/mL). Measurements were taken at time 0 and time 24 hours. Settings for image analysis were as follows: Variance was measured with a filter radius of 10, a threshold of 50, a radius open of 3 and a minimum size of 10,000. The assay was performed in triplicate on three independent days. Image J Version 1.52n.

4.4 Discussion

Given the severity and velocity at which bacteria are developing resistance to conventional antimicrobial treatments, we must investigate, and bring forward, new drugs capable of changing this daunting situation. Peptides with a multifunctional nature are attractive for therapeutic use, as having multiple cellular targets increases the likelihood to kill MDR bacteria and reduce the selective pressure contributing to the development of resistance (Silva *et al*. 2016). While resistance to immunomodulatory peptides has been described through acylation of membrane molecules, overproduction of efflux pumps, electrostatic shedding and biofilm formation, the resistance mechanisms to traditional antibiotics are far more readily attainable (Bahar and Ren 2013). The action of immunomodulatory peptides is very complex. They can translocate across plasma membranes and/or interact with membrane receptors (G-protein coupled receptors, IL receptors or TLRs) (Scott *et al*. 2007). Post-binding, these peptides can stimulate an array of signal transduction pathways and downstream activation of hundreds of transcription factors essential in immunological response (Bowdish *et al*. 2005). Given the vast number of cells, receptors and pathways involved, the actions of peptides can influence a plethora of immunological responses. While structural conformations conferring immunomodulatory activity are difficult to determine, certain features overlap with antimicrobial activity such as an amphipathic conformation with >50% hydrophobic residues and a length of 12-50 mer (Mansour, Pena, and Hancock 2014; Saint-Sauveur *et al*. 2008; Kong *et al*. 2008; Rodríguez-Carrio *et al*. 2014; Haney and Hancock 2013). Considering this, we discuss our newly discovered AMP, NuriPep 1653, and explored if it can also modulate the host immune system.

Human cathelicidin LL-37 is an example of a classical host defense peptide. LL-37 has antimicrobial activity at high concentrations *in vitro*, and this anti-infective action is disrupted in physiologically relevant conditions. However, at the concentrations in which it is released from epithelial cells and neutrophils at sites of infection (< 5 μg/mL), it can induce immunomodulatory effects on various cells (Bowdish *et al.* 2004). As the concentrations of host defense peptides that induce immunomodulatory functions are usually quite low, we conducted infection assays in macrophages pre- and post-peptide treatment at a range from 0.5 - 500 μg/mL, staying below the cytotoxic concentrations as determined by the MTT assay. Based on the limited sensitivity, potential toxic effects on cells after long periods and the fact that tetrazolium reflects cell metabolism rather than cell number, further toxicity testing, incorporating more sensitive techniques, such as live/dead staining and flow cytometry, should be performed in the future.

The infection assays represent two very different situations, which can reflect different health status. In Fig. 4.2A and Fig. 4.2B, the macrophage was not stimulated by bacteria, and represents a "healthy" state of the differentiated THP-1 cells. In this case, when the peptide is applied for 2 hours to the cells at a concentration of 100 μg/mL, no bacterial colonies were identified after plating. Possible reasons for this have been hypothesised. Firstly, that NuriPep 1653 may bind, specifically at this threshold concentration, to receptors or proteins on the macrophage and inhibits the internalisation of bacteria in the first place, so they are likely eliminated through washing or addition of the antibiotic. Similarly, the peptide may have stimulated a mechanism within the macrophages that directly killed the bacteria. Alternative to this theory, the peptide may have been up taken into the macrophages and killed the bacteria once internalised. This hypothesis is supported by the fact that it is only at a concentration of 100 μg/mL that this occurs. Mohan *et al*. (2019) previously determined this CE concentration against *S.* Typhimurium. No effect was seen in the cells that were activated through pathogenic stimulus that represent a "diseased" state, and so perhaps the peptide was inhibited from entering these cells and therefore cannot exhibit its activity. If the activity was based on stimulating the healthy THP-1 cells and enhancing phagocytosis, then this may be blocked in activated cells. NuriPep 1653 may therefore function prophylactically by priming the macrophage before infection or enter healthy cells and assist phagocytosis by killing bacteria from within the macrophage. In order to investigate further the action of the peptide in the macrophages, we suggest future work could be carried out using *Salmonella* constitutively expressing mCherry in order to conduct fluorescent microscopy (Starr *et al.* 2018). Alternatively, the peptide could be tagged with this fluorescent protein or with green fluorescent protein (GFP) and its movement followed when in contact with the cells.

The results obtained in the ELISA assay indicate that NuriPep 1653 can impact inflammation regulated immune functions. Inflammation is a carefully regulated process in the body, essential in healing and repairing damaged tissue as well as protecting against foreign invaders. Dysregulated inflammation is associated with hundreds of diseases from cancer to arthritis, and sepsis (A. Hilchie, Wuerth, and Hancock 2013). Infection can trigger inflammation and stimulate the generation of many factors chemoattracting immune cells and the production of pro-inflammatory cytokines; TNF-α being one of the most prevalent (Sun *et al.* 2018). Therefore, reducing TNF-α can have a marked effect on decreasing the severity of various chronic inflammatory disease (Roubille *et al.* 2015). NuriPep 1653 was shown to significantly reduce TNF-α in LPS stimulated cells. Concentration dependency

was not determined using the two concentrations tested, and very similar reductions were observed both with 0.5 and 5 μg/mL of the peptide. It may be the case that the receptors on the cells are saturated at 0.5 μg/mL and further binding is inhibited. However, a larger concentration range should be tested to confirm this.

Complimentary to the results above, NuriPep 1653 exhibited anti-inflammatory activity using the THP-1 Blue cells. PRR in the immune system recognise pathogenic stimuli and initiate signalling pathways that trigger immunological responses. NF-κB is one of the most important transcription factors and is responsible for the activation of the host immune response and stimulation of inflammatory mediators, such as TNF-α, growth factors and chemokines during infection (Zuliani-Alvarez, Piccinini, and Midwood 2017). After cell stimulation with FLA-ST and subsequent peptide treatment, the secretion of the SEAP reporter was quantified in the cells which correlates with modulation of the NF-κB pathway. Interestingly, NuriPep 1653 was shown to modulate NF-κB through a reduction in its production when stimulated with FLA-ST in a concentration dependant manner. In this way, NuriPep 1653 modulated the TLR5 pathway, which recognises flagellin from both Gram-positive and Gram-negative bacteria. Blocking the NF-κB pathway causes IκB to become sequestered in the cytoplasm and prevents induction of the NF-κB target genes. As a result, this leads to a reduction in inflammation.

As the allergic response is involved in activating cells of the immune system and stimulating inflammation, the allergenic potential of NuriPep 1653 was determined. Furthermore, it was particularly pertinent to assess the predicted allergenicity of NuriPep 1653 as a clinically relevant cross-reactivity has been previously determined to exist between *P. sativum* and *Arachis hypogaea*, better known as the common peanut, on the basis of common vicilin homologues (Ara h 1) (Wensing et al. 2003). Despite this, multiple prediction approaches including mapping of the IgE epitopes, SVM models, Blast searching, and motif alignment tool, MAST were all conclusive in their findings that NuriPep 1653 was non-allergenic.

Finally, the wound-healing properties of NuriPep 1653 were investigated. Wound-healing is a complex biological process linked to the immune system through inflammation and cell regeneration, which can be promoted by immunomodulatory peptides. Throughout wound-healing, natural host defense peptides, mainly defensins, can be induced in keratinocytes which can reduce the pathogenic load surrounding the wound, as well as increase macrophage recruitment, promote growth factors, re-epithelialisation and angiogenesis (A.

Hilchie, Wuerth, and Hancock 2013; Brogden, Bates, and Fischer 2016; Ramos *et al*. 2011). Examples of wound-healing peptides which function through various different mechanisms include HB-107 (P. Lee *et al.* 2004), AG-30 (Kanazawa *et al.* 2016), ll-37 (Ramos *et al.* 2011) and IDR-1018 (Steinstraesser *et al.* 2008). In the case of NuriPep 1653, the peptide was not shown to induce a significant difference in the area of the wound created in the layer of HaCat cells *ex vivo* compared to the untreated control in basal media. We anticipate that the exposure time with the peptide was not long enough to establish a difference from the control cells where natural cell migration was observed. It may be the case that if the cells were exposed to NuriPep 1653 for a further 24 hours, a significant difference may have been obtained with a greater migration stimulated by the peptide than that seen naturally. Given the anti-inflammatory results observed in other assays, it is likely that NuriPep 1653 may indeed have some beneficial role in wound-healing and this will be explored in the future.

Taken together, our results indicate that NuriPep 1653, in addition to its potent antimicrobial activity, may also exhibit immunological functions. Zhang *et al.* (2019) describe the effects of peptides from *Nibea japonica*, a marine commercial fish, at effectively increasing the production of the pro-inflammatory cytokines IL-1β, IL6 and TNF-α in murine macrophages and activating the NF-κB signalling pathway. Immunomodulatory peptides are predominantly associated with enhancing pro-inflammatory immune responses, rather than reducing them, as we have shown. However, authors such as Herrington, Carmody, and Goodyear (2016) and Tak and Firestein (2001) explain how peptides can modulate the NF-κB signaling cascade and that this can be an effective therapeutic target in autoimmunity. It is important to consider that peptides with immunological roles are neither pro- nor anti-inflammatory but rather selectively modulate the immune system. NuriPep 1653 has shown significant anti-inflammatory properties that are not only of paramount importance in microbial infection, but also in several other diseases. NuriPep 1653 is an attractive candidate as an antimicrobial alternative, which can directly kill MDR pathogens, while reducing inflammation and potentially enhancing the phagocytotic potential of macrophages. Our results reinforce that natural, food-derived peptides can have multifaceted functionalities and therefore, a wide array of therapeutic applications.

Chapter 5
General Discussion and Closing Opinions

5.1 Context of this Study

The primary aim of this study was to "unravel a food derived bioactive peptide" from a natural protein source. Therefore, there was a heavy focus on the discovery, characterisation, activity and initial mechanism of NuriPep 1653. However, it is important to firstly discuss the context of our investigation and reveal why the discovery of novel peptides is so important in this day and age.

Firstly, we reviewed the challenges currently facing the food industry, as they struggle to find suitable alternatives to chemical preserving systems in food products. After the second World War, food habits changed dramatically, as ingredients such as fat and sugar, which were previously rationed, came into abundance (Cordain *et al.* 2005; Aschemann-Witzel 2015). This sparked the "fast-food" era. Shortly after this in the 1970's, artificial ingredients, colours, flavours and preservatives were popularised which expanded the realms of food, at the time (Food and Drug Administration 2010). Present day food habits have almost come full circle. The dangers of these artificial ingredients have been exposed as well as the undeniable association between poor diet and an array of diseases, primarily diabetes and obesity. As public education on the impacts of diet and overall health improves, there is now a significant growth in the popularity of plant-based diets, natural ingredients and clean labels among the public (Watrous 2015; Beverland 2014). This has forced producers to re-invent their formulations, ensuring that 1: sensory properties aren't altered, 2: the product does not become prohibitively expensive to produce, and 3: microbial populations involved in spoilage and foodborne disease are controlled.

Bacteriocins are ribosomally synthesised AMPs produced by bacteria as a defence mechanism and have shown to be antimicrobial against both Gram- positive and -negative strains (Cotter, Hill, and Ross 2005). Nisin is an example of a bacteriocin produced by *Lactococcus lactis* which has been recognised since the 1960's for its preserving properties and activity against food pathogens. It is one of the only bacteriocins approved for use in commercial products and is known as E-234 in Europe (Ross, Morgan, and Hill 2002). Nisin has also been acknowledged as "Generally Regarded as Safe" (GRAS) by the FDA and WHO (Leverentz *et al.* 2003). Nisin has been used for 60 years in food and no major development of bacterial resistance has been reported. This bodes well and may be extrapolated for other peptides, which are known to have a limited propensity for inducing resistance (Yeaman and Yount 2003a).

Protein hydrolysates and the peptides contained within, such as PSH described here, may be more suitable as bio-preservatives as they are directly obtained from plant-based proteins. Antimicrobial protein hydrolysates are cheap, environmentally friendly, non-toxic and natural therefore meet the needs of both the consumer and the food producers. The effects of protein hydrolysates at reducing the levels of pathogenic bacteria and preventing spoilage has already been described in complex food models (Osman, El-Araby, and Taha 2016). The activity of protein hydrolysates is attributable to the network of peptides contained within it, which can often be fractionated and purified to enhance activity. The PSPep peptides (Table 2.2) are examples of such peptides, which may contribute to antimicrobial activity either individually or in synergy. PSH was shown to have bactericidal activity against *E. coli* O157:H7, a relevant food pathogen which is easily transmitted through contaminated soil and water and has recently been associated with serious outbreaks of foodborne illness (CDC 2018). The conserved activity of PSH extended to a lettuce leaf food model which represents a frequently contaminated source of bagged "ready to eat" lettuce pieces (CDC 2019; Tirpanalan *et al.* 2011). The MIC of 8 mg/mL was below the cytotoxic concentration indicating a good safety profile. Furthermore, once ingested, the hydrolysate will be subjected to further breakdown as part of the natural proteolytic digestion (Schaafsma 2009). While we concentrated on *E. coli* O157:H7 as the target bacterium, the diverse nature of the peptides contained within protein hydrolysates mean that broad spectrum activity is likely attainable. Here, we highlight just one example of bio-preservation using hydrolysed *P. sativum* protein. However, countless other protein hydrolysates from sustainable, natural sources may play an important part in the revolution of "clean labels" and healthy eating. Protein hydrolysates may replace chemical washing treatments, such as chlorine, in the near future and may also be developed further as preserving ingredients. We discuss the benefits of protein hydrolysates and the challenges in their development in greater detail, later in this chapter. In an attempt to elevate the potential of our findings beyond food preservation we explored individual peptides cleaved from within *P. sativum* proteins and evaluated their potential against MDR pathogens.

By scrutinising the peptide content of PSH we identified NuriPep 1653 as an individual sequence which, when produced synthetically and tested against MDR pathogens, exhibited remarkable antimicrobial activity. Nuritas proprietary software, which comprises of algorithms capable of extensively searching publications, databases and patents, was used to determine that NuriPep 1653 was a novel sequence, and not previously reported elsewhere.

Considering this, the sequence of NuriPep 1653 was patented under submission number 7783813 and application number EP19192678.1 as a novel natural plant derived peptide, with action against MDR pathogens *in vitro*.

Multidrug resistance represents one of the most formidable challenges of the twenty-first century. As the antibiotic pipeline runs dry, MDR pathogens gain a foothold worldwide. With so few new anti-infective compounds approved in the last decade, we are predicted to face a crisis in the coming years, with deaths from antimicrobial resistance to exceed those from cancer (O'Neill 2015). This emergency has been long recognised in the healthcare sector and research in the area of novel antimicrobials has flourished as a result. In particular, AMPs have come to the fore which are capable of addressing many aspects of multidrug resistance where antibiotics have failed (Antosova *et al*. 2009). Considering this, we explored NuriPep 1653 in the context of the multidrug resistance crisis and many of our findings were particularly pertinent. Particularly, we call attention to the low MIC value (12 μg/mL) against a clinical isolate of *A. baumannii* that was shown to be completely resistant to all classes of antibiotic drugs tested. NuriPep 1653 was capable of synergising with colistin and reversing resistance of the pathogen to this lipopeptide reducing the MIC by 8-fold. This is a promising result, which may have important significance in the medical sector. Peptides which function *via* dual mechanisms, decelerate further resistance and potentially provide effective treatment options where they were previously non-existent for patients. As regards the mechanism of action of NuriPep 1653, electrostatic attraction and membrane disrupting activity was evident, as is common for most cationic AMPs. Furthermore, when used in combination, NuriPep 1653 and colistin showed additive effects, where the activity of each compound was potentiated in the presence of the other. Peptide synergism with antibiotics has been previously described and considered an attractive therapy as the peptide induces permeabilisation and facilitates entry of the antibiotic directly into the cell (Hollmann *et al*. 2018). In this case, both colistin and NuriPep 1653 are peptides, which appear to function *via* the same initial mechanism. It may be the case, that post membrane disruption, one or both of the peptides function *via* an intracellular mechanism thereby enhancing killing. However further experimental work, for example with fluorescently tagged peptides and microscopy, would be required to analyse this. It may also be the case, that the synergy observed between NuriPep 1653 and colistin reflects co-operative interaction of the peptides with the bacterial outer membranes. This has been shown in the case of mammalian peptides from different structural classes which showed additive effects

against *P. aeruginosa, E. coli* and *E. faecalis* compared to their activity alone (Yan and Hancock 2001).

NuriPep 1653 was also shown to be non-toxic. In general, AMPs show selectivity for prokaryotic cells over eukaryotic cells based on their negative surface charge which attracts the peptide. Some peptides such as mastoparan, an AMP released as a secondary metabolite from wasp venom exhibit significant cytotoxicity. Irazazabal *et al*. (2016) describe how they modified the peptide by substituting an alanine residue at position 5 and 8 in the sequence. They conclude that the amino acid switch modified the hydrophobic moment which in turn enhanced selectivity and reduced haemolytic and cytotoxic effects. Many papers highlight the rational design of peptides and sequence substitutions based on the structures of existing natural AMPs. In particular, there is a focus on those which adopt an alpha helical conformation upon interaction with the bacterial membrane, as these show potent activity and reduced cytotoxicity (Zelezetsky *et al*. 2005; Giangaspero, Sandri, and Tossi 2001). While the conformation of NuriPep 1653 was not determined experimentally to elucidate the structure when in contact with the membrane, the viability and haemolytic assays we performed reveal a lack of toxic effects in macrophages, liver cells and epithelial cells.

Our studies indicated that NuriPep 1653 did not induce significant resistance when passaged at a sub-inhibitory concentration over 14 days in an XDR strain of *A. baumannii* which already had genetic modifications and virulence factors contributing to its antibiotic resistant character. The general mechanism of action of AMPs mean that development of resistance is far less likely than for antibiotics. This is a particularly attractive feature of peptides, both natural and synthetic (Omardien *et al*. 2018). In addition, peptides often act rapidly on bacterial membranes. When a threshold concentration is reached, the membrane integrity is completely compromised, and cell death ensues. This was observed in our flow cytometry results through the large amount of cell debris and dead cells post-peptide exposure. The killing kinetics assay revealed that at the highest concentration tested (192 µg/mL), which was still below the toxic concentration, it took just 10 and 20 minutes to kill 10^8 CFU/mL of colSAB and colRAB, respectively. Similar to the killing kinetics of NuriPep 1653, SET-M33, a well studied AMP, was shown to eliminate colistin resistant *P. aeruginosa* in a concentration and exposure time dependant manner clearing 10^6 CFU/mL after 2 hours exposure to 8 µg/mL of the peptide (van der Weide *et al*. 2017). As regards its activity, NuriPep 1653 appeared to be specific for Gram-negative pathogens. This may be due to the availability of certain components, such as LPS in the Gram-negative cell wall architecture

to which the peptides are more attracted. This is notable as 5 out of the 6 ESKAPE pathogens, which are considered as the most urgent MDR threats to human health, are Gram-negative. Given that many AMPs are also host defence peptides and that a commonality observed between both is valence sensitivity, which was shown for NuriPep 1653, we continued investigating the peptide in the context of immunomodulating properties.

Peptides which not only directly target a pathogen, but which also modulate aspects of the immune system such as enhancement of the phagocytosis; regulation of the secretion of cytokines and ILs; stimulation of B-lymphocytes and promotion of NK cells to the active site among more, represent an interest approach to tackle the complex mechanisms of resistance and virulence in MDR bacteria (Chalamaiah, Yu, and Wu 2018; Duarte, Vinderola, Ritz, Perdigón, and Matar 2006; Ahn, Cho, and Je 2015; D. Huang *et al.* 2014; Ling-Ling *et al.* 2017). The results from the macrophage infection assays where cells were pre-treated with the peptide, revealed that NuriPep 1653 may enter macrophages and enhance killing of internalised bacteria from within. This theory is supported by the fact that the MIC against *S.* Typhimurium is 100 μg/mL, and it is only at this concentration, that no bacteria were recovered from the lysed cells. We hypothesise that the peptide is up taken in all cases, but it is only at a threshold concentration of 100 μg/mL that bactericidal action is observed. In this way, NuriPep 1653 may enhance the phagocytic activity inside the macrophages. This has been shown in the case of many immunomodulatory peptides including human LL-37 (Tang *et al.* 2015) and bovine cathelicidin Bac7(1-35) (Pelillo *et al.* 2014), which were both up taken by THP-1 macrophages and enhanced the killing of internalised *S. aureus* and *S.* Typhimurium, respectively. One way to verify if NuriPep 1653 is indeed being internalised, would be to add a fluorescent label to it and conduct fluorescent microscopy to follow its "movement" during an infection assay. While many host defense peptides are associated with inducing a pro-inflammatory response, to promote the recruitment of dendritic cells and monocytes and to enhance phagocytosis (D. Yang *et al*. 2004; Auvynet and Rosenstein 2009), NuriPep 1653 was shown do the opposite, and significantly reduce TNF-α in LPS stimulated macrophages. Moreover, upon stimulation with FLA-ST, NuriPep 1653 was shown to reduce NF-κB production *via* a TLR-5 dependant pathway. This is also associated with anti-inflammatory response and modulation of the immune system. While having features common to other host defence peptides such valence sensitive, amphipathic in nature, positively charged and length 22 mer (Mansour, Pena, and Hancock 2014; Saint-Sauveur *et al.* 2008; Kong *et al*. 2008; Rodríguez-Carrio *et al*. 2014; Haney and Hancock 2013), we highlight that NuriPep 1653 exhibits different actions on cells

of the immune system when compared to endogenous host defense peptides, such as LL-37 or HBD1 (Nijnik and Hancock 2009). Host defense peptides are produced by the body in response to certain stimuli and pathogenic "insult", (Bowdish *et al*. 2004; Jenssen and Hancock 2010). Conversely, NuriPep 1653 is a fragment of a non-antimicrobial storage pea protein, and at present, its reason for existence within the plant, or its function remains unknown. While a careful balance must exist between pro- and anti-inflammatory responses of the immune system, NuriPep 1653 has shown interesting activity in its abilities to protecting from infection while modulating inflammation which may be associated with diseases such as cystic fibrosis and gastroenteritis, where inflammation plays a crucial role in the disease progression (Brunetti *et al.* 2016; Valledor *et al.* 2010).

5.2 Non-Traditional Peptide Discovery

Peptides have been divided into various classes over the years as scientific knowledge on structure-function relationships improved. In the case of mammalian and insect AMPs these include secondary metabolites such as defensins, cathelicidins (De Smet and Contreras 2005), cecropins (LJ Zhang and Gallo 2016) and many more. Examples of plant AMP families include the thionins, cyclotides, and snakins (Tam *et al*. 2015). A similarity search was conducted using the PhytAMP database to compare the sequence of NuriPep 1653 to the reported plant peptides, however significant results were not observed and the peptide and did not fall into any class of previously described plant AMP family. NuriPep 1653 does not contain a cysteine rich motif or contain any disulphide bonds which have previously been described as two defining features of plant AMPs (Castro and Fontes 2005). The peptide is however, cationic, and made up of ~50% hydrophobic residues, which have been reported as common features conferring antimicrobial activity through amphipathic conformation and attraction to the negatively charged bacterial membrane components (Yeaman and Yount 2003a). We therefore, describe NuriPep 1653 as a non-traditional AMP derived from a plant source.

We speculate that research into plant AMPs has become somewhat unattractive in recent years, possibly because none of the 271 discovered to date, have progressed very far. That said, it is described above in section 1.2.1 that a bias appears to exist in peptide discovery from plants, as very few have been reported which do not fit the criteria mentioned above. Our approach to discover plant AMPs expands on those produced as secondary metabolites. We anticipate that thousands of potent peptides, with a whole host of biological functions,

may be encrypted within proteins and have yet to be discovered. These peptides may exist for a reason and impart some function to the plant during its lifecycle, or they may exist to impart a beneficial effect in humans when consumed and digested, as in the case of mother's milk. AMPs were initially derived from the secretions and glands of animals, such as for insulin in the 1920's (Banting *et al*. 1922). Later, sequence-based alignment and homology searching became popular as a means to identify new AMPs (P. Wang *et al*. 2011). Current methodologies to construct AMP libraries include PCR techniques, and DNA shuffling where randomly generated nucleic acid libraries encoding for AMPs are expressed biologically (Guralp *et al*. 2013). Synthetic combinatorial methods are also popular as sequences can be custom designed (Hilpert *et al*. 2005). Newer strategies involve activity models based on the predicted 3D structure of AMP molecules (Liu *et al*. 2018). Furthermore, other computational methods have helped accelerate prediction and classification of AMPs. Machine based learning techniques are rapid, economical and highly efficient ways of dealing with large scale prediction tasks. Popular methods include: SVM (P. Meher *et al*. 2017); random forest (Joseph *et al*. 2012); decision tree (Lira *et al*. 2013); hidden Marvok models (Fjell, Hancock, and Cherkasov 2007); and neural network models (Veltri, Kamath, and Shehu 2018) among more. In the case of NuriPep 1653, we used the MS data obtained post-hydrolysis as a library through which we could rank peptides. The ranking system was designed based on a non-discriminatory set of features based on a positive and negative data set of AMPs. We hope that our findings shed a new light on AMP discovery from plants, and spark research to consider peptides which fall outside known families for investigation.

5.3 Opportunities and Challenges of Protein Hydrolysates, Antimicrobial Peptides and Immunomodulatory Peptides

Throughout this work, we addressed the desperate need for novel antimicrobial compounds and propose protein hydrolysates and natural plant derived peptides as promising solutions. In the following section the advantages and challenges in the development of hydrolysates, AMPs and immunomodulatory peptides are discussed, as well as the future prospects of these compounds in the food and pharmaceutical industries.

5.3.1 Advantages and Challenges of Protein Hydrolysates

5.3.1.1 Benefits of Protein Hydrolysates

Protein hydrolysates offer several advantages over conventional antimicrobial molecules due to their wide spectrum of therapeutic action, limited toxicity, and diverse structural properties (M. Nasri 2017). If by-products are used as protein sources, the global annual volume of processing waste could be significantly reduced and help sustain animal and plant agriculture (Hou *et al.* 2017). In addition, microbial and enzymatic hydrolysis can be an environmentally friendly way of producing bioactive peptides, which have vast functionalities across the food and pharmaceutical industry in an economically viable way. Another benefit of hydrolysed proteins is their biochemical stability during processing. Bioactivities are attributable to certain physicochemical properties of the matrix including charge, hydrophobicity and conformation, amongst others. While many isolated peptides may demonstrate reduced antimicrobial activity when tested alone and require encapsulation to avoid sequence deterioration during processing, protein hydrolysates appear to withstand processing conditions better, possibly due to protection within the matrix (Banan-Mwine Daliri, Oh, and Lee 2017). Moreover, bioactive peptides in protein hydrolysates often exhibit multi-functional activities as a result of synergistic effects with other components in the matrix (Chakrabarti, Jahandideh, and Wu 2014). A recent study highlighted the beneficial effects of processing on lentil hydrolysates where high pressure induced further breakdown and release of antioxidant peptides (Garcia-Mora *et al.* 2015). In another study, the heat resistance of AMPs in sausages was confirmed at 70°C and only a partial loss of activity was observed at 115°C (Reunanen and Saris 2004). The successful scaling of hydrolysates was also reported by Contreras *et al.* (2011), whereby the bioactivity was retained through atomisation, homogenisation and pasteurisation. Regarding toxicity, as oligopeptides are readily degraded by proteases in the GI tract, toxicity is drastically reduced as the hydrolysate experiences natural digestion and absorption (H Korhonen 2009).

5.3.1.2 Challenges in Developing Protein Hydrolysates

The stability of bioactive peptides and their ability to resist processing can be regarded as a drawback to their development. Without the intact sequence, the physiological benefits may not always translate from *in vitro* to *in vivo* situations, which often require isolation and modification to resist further proteolysis (H Korhonen 2009). This loss of bioactivity, once

ingested, is not a concern if the hydrolysate is to be used in food preservation where the action is carried out prior to consumption. Instead, it is an effective way of limiting the action of the hydrolysates to preserving the food rather than negatively affecting the gut microbiota.

Other challenges also exist in the development of protein hydrolysates such as batch to batch variation; bitterness and allergenicity. These factors should be carefully considered before production. Inconsistencies may stem from natural variation of the enzymes used in the hydrolysis, producing slightly longer or shorter peptides. This could affect synergies in the matrix or an individual functionality. The situation is compounded in microbial fermentation where reproducibility can be affected by the uncontrollable actions of the live cells, their metabolism and enzyme levels (Banan-Mwine Daliri, Oh, and Lee 2017). Furthermore, peptides released during fermentation and enzymatic hydrolysis remain susceptible to further breakdown, unless the enzymes are inactivated (Lammi *et al.* 2016). Terminating proteolysis in the hydrolysis process, using industrial enzymes, and continuous MS analysis can help limit continuous degradation, mitigate variability and measure the consistency in released peptides from batch to batch.

Another important concern, depending on the application, may be the sensory profile and flavour attributes of a hydrolysate. Microbial hydrolysis has been shown, in some cases, to release bitter tasting peptides (Maehashi and Huang 2009b). A low solid content in the hydrolysis mixture, low molecular weight peptides, bulky hydrophobic amino acids at the C-terminus and basic amino acids at the N-terminus have also been implicated in the generation of bitterness (Lafarga and Hayes 2016; Maehashi and Huang 2009a; Hou *et al*. 2017). According to some studies, leucine, tyrosine and phenylalanine in high proportions in the sequence may contribute to bitter taste (Ishicashi *et al*. 1987). Fourier Transform Raman spectroscopy, *in silico* predictors such as BitterDB[14] and QSAR approaches are useful in exploring and overcoming attributes related to bitterness in peptides (Lafarga and Hayes 2016; Tu *et al*. 2018). Despite their organoleptic profile, protein hydrolysates have already successfully been implemented into many foods on the market in products such as Calpis™ and Evolus® fermented milk, which contains the hypotensive tripeptides VPP and IPP (Hata *et al*. 1996). Therefore, in the future, sensory impacts may not present a significant

[14] http://bitterdb.agri.huji.ac.il/dbbitter.php

hurdle, which would limit the saleability of foods when weighed up against their biological benefits.

Regarding safety, host defence peptide metabolites from animals such as melittin present in bee venom can be incredibly toxic *in vivo* (Unger, Z, and Shai 2001). A study using an *in silico* predictor for peptide toxicity named ToxinPred[15] summarised that peptides with numerous cysteine, histidine, proline and asparagine amino acids in their sequence are more toxic than those with a limited number of these residues (Gupta *et al*. 2013). As described above, protein hydrolysates are rarely toxic and have GRAS status as they constitute a part of foods that have been consumed for hundreds of years before any bioactivities were recognised (Schaafsma 2009). A possibly more pressing concern with hydrolysates is their allergenic potential. In general, allergies involve a host immune response to a protein *e.g*. in Coeliac's disease, this response is against the wheat protein, gluten. As performed above for NuriPep 1653, allergenicity can be determined by comparing the sequence similarity and characteristics with those outlined in databases of allergenic proteins, such as AlgPred[16] (Tu *et al*. 2018). Generally, the conditions involved in hydrolysis, particularly enzyme treatment, destroy the allergenic proteins in food which dramatically reduces any allergenicity concerns however allergy testing should still be performed (K. Kim *et al.* 2011; Schaafsma 2009).

5.3.1.3 Future Directions in Protein Hydrolysates and Application of Bioinformatic Technology

Over the last decade, the rapid advances in technology and bioinformatics have greatly aided the mining of the vast number of potentially bioactive fragments locked within parent proteins of different foods as sources. Genome sequencing ignited *in silico* driven peptide discovery as it paved the way for "omics" studies, particularly proteomics, which relies heavily on gene model annotations for accurate protein identification (Valdés, Cifuentes, and León 2017). Computational methods encompassing software, online tools, databases and patents can be applied to manage, curate and interpret massive amounts of data related to biological systems (Li Chan 2015). The number of possible peptides contained in the proteome of just one food source can vary from 1 to 30 billion. This figure depends on the

[15] http://www.imtech.res.in/raghava/toxinpred/

[16] http://crdd.osdd.net/raghava/algpred/

genetic complexity of the species, *i.e.* the amount of proteins, their length and the number of PTMs of the individual amino acid residues. Bioinformatic tools can allow such vast data to be analysed, and drastically improve methods for identifying functional peptide hydrolysates, that can resist proteolysis and therefore exert their physiological activities (Agyei *et al*. 2016). By employing a multi-faceted approach combining MS, "omics" technologies, bioinformatics tools such as molecular docking and QSAR as well as *in vitro* hydrolysis, potent protein hydrolysates can be identified and many of the limitations discussed in the previous section may also be overcome (Tu *et al.* 2018; Valdés, Cifuentes, and León 2017; Nongonierma and FitzGerald 2016).

The main tools currently used to explore the proteome of the protein substrates are UniProtKB[17], NCBI[18] (National Centre for Biotechnology Information), and BIOPEP[19]. Following this, ExPASy-PeptideCutter can be a useful tool to determine the enzyme cleavage sites and thus, the subsequent peptides which will be contained in the hydrolysate (Tu *et al*. 2018). The peptides predicted to be released post *in silico* hydrolysis can then be compared against known peptides, described in databases such as Pepbank[20] and PeptideDB[21] (Shtatland *et al*. 2007). While peptide hits determined *via in silico* hydrolysis still need to be confirmed *in vitro*, it can vastly reduce the experimental load. It also allows for more high-throughput screening of protein hydrolysates in a cost-effective way (Dziuba and Dziuba 2014).

To date, many protein hydrolysates show physiological effects in the human body, however the specific functional peptide or combinations in the matrix remain unknown. Computational tools are driving the generation of smart protein hydrolysates with represent a rich source of bioactive compounds. These tools aid our understanding of conformation, prediction of functionalities, exemplifying molecular interactions and improving peptide properties (Tu *et al.* 2018). There is still much room for progress in the application of bioinformatics with protein hydrolysates. As knowledge on biomarkers of disease, receptors,

[17] https://www.uniprot.org/help/uniprotkb

[18] https://www.ncbi.nlm.nih.gov/

[19] http://www.uwm.edu.pl/biochemia/index.php/en/biopep

[20] http://pepbank.mgh.harvard.edu/

[21] https://omictools.com/peptidedb-tool

and molecular mechanisms expands, predictions and identification of active hydrolysates will improve thus, reducing the gap between simulated and actual hydrolysis (Tu *et al*. 2018).

Protein hydrolysates are already available on the market, *e.g.* hydrolysed whey protein however, we predict that they may feature more commonly very soon in the future of food. There has been a stark increase in the number of products incorporating novel ingredients and alternative sources of protein introduced to the market in the last few years. As discussed above, the bioactive peptides in protein hydrolysates can be multi-functional. Therefore, on top of their food-safety and preservation properties, protein hydrolysates may be incorporated as functional ingredients with specific health claims such as for lowering cholesterol. The nutraceutical industry is in its infancy in Europe, however, in other parts of the world, the value of functional foods in promoting health has been recognised and already incorporated into the diet. The Japanese ministry for health has implemented a policy for claims relating to functional foods with verified activity as Food for Specified Health Uses (FOSHU). To date, there are more than 1000 FOSHU approved foods with health claims on the Asian market incorporating bioactive peptides from soybeans for cholesterol reduction, milk peptides for osteogenesis and sardine peptides for anti-hypertension among more (Ministry for Health Labour and Welfare, Japan 2019). Moreover, the Foods with Functional Claims (FFC) regulation instigated in 2015, brought 400 new products onto the Japanese market, which has an estimated worth of $70 billion United States Dollars (USD) (Scattergood 2018). If we compare this with European regulations, it is considerably more difficult to obtain approval from the European Food Safety Authority (EFSA) for a nutraceutical product as detailed dossiers outlining full characterization and substantiation of the novel ingredients are required, as well as considerable scientific data (Aggett 2009). LFC24 is a bovine milk-derived casein hydrolysate which, as part of a Food for Health Ireland (FHI) project, was shown to reduce post-prandial blood glucose responses. Despite substantial data and sufficient characterisation, the EFSA rejected their claim, as the human trial was performed in healthy volunteers as opposed to diseased – therefore a cause and effect relationship was not determined (European Food Safety Authority 2016). As health epidemics worsen, it is likely that the value of functional foods and the rigid criteria assessing them will be re-evaluated in Europe and nutraceutical products with protein hydrolysates and bioactive peptide ingredients will be more readily available on the supermarket shelves.

5.3.2 Advantages, Disadvantages and Future Outlook of Antimicrobial Peptides

Over the last decade, the potential of peptide therapeutics as candidates and/or templates for novel antimicrobials has gained momentum in research (Suarez *et al*. 2005). While there are numerous benefits for the use of AMPs in biotechnological applications, challenges also exist which need to be overcome before they can be established as new antimicrobial drugs. The following section pertains specifically to advantages and disadvantages of pure peptides as opposed to protein hydrolysates, that were previously discussed.

5.3.2.1 Drawbacks Associated with the Development of Antimicrobial Peptide Therapeutics

The activity of most peptides is largely dependent on their intact sequence. Any peptidases or enzymes encountered either in the digestive tract or in the blood can have adverse effects on sequence integrity and thus, have a detrimental impact on their ability to reach host targets and exert biological functions (FitzGerald, Murray, and Walsh 2004). Nevertheless, in the case of some larger peptides and proteins, this can be advantageous and lead to the release of novel, more potent, shorter peptide sequences as described for certain hydrolysates above (M. Nasri 2017). Shorter peptides are more stable and resistant to proteolysis. Evidence suggests that di-, tri- and short polypeptides can easily cross the intestinal barrier *via* paracellular proton-driven transport. These peptides can exert their actions at the intestinal level, where generated signals can be transmitted to the brain, endocrine and immune system (Hou *et al*. 2017). Larger peptides, however, are likely subject to breakdown before absorption, which may hamper bioactivity depending on the active site (Gardner 19844). Besides proteolytic stability, peptide structure can be compromised through excessive heating. Furthermore, toxic by-products can be released as well as unwanted interactions/binding with other compounds during processing (M. Nasri 2017).

Toxicity can be a substantial hurdle in developing AMPs as therapeutics. One well known example, is the polypeptide colistin which was withdrawn as an antibiotic in the 1970's due to significant renal and neurological side effects (Spapen *et al*. 2011). In this desperate climate of antimicrobial resistance, colistin has returned on the market as a salvage treatment, but alternatives are sorely needed. The pharmacodynamics and pharmacokinetic profiles of AMPs are poorly understood. Once this is better investigated, it will allow dosing regimens to be designed where a balance between activity and toxicity is achieved. Topical

AMP agents can be desirable as frequent high doses can be achieved and metabolised slowly, minimising toxicity (Haney, Mansour, and Hacock 2017).

The cost involved in peptide production and development is a major limiting factor for their use in the biotechnology industry. The complex structure of defensins and cyclotides, which rely heavily on disulphide bonds to create certain folding patterns, can further complicate synthesis, and this increases cost (Cândido *et al*. 2011). The benefits and drawbacks of various production methods influencing costs are discussed above. Ion sensitivity is an important consideration in the development of AMP therapeutics. The mechanism of action which relies heavily on electrostatic interactions, can be inhibited by physiological anionic salts and serum. Various studies have shown that a reduction in activity was directly proportional to the presence of mono- or di-valent cations in the test media (Menzel *et al*. 2016; Bowdish, Davidson, and Hancock 2005).

While peptides have a reduced propensity for inducing resistance, bacteria have not been passive to the surging progression in AMP research. Microorganisms make considerable biological efforts to express countermeasures against AMP action to enhance survival in their presence (Yeaman and Yount 2003a). Resistance can occur through constitutive or inducible mechanisms. The former refers to the intrinsic ability of an organism to induce resistance and are constitutively expressed even when the peptide is not present. The molecular mechanisms behind passive resistance are not fully understood. Certain strains may naturally possess weakly charged, unsaturated membranes, altered cell energetics and other constitutive characteristics in cytoplasmic structure/ and/or functionality that enhance resistance (Bayer *et al*. 2000; Yeaman and Yount 2003a). The formation of microbial biofilms can also create a shield against electrostatic interactions. This is common in mucosal pathogens which form complex biofilms contributing to their virulence (Yeaman and Yount 2003a). Inducible resistance arises as a result of the peptide exposure or the cell stresses it causes. In an attempt to survive the effects of AMPs, cells can either activate virulence factors to subvert the immediate threat or evolve by means of permanent PTMs (G. Wang *et al*. 2015). Various regulons can be activated which modify the lipid bi-layer and/or envelope which subsequently alters the ability of the peptides to attach to the membrane (Gunn and Miller 1996). Specifically, PmrA/PmrB, the two-component regulatory system operon, can aid in conferring AMP resistance in *Salmonella* and other Gram-negative species (Gunn *et al*. 1998). Yeaman and Yount (2003) provide a

comprehensive and detailed review on possible surface modifications and resistance strategies employed by microorganisms to subvert AMP action.

5.3.2.2 The Potential of Antimicrobial Peptides in Biotechnology Industries

AMPs have vast potential in biotechnology. They can be used as individual broad spectrum antimicrobial agents; as potentiators in combination with antibiotics; as immunomodulatory agents; and/or as endotoxin neutralisation agents (Michael Zasloff 2002). Of importance, the concentrations of AMPs required to kill susceptible pathogens is often higher than for traditional antibiotics, however uniquely, they are often able to rapidly kill multidrug resistant bacteria at equivalent concentrations (Marr, Gooderham, and Hancock 2006; Brogden 2005) Beyond therapeutics, AMPs may have potential in the food and agricultural industries, but as margins are often tight, this is only feasible if production costs can be kept to a minimum (Yazdi, Mahai, and Zardini 2013; Santana *et al*. 2015).

The proteolytic stability and short half-life of AMPs can be drastically improved by N-terminus acetylation, blocking protease action or by cyclisation with disulphide bridges or backbone C- and N- terminal joining (Nguyen *et al*. 2010; Haney, Mansour, and Hacock 2017). Other strategies involve switching non-natural D-amino acids and/or tryptophan into the sequence to improve peptide stereochemistry and metabolic stability (Wade *et al*. 1990). Although, careful consideration must be given to any residue replacements, as it may hamper activity. AMPs can be directed to specific target sites using delivery systems. Liposomal carriers work well for this application, as AMPs are attracted to biological lipid bi-layers however, nanoparticles have also been investigated for controlled AMP release (Li, Nielsen, and Müllertz 2012; Oey *et al.* 2009).

Resistance to biological agents is inevitable in the evolution of species. As discussed, certain microorganisms have shown mechanisms to evade the killing actions of AMPs, however, when compared to antibiotics, the propensity for the development of resistance is far less (Yeaman and Yount 2003a). Complete resistance is unlikely as AMPs have an obligatory interaction with microbial membranes and the consequent requirement to reconstruct this membrane (Marr, Gooderham, and Hancock 2006). Peptides are also likely to have multiple targets making bacterial mutations against AMPs difficult. In cases where resistance was reported, findings were modest with only an increase in the MIC of two to four-fold observed after 30 passages of the bacteria in the presence of the AMP (L Zhang *et al.* 2005). In a stark

contrast, gentamycin caused a 190-fold increase in the MIC under the same experimental conditions (Steinberg *et al.* 1997) .

The synergistic effects of peptides with conventional antibiotics may help overcome the burden of multi drug resistance and make it more difficult for further mutations to happen by working through combined mechanisms (Marr, Gooderham, and Hancock 2006). Of concern is the fact that therapeutically administered peptides may accelerate the evolution of resistance to self-proteins and innate host defence peptides and thus render humans more susceptible to peptide resistant infections (Bell and Gouyon 2003). Hancock *et al*. believe this is a harsh estimation as peptide use has been widespread for several years in pharmaceuticals (polymyxin B) and in food (nisin) without impacting resistance in any organisms or innate ability to fight infection (Hancock 2003; Marr, Gooderham, and Hancock 2006). As we fall behind in this race of multidrug resistance, AMPs represent a prosperous solution when few others come to the fore.

5.3.2.3 Future Outlook for Antimicrobial Peptide Therapeutics

The current regulatory framework governing novel AMPs as pharmaceutical drugs is archaic. Firstly, we present peptides as a whole group. At present, protein and peptide drugs make up approximately 10% of the expansive pharmaceuticals market and was valued at $23.05 billion USD, and predicted to accelerate and generate almost double ($43.26 billion USD) by 2024 (GlobalNewsWire 2018). Currently, 254 protein-based drugs, of which ~60 are marketed peptides, have FDA approval for use as therapeutics across a number of disease areas (Fosgerau and Hoffmann 2015). THPdb[22] is a comprehensive database providing information on the FDA-approved proteins and peptides (Usmani *et al*. 2017). Combined, peptides with immunological and antimicrobial activity represent 25% of the proteins and peptides approved. In their early development, the majority of peptides which were brought forward for clinical development, had fewer than 10 amino acids in their sequence. Improvements in technology mean length is no longer a limitation, and sequences with up to 40 resides are easily produced and considered for development (Lau and Dunn 2018).

[22] http://crdd.osdd.net/raghava/thpdb/

Focusing now on AMPs specifically, despite the abundance of knowledge on thousands of sequences, none have successfully made it to market. However, the diminished antibiotic reserves are forcing a modernisation of the global approval criteria and regulatory hurdles in the testing and launching of new antimicrobial drugs including AMPs. Pexiganan, a 22 amino acid analogue of magainin, can be considered as a "victim" of this rigid system where despite substantial data indicating adequate safety and effectiveness in a number of models, it was rejected by the FDA as it was considered to be no more effective than the current standard of care (Marr, Gooderham, and Hancock 2006; Baltzar and Brown 2011). In the current climate, the propensity to develop resistance, and activity against MDR and XDR strains must now be strongly considered in the evaluation of new drugs against antibacterial agents. With this in focus, a number of global initiatives aimed at incentivising antibiotic Research and Development (R&D) have been implemented such as the Joint Programming Initiative on Antimicrobial Resistance (JPIAMR); the Innovative Medicines Initiative's (IMI's); New Drugs for Bad Bugs (ND4BB) programme; Biomedical Advanced Research and Development Authority's (BARDA); Broad Spectrum Antimicrobials Program and Combating Antibiotic Resistant Bacteria Biopharmaceutical Accelerator (CARB-X) (Simpkin, Renwick, and Mossialos 2017). Supporting a variety of different development stages, these incentives aim to help overcome some of the unattractive scientific, regulatory, and economic hurdles which have deterred pharmaceutical interest in exploring novel antimicrobials and turn high level commitments into real tangible actions by creating new antibiotic solutions (Luepke *et al*. 2017). If we are to stay one step ahead of the MDR crisis, it must be taken more seriously, and antibiotic stewardship programmes incorporating leadership commitment, accountability, drug expertise, tracking, reporting and education must be implemented firmly by global governments in hospitals and healthcare settings (Nori, Bartash, and Ostrowsky 2017).

As discussed above, enthusiasm for peptide-based drugs has been somewhat hampered by their short plasma half-life, negligible oral bioavailability and physicochemical instability which can restrict ease of administration. Many peptide drugs such as Byette and Victoza, glucagon-like peptide-1 (GLP-1) receptor activators, are currently only available as injectables which is unappealing for those requiring chronic outpatient therapy (Lau and Dunn 2018; Shields 2017). As research into suitable vectors for delivering peptides and ways to improve their lability excels, we can expect to see an increase in the number of drugs which are delivered orally in the near future as this is viewed as attractive in supporting patient compliance (Baptista *et al*. 2018; Lau and Dunn 2018).

Various strategies have been proposed to improve the undesirable characteristics of peptides such as glycosylation, PEGylation and sequence manipulation with non-natural amino acids (Fosgerau and Hoffmann 2015). In addition, the advent of impressive advancements in data mining, bioinformatics and computational biology supports peptide discovery, aiding in sequence design and optimisation. *In silico* discovery methods allow unique structural features to be identified which are generated by uncommon PTMs of non-ribosomal synthesis (Ling *et al*. 2015).

5.3.3 Advantages and Challenges in the Development of Immunomodulatory Peptides

5.3.3.1 Therapeutic Potential of Immunomodulatory Peptides

Naturally occurring immunomodulatory peptides from plants, microbes or animals can serve as templates for the synthesis of novel immune enhancing drugs (Haney and Hancock 2013). IDR-1 is one such example, which was designed based on the structure of the natural bovine peptide bactenecin, however with two proline residues incorporated in the sequence to deplete antimicrobial activity (Scott *et al.* 2007). Scott *et al.* revealed that while no direct bactericidal effects were observed, IDR-1 protected mice against various infections (*Salmonella, Enterococcus* and *Staphylococcus spp.*) as a result of macrophage recruitment, enhanced chemokine expression and suppressed inflammation. This represents an important finding as MDR infections may be targeted indirectly by using immunoregulatory agents. Peptides, with both functions, *i.e.* anti-infective and immunomodulatory activities, can therefore be employed as a dual pronged strategy to combat resistant pathogens. Not only do the bacteria experience a direct reduction in population but the host's own defence system becomes heightened to clear infection. Additionally, inflammation may be reduced as a result of the molecular mechanisms stimulated by the peptide, extending the therapeutic potential of these compounds even further (Garlapati *et al.* 2012).

The various challenges of AMPs are well described, therefore, mimicking the structural features of endogenous immunomodulatory peptides and boosting host defence against invading pathogens acts as a prophylactic strategy rather than tackling an established infection. An example of this can be seen with the milk peptide hLF1-11, which was initially under development as an antimicrobial by AM pharma. Once hLF1-11 exhibited immunological functions, the company concentrated their efforts on evaluating the

protective effects of the peptide against onset of infection during transplantations (Jenssen and Hancock 2010; van der Does *et al*. 2010).

5.3.3.2 Challenges in Developing Immunomodulatory Peptides as Therapeutic Agents

A commonly encountered hurdle in the development of peptides across various therapeutic applications is their poor pharmacokinetic profile, limited oral availability and high production cost. Industries are shifting towards hybrid and solution phase synthesis methods in an attempt to overcome this (Jenssen and Hancock 2010). Compounding the difficulties in developing immunomodulatory peptide drugs is the fact that their exact mechanisms are often not elucidated and often a structure-function relationship cannot be defined (Chalamaiah, Yu, and Wu 2018; Haney and Hancock 2013). That said, QSAR modelling approaches as well as iterative design studies are aiding the successful design of novel immunomodulatory peptides (Haney and Hancock 2013). Furthermore, specific tests can be performed to validate the predicted immune response such as anti-inflammatory activity reducing LPS-induced TNF-α production (Wieczorek *et al*. 2010).

The safety and toxicity are much the same as described for AMPs above, however concerns have been raised in the case of amino acid substitutions or non-natural immunoregulatory peptides which persist in the system and resist degradation that it may lead to hyperstimulation of the immune system and ultimately lead to inflammation (Jenssen and Hancock 2010).

Another limitation pertaining to immunomodulatory peptides is the lack of subsequent investigation after initial *in vitro* and *in vivo* assay. Despite peptides showing successful results in healthy animals, few are evaluated beyond this point, for their potential health benefits in a diseased population (Chalamaiah, Yu, and Wu 2018). Furthermore, of the studies which have investigated the immunological functions of protein hydrolysates, vital parameters are often omitted such as cell viability in *ex vivo* studies and body weight, haematology and food intake in *in vivo* studies (Chalamaiah, Yu, and Wu 2018).

5.4 Future prospects for NuriPep 1653

As highlighted in this work, we indicate with just one example, NuriPep 1653, that an inconceivable amount of biological data is yet to be unearthed and explored from nature. Computational approaches are essential to manage this data and help concentrate the number

of potentially potent peptides for testing and validation. This has a great economic benefit, as it reduces the experimental load needed to validate hypotheses. As in almost all aspects of life, technology is helping us to develop and reach new heights of what is possible. This is especially interesting in the pharmaceutical landscape as having the tools to help identify other peptides like NuriPep 1653, with potent activities, can help resist the MDR crisis, and a host of other diseases such as cancer, autoimmunity, Alzheimer's and more.

Our work largely focuses on the discovery and *in vitro* validation of the antimicrobial properties of PSH and NuriPep 1653. In the case of PSH, steps for the further development to bring the protein hydrolysate closer to a marketable preservative would include:

- Analysing the proteolytic and salt stability to determine the suitable food products.
- Conducting studies in a wider range of microorganisms including other bacteria, fungi and mould, frequently associated with spoilage and disease.
- Identifying the suitable inclusion rate in foods and determining if the production and filtration method is still cost effective.
- Testing in a wider range of food products included as an ingredient and a wash in other fruits and vegetables.
- Conducting a sensory analysis to identify any changes induced by PSH on the organoleptic profile of foods.
- Synthesise PSPep peptides to identify the peptides responsible for activity. This fraction of the hydrolysate could be concentrated using techniques like SEC.

In the case of NuriPep 1653, the next steps for development would include:

- Making modifications (such as incorporating D-amino acids) in the peptide to identify if it enhances the salt stability of the peptide.
- Identify potential vectors which may be used to transport the peptide to the active site (infection) without being degraded.
- Perform a multiplex array to determine the effects of NuriPep 1653 on a wider range of cytokines to determine other potential immune modulating actions.
- Perform infection assays using another bacterium to identify if the actions on/in the macrophage were specific to intracellular *S*. Typhimurium.
- Determine the serum stability of the peptide.
- Perform PK/PD studies to evaluate the suitability for a trial in a murine model

- Identify a suitable delivery system for the peptide, *i.e.* inhalant, intra-venous, oral delivery, *etc*.
- Perform studies on a wider range of clinical isolates with different antibiotic resistance profiles.
- Identify if the peptide can potentiate the action of other types of antibiotic to expand its therapeutic potential.

5.5 Concluding remarks

Overall, our work represents a new era for antimicrobial compounds. We explore the abundance of peptides in plants and reveal extra-ordinary compounds from those previously thought to be ordinary. PSH is an example of a "from food – for food" approach to preservation, ensuring food safety against notorious pathogens such as *E. coli* 0157:H7, while keeping in line with the clean label, plant-based revolution. Moving towards the devastating challenge multidrug resistance poses to health, we reveal the potential of a potent non-traditional pea peptide, NuriPep 1653, which was shown to effectively kill XDR *A. baumannii* and other pathogens through electrostatic and membranolytic mechanisms. Moreover, beyond its anti-infective action, NuriPep 1653 exhibits a host of other activities including modulating aspects of the immune system and regulating inflammation, which vastly expands its therapeutic potential. Our discovery approach breaks the mould associated the more traditional characterisation of plant AMPs and therefore, unearths enormous potential for the discovery of peptides with remarkable bioactivities from natural sources that could transform the future of food and health.

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Appendices

Predecessor peptides of NuriPep 1653

NuriPep 1653 was identified after optimisation of the enzymatic hydrolysis process and assay conditions. Various other peptides were identified and explored in this optimisation process. Despite the CE values being excessively high for commercial development, some preliminary studies were conducted on these peptides for learning purposes. These peptides were identified in the same was as described above for NuriPep 1653 however, alternative hydrolysis conditions meant different peptides were identified in the earlier stages of the work.

Sixteen peptides were identified from the MS analysis and synthesised to 95-99% purity (GenScript, USA) and tested *via* the CE drop method for antimicrobial activity against a range of Gram-negative and -positive bacteria, as outlined in Table 6.1. The concentration range used for screening was between 100-1000 μg/mL. The results revealed that antimicrobial effects were obtained between 700 - 875 μg/mL. Five peptides out of sixteen were shown to have activity against at least one strain tested. NuriPep 1546 showed antibacterial effects against both a Gram-positive and -negative bacterial strain indicating potential broad-spectrum activity.

Table Appendix 1. Antimicrobial activity of NuriPep peptides against a range of Gram-negative and Gram-positive bacteria.

NuriPep ID	*S. aureus*	*E. coli*	*S.* Typhimurium	*P. aeruginosa*	*K. pneumoniae*
1546	**800**	NI	NI	NI	**750**
1547	NI	NI	NI	NI	NI
1548	NI	NI	NI	NI	NI
1549	NI	NI	NI	NI	NI
1550	NI	NI	NI	**875**	NI
1551	NI	NI	NI	NI	NI
1552	NI	NI	NI	NI	NI
1555	NI	NI	NI	**875**	NI
1557	NI	NI	NI	NI	NI
1558	NI	NI	NI	NI	NI
1559	NI	**700**	**800**	NI	NI
1560	NI	NI	NI	NI	NI
1561	NI	NI	NI	NI	NI
1562	NI	NI	NI	NI	NI
1563	NI	NI	NI	NI	NI
1565	NI	NI	NI	**875**	NI

Legend: Activity was determined using the CE drop plate method. The CE values are shown in μg/mL. A lack of antimicrobial activity at concentrations below 1000 μg/mL is shown as NI. The experiment was conducted in triplicate on three independent days.

Antimicrobial activity of NuriPep -1565, -1555 and -1550 in pairwise combination

As three peptides, NuriPep -1550, -1555 and -1565 exhibited antimicrobial activity against *P. aeruginosa*; we investigated if these peptides could interact with each other to exert synergistic effects. The length, charge, percentage hydrophobicity, and PI of these three peptides are shown in Table 6.2. *P. aeruginosa* was incubated with each peptide individually or in combination at sub-inhibitory concentrations for 90 minutes, then plated on MH agar and allowed to grow for 18 ± 2 hours after which, the CFU/mL were determined. In the case of NuriPep 1550, there was no reduction in bacterial populations seen until a threshold concentration was reached, after which all bacteria were eliminated (Fig 6.1). NuriPep 1565 induced a 2-log reduction at 550 µg/mL however a concentration of 875 µg/mL was required before bactericidal effects were obtained. The combination index score of these two peptides does not indicate synergistic effects according to the method outlined by Chou (2010). However, NuriPep 1550 and NuriPep 1565 in combination at sub-inhibitory concentrations did affect the viability of *P. aeruginosa* at lower concentrations than that achieved by either peptide alone reducing the CE concentration from 875 to 700 µg/mL (Fig.6.1A). NuriPep 1555 had reduced antimicrobial activity compared to the other two peptides. It did not interact synergistically with any other peptide against *P. aeruginosa* (Fig. 6.2B) suggesting that additive effects are peptide specific. From these results, we hypothesise that there are many peptides functioning *via* additive effects within protein hydrolysates and this contributes to their multi-functional actions.

The killing concentrations of these peptides, alone or in combination were too high for them to be considered commercially interesting and so were not investigated in any great detail in this work. These peptides were part of the feedback-learning loop for the hydrolysis procedure and optimisation of the antimicrobial assays. When compared to NuriPep 1653, which is the main peptide of focus in this thesis, the peptide characteristics are sub-optimal. Only NuriPep 1565 is charged, and even so, only + 2. None of the other peptides showed a percentage hydrophobicity above 40% in their sequences and the PI values were below 6 for both NuriPep 1550 and -1555. In this way, we can justify the reason for the relatively poor activity of these peptides compared to NuriPep 1653 which has a higher charge, percentage hydrophobicity and PI (Tossi, Sandri, and Giangaspero 2000). These initial assays were however, of integral importance in understanding the important features conferring antimicrobial activity and guided the discovery of more potent peptides.

Table Appendix 2. Features of predecessor peptides before the discovery of NuriPep 1653

	Peptide Characteristics			
	Length	Charge	Hydrophobicity	PI
NuriPep 1550	5	0	40%	5.96
NuriPep 1555	8	0	25%	5.27
NuriPep 1565	11	2	18%	9.99

Legend: Features of NuriPep 1550, -1555 and 1565 including length, net charge, % hydrophobicity and PI.

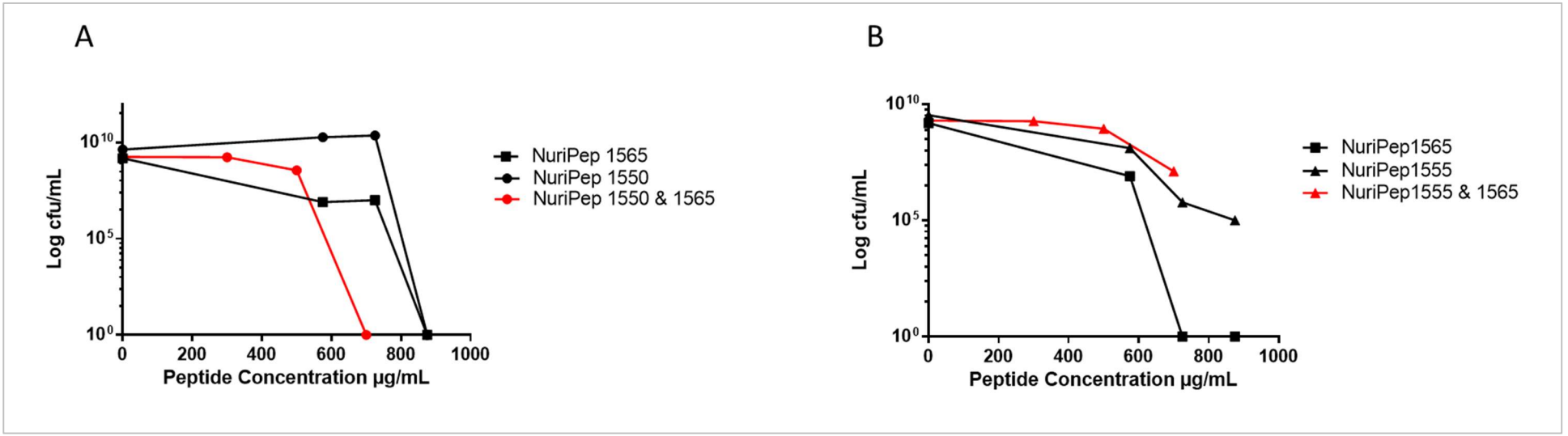

Figure Appendix 1. Reduction in *P. aeruginosa* populations after peptide treatment either alone or in pairwise combinations: Effect of NuriPep-1550 and -1565 (**A**) or NuriPep-1555 and -1565 (**B**) when tested alone at 875, 725 and 575 µg/mL and then in combination (1:1 ratio) against *P. aeruginosa* at 3.38 x 10^9 cells/mL. Experiments were conducted in triplicate on three independent days.

Mass Spectrometric analysis of NuriPep 1565

As part of the learning and optimisation of the methods used to further explore protein hydrolysates the peptide sequences contained within NuriPep 1565 were analysed using tandem MS. The mass spectrum of the single peptide with its respective charges is shown (Fig. 6.2A). Peptide molecular ions fragment preferentially at certain points along the backbone. This allows identification of individual amino acids. By performing liquid chromatography mass spectrometry (LCMS) on the PSH, we determined if NuriPep 1565 was among one of the peptide masses present in the mixture. Furthermore, by performing MS/MS we could select a parent mass from the LC-MS result, separate out that parent, fragment it, and determine the "daughter" ions from that mass. The chromatogram of the entire hydrolysate is shown (Fig. 6.2Bx) where total intensity is shown *versus* time. The analysis of the peptides was performed by in gel digestion and nano LC/MS/MS by MS Bioworks using a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. Peptides were loaded on a trapping column and eluted over a 75 μm analytical column at 350 nL/min. Both columns were packed with Luna C18 resin (Phenomenex). A 1-hour gradient was employed. The mass spectrometer was operated in data-dependent mode, with MS and MS/MS performed in the Orbitrap at 70,000 FWHM and 17,500 FWHM resolution, respectively. The fifteen most abundant ions were selected for MS/MS. The molecular weight of NuriPep 1565 was identified in the MS spectra and was extracted using SeeMS software. It is shown in high abundance with its +1 and +2 charges respectively, in Fig. 6.2By and Fig. 6.2Bz. The ionisation patterns indicate increased confidence of the presence of the ions. Figure 6.3 shows all of the identified peptides within the PSH. The location of NuriPep 1565 among them is highlighted in red. The major peaks in MS/MS spectrum are b ions, where the charge is retained on the N-terminus, and y ions, there is the charge is on the C-terminus. Using Peaks[23] software individual ion analysis of the residues confirmed the sequence of NuriPep 1565 within the hydrolysate mixture. MS analysis performed by Dr. Savage in Nuritas Ltd .

[23] http://www.bioinfor.com/peaks-studio/

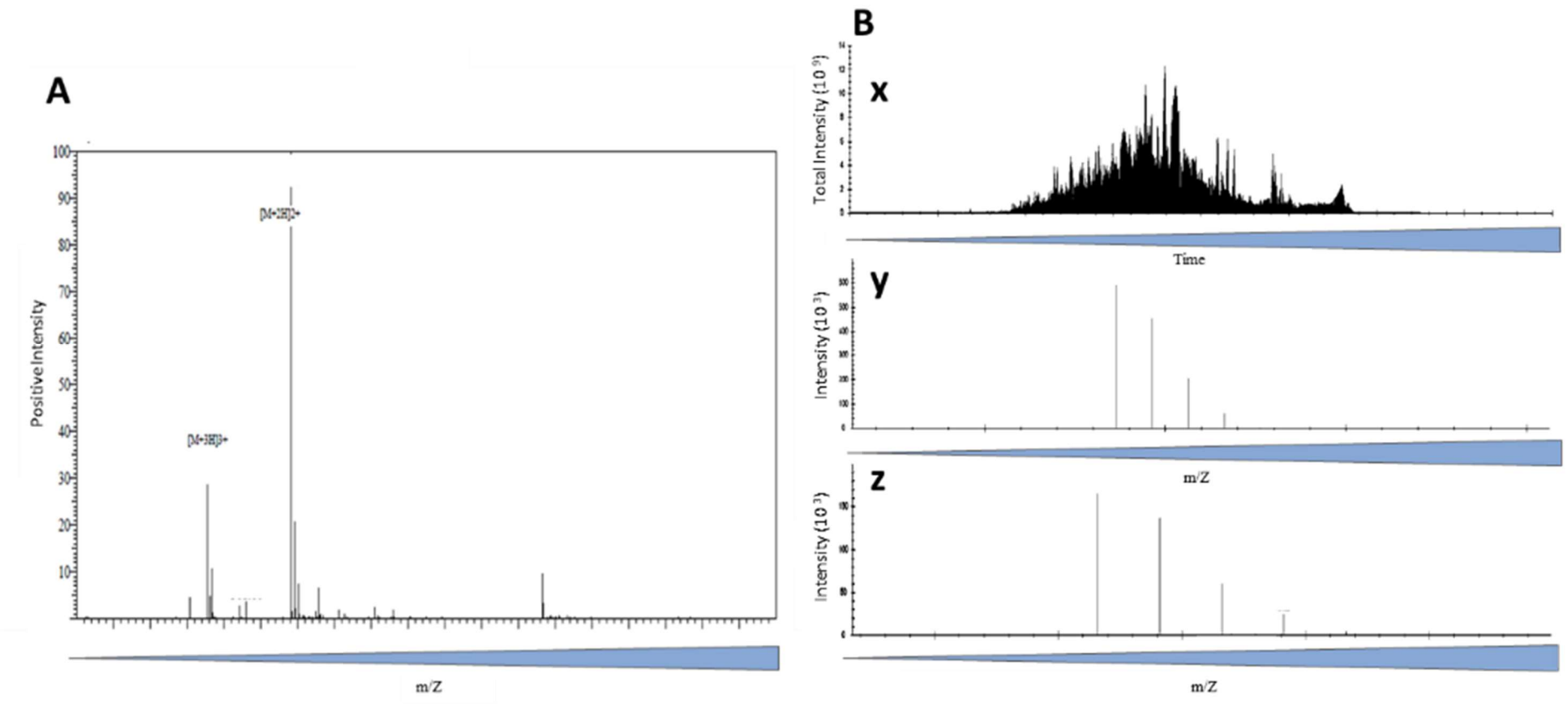

Figure Appendix 2. Mass Spectroscopy analysis of NuriPep 1565 and PSH. MS/MS spectrum of NuriPep 1565 indicating the molecular weight of the parent ion (**A**). (**Bx**) is the complete MS chromatogram of the hydrolysate. The +1 charged parent ion is present in high abundance (**By**) and a +2 ion (**Bz**). Analysis performed using SeeMs (ProteoWizard Tools). MS analysis performed by Dr. Savage in Nuritas Ltd.

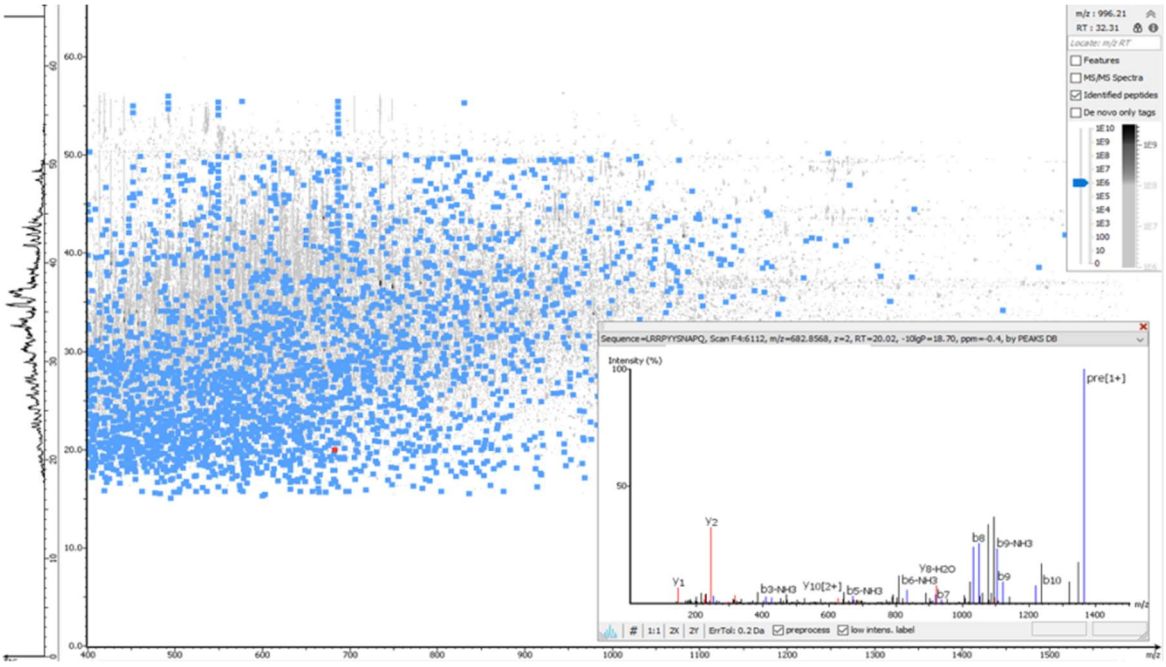

Figure Appendix 3. MS/MS analysis of NuriPep 1565. Tandem MS of all the ionised peptides from PSH. NuriPep 1565 is highlighted (red) and the subsequent ionisation profile and molecular weight of the individual residues are shown. Analysis was performed using PEAKS software. MS analysis performed by Dr. Savage in Nuritas Ltd.