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**Dimerization as a strategy to engineer antimicrobial peptides for
application against enteric bacterial pathogens of poultry**

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Abstract

This study aimed to investigate the use of phage display in discovering novel antimicrobial peptides (AMPs), and to test the effectiveness of improving AMP activity by dimerization. AMPs are an emerging avenue of research showing potential to circumvent the antibiotic resistance crisis that is developing. These peptides offer an alternative therapeutic, one that can be used to replace/supplement current antimicrobial therapies. Characterisation studies using both standardised techniques such as broth microdilution, and others identified in the literature were used to investigate a panel of AMPs discovered by bioinformatic analysis of a phage display library. Recombinant methods were then attempted to express dimeric forms of AMPs in *E. coli* and inverse PCR used to insert both known AMP sequences and libraries of randomised sequences into plasmids for expression for antimicrobial susceptibility testing. Finally commercially synthesised dimeric AMPs were tested in mixed culture models and by PMA-based qPCR to investigate their activity in more realistic matrices. Characterisation studies discovered five novel AMPs with activity as low as 8 mg L⁻¹, but it was clearly evidenced that the antimicrobial activity of these peptides is matrix-dependent, and agar-based assays are not appropriate for AMP screening. Investigations into recombinant production of AMPs highlighted some of the difficulties in expressing small, potentially toxic peptides. By conjugating the phage-derived AMPs with the potent AMP C-BF, robust and selective killing of pathogenic *E. coli* serotypes could be achieved within mixed cultures. Even in highly dense cultures, selectivity was retained thereby suggesting that the AMPs identified, and improved upon, here have potential for therapeutic use within poultry farming but more research into their effectiveness is needed.

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List of Abbreviations

aMHB	– adjusted Mueller Hinton broth
AMP	– Antimicrobial peptide
AMR	– Antimicrobial resistance
ARG	– Antibiotic resistance gene
APEC	– Avian pathogenic <i>E. coli</i>
AUC	– Area under curve
BSA	– Bovine serum albumin
CLSI	– Clinical & laboratory standards institute
Cq	– Quantification cycle
DBAASP	– Database of antimicrobial activity and structure of peptides
DMSO	– Dimethyl sulfoxide
DNA	- Deoxyribonucleic acid
EFSA	– European food safety authority
EU	– European Union
ExPEC	– Extraintestinal pathogenic <i>E. coli</i>
FBC	– Fractional bactericidal concentration
FIC	– Fractional inhibitory concentration
GBS	– Guillian-Barre syndrome
GI	- Gastrointestinal
LPS	– Lipopolysaccharide
MAP	– Multiple antigenic peptide
MBC	– Minimum bactericidal concentration
MHA	– Mueller Hinton agar
MHB	– Mueller Hinton broth
MIC	– Minimum inhibitory concentration
NGS	– Next generation sequencing
NTS	– Non-typhoidal serovars
ORF	– Open reading frame
PBMC	– Peripheral blood mononuclear cell
PBS	– Phosphate buffered saline

PDS – Peptide dilution solution
PEG – Polyethylene glycol
PMA – Propidium monoazide
PMF – Proton motive force
qPCR – Quantitative polymerase chain reaction
QSAR – Quantitative structure activity relationship
RBC – Red blood cell
RF – Replicative form
RNA - Ribonucleic acid
RO – Reverse Osmosis
SD – Standard deviation
sMHA – soft Mueller Hinton agar
SPPS – Solid phase peptide synthesis
TI – Therapeutic index
TUP – Target-unrelated peptide
v-PCR – Viability polymerase chain reaction

1 General Introduction and aims

1.1 Current therapeutic interventions in agriculture

1.1.1 Introduction to antibiotics and antibiotic resistance

Current clinical interventions for diseased animals involves antibiotic administration. Antibiotics are drugs which can inhibit or kill bacteria (bacteriostatic or bactericidal, respectively). In the literature, an antimicrobial is often used interchangeably with the term antibacterial. For clarification in this study, antibiotics are a class of antimicrobial with an activity specific against bacteria. Therefore, the term antimicrobial can refer to any inhibitory or killing effect against a range of organisms such as fungi to viruses whereas antibacterial pertains only to bacteria-specific effects. Antibiotics can be separated into classes, dependent on their chemical structure, mode of action or the range of effectiveness across bacteria (Schwalbe, Steele-Moore, and Goodwin 2007). For example, perhaps the most well-known antibiotic penicillin, is a β -lactam. This is a major class of antibiotics which are characterised by an eponymous β -lactam ring. All β -lactams interfere with the synthesis of peptidoglycan, an integral part of the bacterial cell wall. Apart from penicillins, other prominent members of the β -lactams are cephalosporins, carbapenems, and monobactams. Other classes of antibiotics can be separated by their type of antimicrobial activity, mechanism of action, and structure (Table 1-1).

Table 1-1. Antibiotic Classification

The various antibiotic classes that have been discovered to date can be differentiated by whether they have bactericidal or bacteriostatic activity, how they affect the cell, what indications they are used for, and their structure.

Class	Bactericidal or Bacteriostatic*	Mechanism of action	References
β -Lactams	Bactericidal	Inhibits cell wall synthesis	(Kong, Schneper, and Mathee 2010)
Aminoglycosides	Bactericidal	Inhibits protein synthesis	(Krause <i>et al.</i> 2016)
Quinolones	Bactericidal	Inhibits DNA replication	(Aldred, Kerns, and Osheroff 2014)
Tetracyclines	Bacteriostatic	Inhibits protein synthesis	(Chopra and Roberts 2001)
Sulfonamides	Bacteriostatic	Inhibits folic acid synthesis	(Smith and Powell 2000)
Macrolides	Bacteriostatic	Inhibits protein synthesis	(Kanoh and Rubin 2010)
Glycopeptides	Bactericidal	Inhibits cell wall synthesis	(Blaskovich <i>et al.</i> 2018)
Chloramphenicol	Bacteriostatic	Inhibits protein synthesis	(Balbi 2004)
Oxazolidinones	Bacteriostatic	Inhibits protein synthesis	(Diekema and Jones 2000)
Ansamycins	Bactericidal	Inhibits RNA synthesis	(Fan <i>et al.</i> 2018)
Streptogramins	Bactericidal	Inhibits protein synthesis	(Wright 2007)
Lipopeptides	Bactericidal	Disrupts cell membrane	(Schneider <i>et al.</i> 2009)

In a prescient warning from as early as 1945, Alexander Fleming cautioned against the overuse of antibiotics, or treatment regimens with short durations, as these may lead to the development of resistance. Resistance has been described as “when bacteria change in some way that reduces or eliminates the effectiveness of drugs, chemicals or other agents designed to cure or prevent the infection” (Bisht *et al.*, 2009). There are four main mechanisms by which a bacterium can gain resistance to antibiotics: modifying the antibiotic, preventing access to the target, changing the target, and “global cell adaptations” (Munita and Arias 2016). Since antibiotic treatment began, after the discovery of Salvarsan in 1910, bacteria have experienced a strong,

widespread selective pressure and have been evolving to overcome it (Davies and Davies 2010; Ehrlich and Hata 1910). While the ‘golden age’ of antibiotic discovery ensured multiple treatment options were available, since then discovery has been slow and intermittent. There were two new classes of antibiotics discovered in 1960, and after that only two more in the early 2000’s (Conly and Johnston 2005). This does not consider new analogues within the same classes, such as methicillin: the first semi-synthetic penicillin resistant to hydrolysis via penicillinases (Fairbrother and Taylor 1961). However, newer mechanisms of antimicrobial resistance can render variants ineffective (Coates, Halls, and Hu 2011). Such novel mechanisms of resistance include the newly discovered NDM-1 metallo β -lactamase conferring resistance to nearly all β -lactam drugs (Pesesky *et al.* 2015). Treatment options for human infections with bacteria harbouring the NDM-1 metallo β -lactamase are extremely limited, with risk assessments needed to balance the toxicity of treatment against its efficacy (Rogers *et al.* 2013). Therefore, there is a decline in the availability of treatments against bacterial infection in humans.

1.1.2 Antibiotic administration in food animals

Antibiotics have not been limited to therapeutic uses in humans, they have also been used in animal husbandry for three main reasons: to treat infections, for prophylaxis, and to promote growth (Landers *et al.* 2012). Despite Fleming’s caution against overuse, in many developed countries food animals can account for 50-80% of total antibiotic use (Cully 2014). It is difficult to estimate how much developing countries use antibiotics, and what for, as they rarely have suitable government-led antibiotic surveillance (Founou, Founou, and Essack 2016). However, based on data available

from 2010, countries such as Brazil, India, and South Africa are expected to almost double their antimicrobial consumption by 2030, with most attributed to increased food animal usage (Van Boeckel *et al.* 2015). This is corroborated by Browne *et al.* (2021), who found large increases in many low-to-middle income countries from 2000 - 2018, with India noted for a particularly dramatic increase.

1.1.2.1 Food animal antibiotic usage and its impact

With such a large amount of antibiotics being used in the agricultural sector, determination of its impact on resistance development is required. Overall, lower occurrence of antimicrobial resistance (AMR) does tend to occur in countries with a lower antimicrobial consumption for food-producing animals, suggesting a possible link between administration and resistance development (Garcia-Migura *et al.* 2014). This link is difficult to prove, due to confounding factors related to farm management, such as hygiene and biosecurity measures, which can influence resistance development and the population of animals harbouring resistance genes (Martins da Costa *et al.*, 2009; Persoons *et al.*, 2011). Despite this, the first demonstration of antibiotic resistance spread was carried out by Levy, FitzGerald and Macone (1976) in chickens inoculated with *E. coli* containing an artificial marker plasmid encoding tetracycline resistance. This plasmid was then later detected in spatially separated chickens (if their diet consisted of tetracycline-supplemented feed) and farm workers' faecal samples. The link between antibiotic use in animals, resistance development in bacteria, and subsequent detection in humans remains controversial, but has been supported by further systematic reviews of the literature (Burow *et al.* 2014; Simoneit *et al.* 2015). Dutil *et al.* (2010) also describes a strong correlation found between isolating Ceftiofur resistant *Salmonella enterica subsp. enterica* serovar Heidelberg

from retail chicken and incidences of the same resistant serovar infecting humans in Canada. Considering the proportion of Heidelberg serovar isolates resistant to Ceftiofur was correlated to the use of the drug, this provides further evidence that antibiotic use in food animals can have an impact on human clinical treatment.

1.1.2.2 The antibiotic resistance problem

With AMR infections predicted to kill 10 million people annually by 2050 and cause \$100 trillion worth of economic loss, efforts to control the development of resistances are needed (O'Neill, 2016). One health strategies take a holistic view not centred on one cause e.g. administration to animals, and these have occurred in developed countries whereas approaches in developing countries are localised to clinical interventions for humans (Nadimpalli *et al.* 2018). Tackling AMR to preserve antibiotic efficacy would require a globally coordinated effort to ensure that antibiotic interventions in all countries are appropriate. For example, banning only specific antibiotics appears to be the least successful intervention for reducing the population of bacteria harbouring resistance genes (Tang *et al.* 2019). Furthermore, resistance determinants arising in one country quickly disseminate across the globe as seen with the colistin resistance gene *mcr-1* (Gelbíčová *et al.* 2019). This is likely to have been highly selected for due to the, then unrestricted, use of colistin in pig feed in China for the countries' approximately 500 million pigs per year (Larson 2015). With poorly managed antibiotic use in many countries, it is likely that policies in specific countries, which have been designed to extend the therapeutic lifespan of these drugs, will not work as effectively as they could.

1.2 Poultry Pathogens

1.2.1 Poultry Farming

In 2020, 134 million tonnes of poultry meat were estimated to be produced globally (OECD and FAO 2021). Poultry continues to be the fastest growing meat sector (Alexandratos and Bruinsma 2012), and OECD-FAO statistics forecasts poultry to account for almost half of all additional meat produced in the next decade (OECD and FAO 2021). With regards to the current climate crisis, it has been suggested that a reduced consumption of meat altogether would help alleviate emissions (Hoolohan *et al.* 2013). Considering the quantity of meat eaten, and therefore desire for consumption, a more consumer-friendly option would be to eat less red meat and more from monogastric origin, e.g. poultry, due to its lower environmental footprint (Vaarst, Steinfeldt, and Horsted 2015). Furthermore, religious proscriptions in poultry consumption are relatively few compared with beef and pork. Poultry are comparatively low in saturated fats and also require comparatively few resources for husbandry. As such, there are numerous reasons for poultry farming to fulfil the predictions forecasting increased meat production in both developed and developing countries.

Small-scale poultry farming is common in rural, poor communities in developing countries and contributes significantly to food security (Wong *et al.* 2017). However, when incomes increase there is a concomitant increase seen in meat consumption (Schroeder, Barkley, and Schroeder 1996). When this happens, small-scale farms make way for industrial, vertically integrated production systems as exemplified in Asia (Ahuja and Sen 2007). These high production systems essentially out compete

small holders mainly due to the economies of scale when demand is high (Mottet and Tempio 2017). In these large-scale farms, with the small nature of the bird, it is rarely practical for individual animals to be treated for an infection; poultry are often treated as a group, resulting in unnecessary medication, a known risk factor for antibiotic resistance development (Gopal Rao 1998). For example, Braykov *et al.* (2016) investigated antibiotic resistance genes (ARGs) present in both large-scale and household broilers and laying hens in rural Ecuador and found significantly higher resistance rates in chickens from the high production systems. This is not a problem limited to developing countries; in Canada 95% of antibiotics administered to broilers between 2013 and 2015 were not targeted to individual animals due to clinical need, but were treated via feed or water (Agunos *et al.* 2017). With use of intensive poultry farming rising, a concern is that sales of antimicrobials could also increase due to the overreliance on antibiotics often present within these systems, and this could lead to accelerated antibiotic resistance development.

1.2.2 Bacterial pathogens

The list of diseases which can cause economic loss in the poultry industry is extensive. The current study will only focus on the predominant Gram-negative, zoonotic bacterial pathogens: *Campylobacter jejuni*, *Salmonella enterica* serovars, and avian pathogenic *Escherichia coli* (APEC). *C. jejuni* and *Salmonella* infections combined number over 300,000 per year in the European Union (EU), as reported to the European Food Safety Authority and European Centre for Disease Control (EFSA and ECDC 2018). *Salmonella* and APEC infections in poultry flocks can also lead to

widespread culling and large economic losses, therefore these three bacterial pathogens represent an ideal target for control measures.

1.2.2.1 *Campylobacter* spp.

Campylobacter is a Gram-negative microaerophilic bacterium, with most of the species able to cause disease in humans (campylobacteriosis) but typically leading to asymptomatic colonisation in poultry (Shane 1992; Silva *et al.* 2011). Since 2005, campylobacteriosis has been the most reported single bacterial gastrointestinal disease in Europe, according to the EFSA, with over 246,000 cases in 2017 (EFSA and ECDC 2018). This figure is considered to be an underestimate due to the under-reporting and under-detection common with self-limiting diseases associated with most food-borne pathogens (Gibbons *et al.* 2014). The true number of infections is believed to be around 9 million a year in the EU with an estimated cost of around €2.4 billion a year due to loss in productivity of workers in the EU (EFSA 2011), and \$1.7 billion in the USA (Hoffman, Batz and Morris, 2012). Approximately 20-30% of human *Campylobacter* infections may be directly related to the preparation and consumption of poultry meat (EFSA 2010b), with milk and broiler meat comprising the two most common sources of food-borne outbreaks in 2017 (EFSA and ECDC 2018). Poultry colonisation is thought to be mostly through a complex transmission process involving environmental sources (Sahin, Morishita, and Zhang 2002).

The two main established species of *Campylobacter* in human gastrointestinal disease are *C. jejuni* and *C. coli*, although others such as *C. concisus* are considered emerging pathogens (Kaakoush *et al.* 2015). Due to the infection usually being self-limiting, antibiotic treatment is not recommended. If the infection persists, or the patient suffers

severe symptoms, administration of a macrolide, generally erythromycin or azithromycin, is used (Eiland and Jenkins 2008). While usually self-limiting, infection can lead to Guillain-Barre syndrome (GBS) in approximately 3 out of every 10,000 cases (Scallan *et al.*, 2015). GBS is an autoimmune disorder in which the peripheral nervous system is attacked by the host's own immune system. The sialylated lipooligosaccharide of *C. jejuni* is antigenically similar to the ganglioside epitopes displayed on human nerves, causing a phenomenon called “molecular mimicry” (Sheikh *et al.*, 1998). This phenomenon causes the antibodies, which are produced in response to the bacteria, to cross-react with the peripheral nervous system leading to demyelination of the peripheral nerves and/or the axons (Willison, Jacobs, and van Doorn 2016). In addition to GBS, reactive arthritis, also known as Reiter's syndrome, and irritable bowel syndrome are also associated with *Campylobacter* and other bacterial agents causing gastroenteritis (Balemans *et al.* 2017; Dworkin *et al.* 2001; Hannu *et al.* 2006).

Possibly due to the increasing trend seen in the EU from 2008 (EFSA and ECDC 2018), the UK Food Standards Agency has been working alongside retail suppliers of poultry to reduce the levels of *Campylobacter* present in fresh, whole chickens. Across the EU, a baseline survey was undertaken to determine the rate of *Campylobacter* contamination in broilers at the slaughterhouse which found that 75.8% of them were contaminated to some degree (EFSA 2010a). Thanks to a concerted effort to reduce this, in the UK there have been significant decreases in the percentage of chickens with *Campylobacter*, with only 37.5% of broiler carcasses testing positive in 2018 (EFSA and ECDC 2018). There has also been a stabilisation of the increasing trend seen in the EU, with regards to *Campylobacter* infections in humans (EFSA and

ECDC 2018). This plateauing highlights a possible link between *Campylobacter* colonisation of poultry and subsequent infection in humans, therefore control measures to minimise the presence of this bacterium in poultry could further decrease infection rates. Reducing intestinal colonisation of chickens by *Campylobacter spp* is the ideal route for decreasing infection in humans, but this is difficult due to the commensal nature of the bacterium in chickens. Successful methods have targeted prevention of colonisation, due to poultry's role as a reservoir of *Campylobacter* being responsible for most human infections (EFSA 2010b). For example, Gibbens *et al.* (2001) found a 50% decrease in poultry colonisation by using increased biosecurity and hygiene measures. However, a similar study found no decrease in colonisation rates across poultry flocks (Ridley *et al.* 2011). One explanation is a lack of compliance, although Gibbens *et al.* (2001) reported <100% of farms in the intervention arm of the study satisfactorily completing biosecurity measures while still achieving lower colonisation rates. Another consideration is the biosecurity measures put into place, perhaps only particular interventions are necessary and those used in Ridley *et al.* (2011) were extraneous. Therefore, more innovative methods may be needed to reduce *Campylobacter* prevalence in poultry or more research is needed to determine which prevention methods are effective in a farm environment.

1.2.2.2 *Escherichia coli*

E. coli is a Gram-negative bacterium ubiquitous in the gastrointestinal tracts of virtually all warm-blooded animals (Tannock 1994), with most of these strains causing the host no harm. Although *E. coli* is a commensal in the intestinal tract, often colonising soon after birth, some strains of *E. coli* that have obtained combinations of virulence factors can cause the disease colibacillosis. When these virulence factors

have been obtained during the colonisation of avian species, they are termed APEC (Dho-Moulin and Fairbrother 1999). APEC causes syndromes of both localised and systemic infections in poultry, leading to large economic losses because of APEC-associated morbidity, either as a primary or secondary infectious agent. Examples of symptoms in APEC colibacillosis range from mostly aesthetically-damaging, such as cellulitis (Peighambari *et al.* 1995), to life-threatening, such as colisepticaemia (Huja *et al.* 2015). APEC infection in chickens is usually treated with antibiotics, but strains have often been found to be resistant (Lutful Kabir 2010).

Colibacillosis in poultry has been attributed to various serotypes (Abd El-Mongy *et al.* 2018; Sojka and Carnaghan 1961), with this diversity being a key problem in treating and the prevention of APEC infections (Guabiraba and Schouler 2015). For example, it has made development of an effective vaccine difficult, as there are numerous antigens associated with disease. However, there are particular serotypes that seem to be more strongly associated with pathogenicity in birds. For example, Schouler *et al.* (2012) found that 56.5% of APEC isolates investigated came from six serotypes: O1, O2, O5, O8, O18, and O78. However, it should be noted that characterisation of *E. coli* can be achieved by various methods not limited to serotyping. For example, investigations into the lineages of *E. coli* splits the bacterial species into four major phylogenetic groups: A, B1, B2, and D (Clermont, Bonacorsi, and Bingen 2000). Within these four groups, APEC is usually associated with D and B2 (Clermont *et al.* 2000; Da Rocha *et al.* 2021). Furthermore, sequence typing offers additional information with which to characterise *E. coli* isolates as described by Mehat, van Vliet and La Ragione (2021), whereby use of whole genome sequencing has explored the lineages of the serotypes O1, O2, and O78 and its impact on current

diagnostics and treatments. While there is a complexity to the APEC pathotype not wholly described by serotyping, grouping by serotype allows further investigations without extensive typing assays while still retaining an element of selectivity against APEC isolates.

In humans, *E. coli* infection is usually limited to the intestines leading to typical food-poisoning symptoms such as diarrhoea or vomiting. Determining the impact of poultry in *E. coli* zoonoses is difficult, as only Vero-toxin producing strains are notifiable. Therefore, strains not encoding this toxin are not recorded in documents such as the UK's annual zoonoses report or EFSA monitoring reports. However, it should be noted that any cases of food-poisoning must be reported to the Environmental Health Department and that links to poultry, e.g. occupation, can be determined by investigations into these cases. With Vero-toxin producing strains mostly found in ruminant animals, data from poultry zoonosis is sparse and transference of APEC isolates is, for the most part, assumed due to isolation of such strains from poultry meat and eggs (Mitchell *et al.* 2015). However, it is generally hypothesised that APEC strains originating from poultry are a likely cause of extraintestinal infections in humans (Mellata 2013). Extraintestinal pathogenic *E. coli* (ExPEC) is a label for subsets of pathogenic *E. coli*, such as APEC, which have the necessary virulence factors to facilitate infection beyond the intestine. The most prevalent disease caused by ExPEC strains are urinary tract infections, of which *E. coli* is the most common causative agent accounting for up to 80% of all uncomplicated cystitis cases (Kang *et al.* 2018). ExPEC strains are also responsible for a third of all bacteraemia cases in England annually (Abernethy *et al.* 2017). While zoonoses are not officially recorded, the link between ExPEC infections in humans and poultry products is deemed

significant because of the similarities in the isolated strains' virulence and antibiotic resistance genes (Cunha *et al.* 2017; Manges and Johnson 2012). Foxman (2010) noted that the number of human ExPEC infections resistant to first line antibiotics is increasing, with the impact from resistant APEC strains unclear. However, it is known that the number of antibiotic resistance genes identified in APEC isolates is increasing and that poultry constitute potential reservoirs for ExPEC infections (Kim *et al.* 2007; Massella *et al.* 2021). Without appropriate control and prevention measures, APEC strains are likely to increasingly harbour both antibiotic resistance and virulence genes, and could contribute to complications in human clinical treatment.

1.2.2.3 *Salmonella* spp

Salmonella is a diverse genus of Gram-negative bacteria. For example, *S. enterica* ssp *enterica* has over 1500 serovars (Fàbrega and Vila 2013). While all species and serovars of *Salmonella* can potentially cause disease in humans, most are host-specific and adapted for infection in just one or two animals leading to only rare cases of zoonosis. For the purpose of this study, both the species *enterica* and subspecies *enterica* can be assumed when the nomenclature is *S. "serovar"* e.g. *S. enterica* ssp *enterica* serovar Enteritidis is contracted to *S. Enteritidis*. There is a distinction made between Typhoidal *Salmonella* serovars such as *S. Typhi* and *S. Paratyphi*, which are highly specialised and cause enteric fever in humans with potential asymptomatic carrier states (Gunn *et al.* 2014), and non-Typhoidal serovars (NTS) such as *S. Hadar* and *S. Typhimurium*. Enteric fever can be a life-threatening, systemic infection in humans (Amicizia *et al.* 2017); NTS infection usually leads to a self-limiting infection located in the gastrointestinal tract. However, on average, 8% of NTS infections result in bacteraemia with age positively correlated with an increased susceptibility (Mandal

and Brennand 1988). Due to Typhoidal *Salmonella* serovars being adapted to humans, their route of transmission is not zoonotic, unlike NTS.

There are thought to be various means by which *Salmonella* is brought onto a flock and disseminated, such as water and feed and, for free-range poultry in particular, wildlife (Hanning, Nutt, and Ricke 2009). From the initial infection, most likely via the oral route, *Salmonella* can colonise the intestinal tract and transmit horizontally and vertically (Yue *et al.* 2021). Indeed, horizontal transmission of *Salmonella* during rearing of broilers and to carcasses at the slaughterhouse were reported to be the main factors determining prevalence of *Salmonella* in poultry meat (Heyndrickx *et al.* 2002). As a result of the zoonotic potential of NTS, Regulation (EC) No 2160/2003 states that each member state within the EU is required to reduce the prevalence of five serovars of NTS to below 1% in breeding hens (*Enteritidis*, *Hadar*, *Virchow*, *Infantis*, and *Typhimurium*). Koutsoumanis *et al.* (2019) reported that the national control programs established to attain this target brought the confirmed cases of salmonellosis, disease in humans caused by *Salmonella*, down from over 200,000 p.a. in 15 member states of the EU in early 2000's, to less than 100,000 in 28 member states between 2012-2015. Of the total human infections within the EU caused by *Salmonella*, *S. Enteritidis* is the most common causal agent (EFSA and ECDC 2018). It is also the most prevalent serovar causing human salmonellosis reported by the world's biggest producer of poultry meat, the USA (CDC 2018; Mottet and Tempio 2017).

While *S. Enteritidis* is the most prevalent serovar in clinical isolates obtained from chicken flocks in the USA (CDC 2018), in the EU it is *S. Infantis* (EFSA and ECDC 2018). Interestingly, *S. Infantis* only accounts for just over 2% of human infections

compared to almost 50% attributed to *S. Enteritidis* (EFSA and ECDC 2018). This suggests that chicken products account for a large proportion of salmonellosis reported in the USA, but might not within the EU. Alternatively, *S. Enteritidis* infection in humans may be more successful than *S. Infantis*. However, according to the EFSA and ECDC, eggs and egg products account for the majority of salmonellosis outbreaks within the EU suggesting that either *S. Infantis* may be less successful or other factors affected the difference in infection rate (EFSA and ECDC 2021). Therefore, to reduce the cases of salmonellosis within the EU further, interventions aimed at reducing *Salmonella* contamination of eggs may help. The USA could also benefit from *Salmonella* reduction in poultry to reduce salmonellosis cases.

Control measures for reduction of *Salmonella* in poultry include hygiene measures to reduce the prevalence within the housing structure of flocks, but these are only effective to a limited degree and should be used in conjunction with other methods (Mead 2004). Vaccination is also recommended for layers, but knowledge of the serovars prevalent in the area is needed and they are also not recommended as the sole prevention measure (OIE 2019). Use of therapeutic antimicrobials to control *Salmonella* infections has so far not been effective, potentially due to its intracellular pathogenesis, and use of antibiotics such as Enrofloxacin has caused AMR concerns (Agunos, Léger, and Carson 2012). Therefore, the most effective strategies may be those targeted at increasing the birds' resistance to colonisation (Vandeplas *et al.* 2010). This has been attempted through dietary modification as nutrition, both *in ovo* and post-hatching, may help modulate the immune system (Klasing 1998).

1.3 Alternatives to antibiotics

With the search for new antibiotics returning few candidates which progress to market (Ventola 2015), research has increasingly turned to alternatives when combating these pathogens to avoid returning to a pre-antibiotic era. Such alternatives have mostly targeted prevention of disease, enabled by vaccination programmes, rather than treatment. While bacterial vaccinations can be effective, as described in multiple studies by Rabie and Amin Girh (2020), there are significant disadvantages of vaccines which depend on the type used and its administration method (Marangon and Busani 2007). This is due to the impracticalities of administering bird-by-bird when there can be ~200,000 chickens per shed in commercial farms, and therefore any mass-administration technique such as via drinking water or spraying require very stringent conditions to reduce the risk of vaccine failure by inadequate dosage. For example, when using the spray method there are many factors to consider such as water droplet size, whether the birds are spread evenly, air flow and evaporation.

The commercially available Poulvac vaccine, which is genetically altered with an *aroA* deletion, has shown good efficacy against *E. coli* O78 infections when the spray method is used but the protection against heterologous serotypes may be limited (Asghar Sadeghi, Shawrang, and Shakorzadeh 2015; Mohamed *et al.* 2016; Sadeyen *et al.* 2015). Attempts to identify a suitable vaccine for *Campylobacter* have yielded varied results (Kobierecka *et al.* 2016). This is thought to be due to a combination of high glycosylation diversity and a number of immune evasion techniques, such as its capsular polysaccharide and phase variation (Poly *et al.* 2019). *Salmonella* vaccination has been more successful in layers and can be administered through either aerosols via spraying or given orally. However, similarly to vaccines against *E. coli*, the protective

nature of vaccinations when challenged with heterologous serovars is limited (Varmuzova *et al.* 2016). Diversity has also hampered vaccination attempts against APEC (Lynne *et al.* 2012). Better understanding of the physiological reasons behind vaccine failure is needed before they can be optimised for the prevention of further use of antibiotics (Sharif and Ahmad 2018), therefore there is still a need for viable alternatives.

1.4 Antimicrobial Peptides

1.4.1 Antimicrobial peptide (AMP) characteristics

An AMP is a very small peptide, usually less than 10 kDa with an overall positive charge and containing both hydrophobic and hydrophilic sections (amphipathic) (Liu *et al.* 2017). However, the term AMP has been applied to a range of peptides that do not necessarily display these characteristics. For example, colistin, a lipopeptide antibiotic in the polymyxin group; vancomycin, a glycopeptide antibiotic; and bacitracin, a mixture of cyclic peptides, have all been classed as AMPs. Furthermore, bacterial AMPs, which can be non-ribosomally produced, are less likely to be cationic or amphiphilic and are more likely to be extensively modified (Hancock and Chapple 1999). Therefore, bacterial AMPs can also be referred to as peptide antibiotics, or bacteriocins, to avoid confusion with the AMPs found in higher organisms. For the peptides used in this study, the term AMP will be used to define cationic and amphiphilic peptides only.

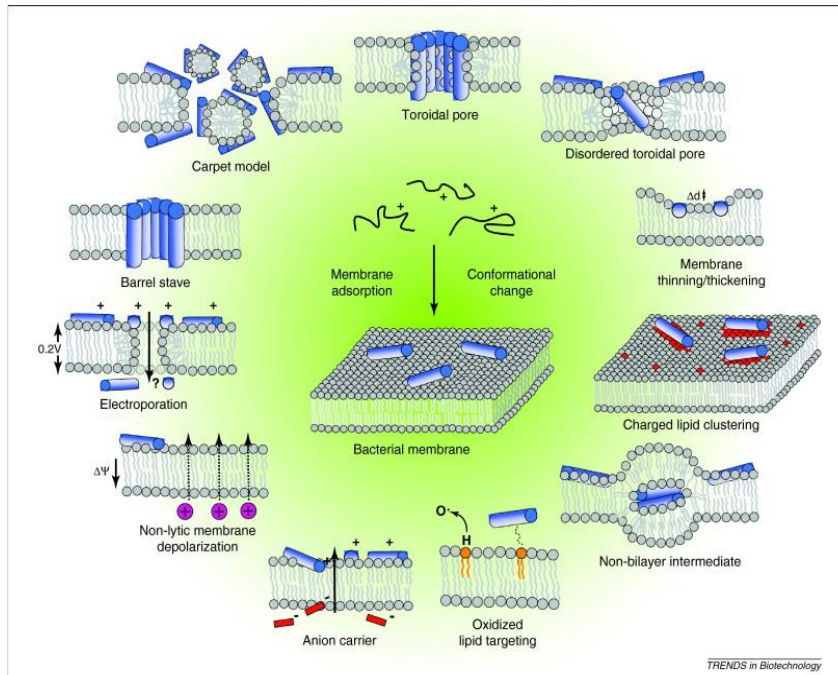


Figure 1-1. AMP mechanism of action.

AMPs bind electrostatically to a bilayer membrane and act in various ways. The three most well-characterised mechanisms include the carpet model, toroidal pore, and the barrel stave actions. In the carpet model, AMPs act in a detergent-like manner, aggregating on the surface of the bilayer until a critical micellar concentration is reached and the membrane is disintegrated. In the toroidal pore and barrel stave models, AMPs insert themselves perpendicularly into the membrane forming a pore. In the former, the phospholipids are able to arrange themselves with the phosphate groups in between the AMPs. This is not permitted by AMPs with a barrel stave mechanism. There are also less well-characterised methods, such as the disordered toroidal pore and membrane thinning which require more structural study.

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1.4.2 Antimicrobial mode of action

The exact mechanism of action for AMPs is unclear, and appears to be peptide-specific, yet characteristics such as their hydrophobicity and cationicity have been used to hypothesise general modes of action. This includes an initial electrostatic interaction of the peptide with a negatively charged bacterial membrane and then integration into the membrane due to its hydrophobicity (Bechinger and Gorr 2017).

In Gram-negative bacteria it is generally thought that AMPs, after membrane adsorption due to the electrostatic interaction, form hydrogen bonds with the phosphate groups within the LPS; once bound to the phosphate groups an AMP displaces the divalent Mg^{2+} and Ca^{2+} cations and this destabilises the membrane (Li *et al.*, 2017). The destabilisation helps the AMP integrate into the membrane, which then diffuses inwards towards the cytoplasmic membrane where the AMP can accumulate until high enough concentrations are reached to disrupt the transmembrane potential and kill the cell. Within Gram-positive bacteria the process is simpler due to the presence of a single membrane, where the negatively charged teichoic acids present can facilitate accumulation of the AMP for adsorption and membrane perturbation (Malanovic and Lohner 2016).

There are specific models which state how integration into the membrane might take place such as the barrel-stave, the carpet, and the toroidal-pore model (Shai, 2002; Figure 1-1). In the barrel-stave model, the AMP aggregates and forms a “barrel” with a hollow core which inserts into the membrane and thereby creates a pore. In the carpet model, the AMP binds to the surface of the bacteria separately and covers it until a threshold is reached and the bilayer is broken down through detergent-like activity of the peptide. The toroidal-pore model is similar to the barrel model, but the inside of the pore is lined with both the peptides and lipid head groups of the membrane. These are rather simplistic models as some AMPs have intracellular targets for their antimicrobial activity, and translocation across membranes without cell lysis is possible (Le, Fang, and Sekaran 2017; Sharma and Nagaraj 2015). In addition, investigations using Maculatin-1, an AMP from an amphibian, showed that even if an AMP compromises bacterial membranes at a similar efficacy, this may not lead to a

similar MIC (Sani *et al.* 2015). There are also further hypothesised membranous mechanism of actions of AMPs, which include non-lytic membrane depolarisation and oxidised lipid targeting (Figure 1-1).

1.4.3 AMP resistance development in bacteria

Resistance to AMPs can occur, as with antibiotics. This can be through expression of proteolytic enzymes, changes in the cells surface, and expulsion via efflux pumps (Joo, Fu, and Otto 2016). The lipopolysaccharide (LPS) surrounding Gram-negative bacteria comprises three elements, lipid A, a core oligosaccharide, and the O-antigen (Erridge, Bennett-Guerrero, and Poxton 2002). In the LPS, lipid A and core oligosaccharides often contain negatively charged residues (Firdich and Whitfield 2005; Gangloff and Gay 2004). Changes to the cell surface to confer resistance to AMPs include reducing the negative charge introduced by Lipid A and the core oligosaccharides, and to saturate the membrane to increase stability and decrease permeability (Nizet 2006). Strategies to reduce the negative charge can include addition of certain moieties to the phosphate groups on Lipid A, both *Pseudomonas aeruginosa* and *S. Typhimurium* have been noted to add the sugar aminoarabinose (Moskowitz, Ernst, and Miller 2004). An example of saturation of membranes comes from *Salmonella spp*, which can add acyl chains to its Lipid A via PagP (Guo *et al.* 1998).

1.4.3.1 Resistance development to AMPs compared to antibiotics

While there have been incidences of resistance occurring to AMPs (Maria-Neto *et al.* 2015), resistance levels to some AMPs have been found to be significantly lower than those developed against antibiotics (Spohn *et al.* 2019). There was also a complete lack of resistance development against one AMP, tachyplesin II, even in bacterial strains with increased mutation rates (Spohn *et al.* 2019). Tachyplesin II is one of three tachyplesin conserved AMPs isolated from horseshoe crabs (Vernen *et al.* 2019). However, in contrast to Spohn *et al.* (2019), Hong, Hu and Ke (2016) discovered that resistance to the similar tachyplesin I was obtainable, but this required the evolution experiment to be performed for four times as many generations when compared to the previous study (Spohn *et al.* 2019). The use of AMPs might therefore result in slower resistance development than has been seen with antibiotics. This could be due to the multimodal mechanisms of action some AMPs have developed, where bacterial resistance to only the peptide's membrane-targeting or intracellular interactions is not enough for survival (Lewies, Du Plessis, and Wentzel 2019). However, it should be noted that resistance to AMPs has been documented and so resilience to resistance development may be a peptide-specific trait (Andersson, Hughes, and Kubicek-Sutherland 2016).

The SOS response is usually the result of DNA damage in bacteria, which leads to a host of induced genes which promote DNA repair, recombination, and mutation (McKenzie *et al.* 2000). It is these induced genes that allow adaptive mutation in unfavourable environmental conditions, e.g. when antibiotics are present. β -Lactam

and fluoroquinolone antibiotics have been shown to induce the SOS response in *S. aureus* resulting in replication and transfer of pathogenicity islands, thereby enhancing the spread of bacterial virulence genes (Maiques *et al.* 2006). Where AMPs translocate across the lipid bilayer, this can then lead to intracellular targets being affected, often resulting in DNA binding and subsequent SOS induction (Gottschalk *et al.* 2013). However, for membrane-targeting AMPs such as LL-37, the human cathelicidin, no increase in the recombination frequency was found after application to *E. coli* (Rodríguez-Rojas *et al.* 2018). This may have an impact on why AMP resistance can be slower than other antimicrobial therapeutics.

Despite this, they may induce mutagenesis through other regulatory pathways. For example, Limoli *et al.* (2014) found that while LL-37 didn't stimulate the SOS response, it did induce *P. aeruginosa* to convert to its mucoid variant by binding non-specifically to DNA at sub-inhibitory concentrations. Interestingly, this study showed that at sub-inhibitory concentrations LL-37 fails to cause membrane damage, but it does facilitate entry of LL-37 into the cytosol to affect intracellular targets (Limoli *et al.* 2014). The conversion of *P. aeruginosa* to a mucoid variant is seen in the later stages of Cystic Fibrosis patient infections, which may result in a poorer clinical prognosis (Fick, Sonoda, and Hornick 1992). The exact mechanism of how this interaction increased the conversion rate to the mucoid variant remains to be elucidated, however it does highlight that more research is needed into the effects of AMPs on clinically important pathogens. This also introduces the concern that there could be unintended side effects of AMPs, which are dependent on the concentration administered. Also of concern is that administration of AMPs, and any resistance development acquired due to this, may lead to co-resistance to antibiotics. For

example, one method of increasing resistance to AMPs is via a capsule polysaccharide which inhibits an AMPs ability to bind to the bacterial membrane (Campos *et al.* 2004). Production of a capsular polysaccharide may increase virulence and resistance to host complement killing, so these possibilities of resistance development affecting antibiotic treatment success should be investigated (Geisinger and Isberg 2015).

1.4.3.2 Cross-resistance against innate immune response

The development of resistance against AMPs may be slower to arise and less prevalent than resistance to antibiotics. However, their role in the mammalian innate immune response might present another unintended side effect if resistance does arise. Through a mechanism called cross-resistance, whereby a resistance mechanism to one antimicrobial confers resistance to another, the development of resistance against therapeutic AMPs might undermine the AMPs in the mammalian immune system. With multiple incidences of AMP resistance mechanisms (Maria-Neto *et al.* 2015), and resistance to colistin positively correlating with resistance to LL-37 (Napier *et al.* 2013), it is vital that it is understood how this phenomenon might influence any use of AMPs as a treatment. Because of this concern, Kintsjes *et al.* (2019) suggested that with appropriate selection of AMPs with different modes of action, cross-resistance to human AMPs will not occur due to the different resistant determinants needed. With the possibility of increasing the virulence of pathogens via reducing the effectiveness of human immunological defences, the effect on severity of disease caused by AMP-resistant bacteria compared to those susceptible to AMPs should be evaluated. Another consideration should also be the effects on resistance towards AMPs that constitute integral parts of immune systems across the natural world, for instance insects host various AMP families with different biological effects (Wu, Patočka, and Kuča 2018).

1.4.4 AMP use in agricultural applications

To begin determining the effect AMPs might have as an alternative to antibiotics, there have been a limited number of studies on AMP use in agriculture, including poultry production. One avenue of research is the use of AMPs as therapeutics. Intensively reared chickens may have very limited acquisition of beneficial microbiota from the environment due to the conditions they are raised in, as well as the short duration from hatching to slaughter (Čermák and Skřivanová 2016). This is linked to increased susceptibility to infection due to a lack of competitive gut flora (Patterson and Burkholder 2003). Chickens are also more susceptible to pathogens during their first week, as their immune system is not fully developed (Ohimain and Ofongo 2013). Therefore, AMPs could have a potential application for poultry without the microflora necessary for effective protection. For example, Kogut *et al.* (2010) administered a non-ribosomal, bacterially-produced AMP (BT) for four days post-hatching and showed that the AMP displayed immunostimulatory properties which significantly decreased *S. Enteritidis* caecal colonisation compared to control birds. However, this study did not investigate whether BT could reduce mortality. Jiang, Sims and Conway (2005) investigated this by using BT as a feed additive for broilers and then comparing mortality rates after a natural colibacillosis outbreak to birds fed the known antibacterial feed additives bacitracin methylene disalicylate and monensin. BT was shown to offer the greatest protection, with a significant decrease in mortality when compared to the no treatment controls (0.051% mortality at 41 days compared to 13.4%). While BT does contain non-standard amino acids, it is only 13 residues long and still retains the amphiphilic and cationic nature that defines AMPs, suggesting the effects could be reproduced with natural amino acid sequences.

AMPs might also help poultry to acquire beneficial microflora, evidenced by Daneshmand *et al.* (2019) whose cLF36-supplemented diet for broilers increased their beneficial microflora significantly while decreasing the number of *E. coli* and *Clostridium spp.* In comparison, antibiotics decreased all bacterial populations indiscriminately. This is thought to be due an increased selectivity of the AMP compared to the antibiotic tested. In *in vitro* assays, cLF36 was shown to have varying levels of activity against both Gram-negatives and Gram-positives, but no beneficial bacteria were tested (Tanhaiean *et al.* 2018). Research into AMPs application as a feed additive is still very limited, with more studies needed on the protective effects *in vivo*.

AMPs may also have a potential role in agricultural settings beyond their direct antimicrobial action, utilising their immunomodulatory properties. Poultry undergoing heat stress are susceptible to intestinal injury, including increased permeability (Goo *et al.* 2019), this leads to decreased welfare and productivity of laying hens and broilers (Lara and Rostagno 2013). Due to known effects on the intestine in growth promotion studies (Bao *et al.* 2009), AMPs may allow animals to physiologically cope better with heat stress. Hu *et al.* (2017) found that AMPs isolated by extraction from swine intestinal tissue helped broilers maintain normal immunological functions and intestinal morphology under heat stress. This was likely by influencing multiple protein pathways associated with mucous production and intestinal protection as both IgA secretion and goblet cell production were improved. Climatic factors, including global warming (Renaudeau *et al.* 2012), are limiting development of the poultry farming industry as heat stress is linked to reduced poultry performances (Nawab *et al.* 2018). Therefore, this research could become increasingly pertinent as the demand for poultry grows and food security becomes more difficult.

While concerns have been raised about repeating the mistakes previously made with antibiotics, with regards to unnecessary use in agriculture, AMPs have been used in growth promotion studies. Intestinal modulation may not just increase resilience to heat stress, (Bao *et al.* 2009) also found intestinal physiological modulation in poultry with a concomitant improved growth performance when pig β -defensin 1 was administered in drinking water. AMPs in feed have also been shown to increase nutrient digestibility as well as increased nitrogen retention and improved metabolizable energy in broilers (Choi *et al.* 2013b, 2013a; Wen and He 2012). Due to the global nature of AMR and the failure of some countries to curb antibiotic use, the use of AMPs in agriculture may be beneficial. Antibiotics are already used widely in human therapeutics, if they can be preserved and their useful lifespan extended, it would provide security against a post-antibiotic era. As well as the potential therapeutic application of AMPs in agriculture, their use as an alternative growth promoter would also likely be easier to implement in countries unwilling, or unable, to move away from antibiotic-induced growth promotion. However, unless there is a reliable method for discovering AMPs, they are not likely to be a cost-effective investment for pharmaceutical companies.

1.5 Phage Display mediated discovery of AMPs

1.5.1 Introduction to phage display

One of the most common ways of discovering new AMPs, or even modifying current ones, is with bacteriophage (phage) through a method known as phage display (Pini *et al.*, 2005; Tanaka, Kokuryu and Matsunaga, 2008; Bishop-Hurley, Rea and McSweeney, 2010a; Sainath Rao, Mohan and Atreya, 2013). Phage display was first

used by George Smith where a gene was inserted into the ORF of the minor coat protein (pIII) in the M13 phage genome, this allowed the display of pIII fused to a peptide (Smith 1985). This system was developed further, allowing modification of the other capsid proteins to display fusion proteins, most notably pVIII (Gao *et al.* 1999; Hufton *et al.* 1999; Minenkova *et al.* 1993). Each phage has about 2700 copies of pVIII, the major coat protein, but only about 5 copies of pIII at one end of the virion (Pratt, Tzagoloff and Beaudoin, 1969; Rodi, Soares and Makowski, 2002; Figure 1-2). The phage used in these systems are all called Ff-phages.

Filamentous phage life cycle

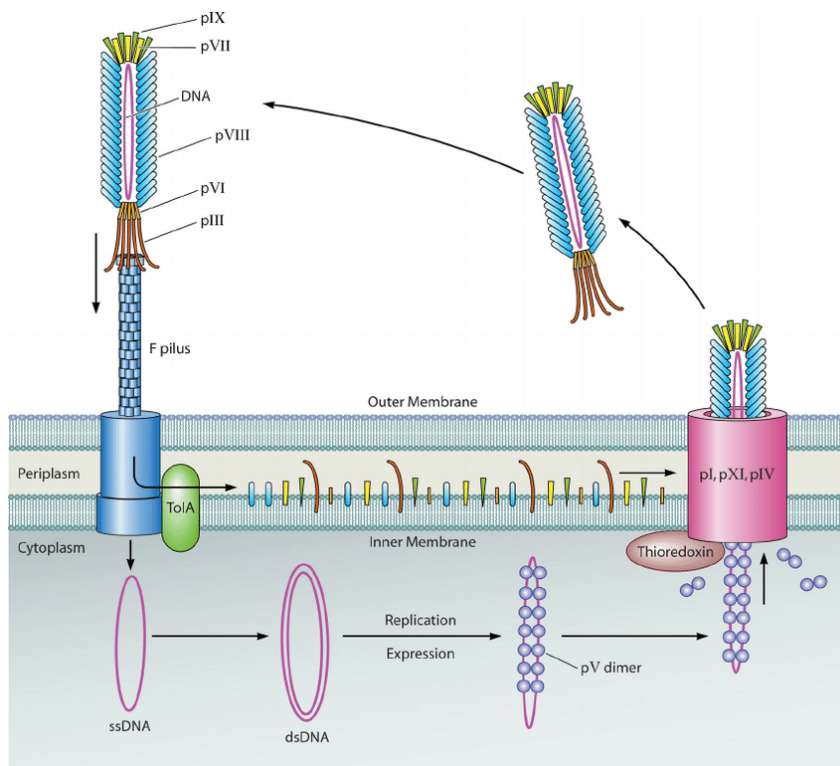


Figure 1-2. The life cycle of a filamentous phage.

The Life cycle of a Ff-phage starts with the attachment of the phage via the F-pilus. This then leads to replication of the phage genome and translation of phage proteins, resulting in the accumulation of protein pV. Once the correct concentration is reached, the ssDNA is secreted to the periplasm ready for integration with the structural proteins. Once the construction of the phage particle is complete, it is secreted from the cell.

Reprinted from, Antimicrobial Agents and Chemotherapy. Volume 56 /Issue 9, Page 4570, Huang, J.X., Bishop-Hurley, S.L., and Cooper, M.A. Development of anti-infectives using phage display: biological agents against bacteria, viruses, and parasites. Copyright (2012), with permission from American Society for Microbiology.

1.5.2 Filamentous phage life cycle

M13, f1, and fd phages all cause a non-lytic infection in *E. coli*, initiated via an interaction between the N-terminus of the pIII protein, and the F-pilus of the bacterium (Figure 1-2). It is due to the dependency of these phages on the presence of an F-conjugative plasmid for infection and their sequence homology, that they are termed Ff-phages (Beck and Zink 1981). Ff-phages contain ssDNA genomes, consisting of

11 genes with an intergenic region containing an origin of replication for synthesising (+) and (-) DNA strands for creation of its replicative form. These genes are split into 3 groups, based on their function. The first group (II, V, X) encode those necessary for replication of DNA, the second (III, VI, VII, VIII, IX) are structural and the third (I, IV, XI) are necessary for virion assembly alongside a packaging signal (Tikunova and Morozova 2009). Only those proteins necessary for replication remain in the cytoplasm, the others are targeted to the inner membrane for translocation into the periplasmic space (Rakonjac *et al.* 2011).

After the interaction between the N-terminal domain of pIII and the F-pilus, the latter is retracted, pulling the phage towards the cell, and the TolA protein specifically (Mai-Prochnow *et al.* 2015). This then allows infection of the cell (Figure 1-2). The host machinery, RNA and DNA polymerases, are manipulated to synthesise the phage's replicative form (RF) from the phage origin of replication (-) strand (Higashitani, Higashitani, and Horiuchi 1997). As RNA polymerase requires dsDNA as a template, the (-) origin forms a hairpin structure, the stem of which mimics a promoter sequence (Higashitani *et al.* 1996). This allows RNA polymerase to initiate synthesis of the (-) strand via construction of an RNA primer which DNA polymerases then extend to create the RF. The protein pII then nicks the (+) strand, creating a primer for host DNA polymerases to synthesise a new (+) strand and thus increases the number of DNA templates for transcription and translation (Rakonjac 2012). These RFs are duplicated to about 50 per cell and are the template for viral protein production (Rakonjac 2012). Packaging is dependent on the concentration of the pV protein; once a certain level of pV has been attained, the ssDNA (+) complexes with the protein and becomes the substrate for secretion into the periplasm for viral assembly (Mazur and Zinder 1975).

Assembly is then initiated by two minor proteins, pVII and pIX, interacting with the packaging signal (Russel and Model 1989). As the DNA complex is secreted through the membrane, pV is dissociated from the DNA and replaced with subunits of pVIII until the DNA is covered (Mai-Prochnow *et al.* 2015). Two other proteins form a terminating cap, pIII and pVI, and release the virion from the cell (Rakonjac, Feng, and Model 1999).

1.5.3 Phagemid-mediated phage display

With the display of peptides on a coat protein, the phage particle may lose infectivity or its ability to undergo normal assembly (Iannolo *et al.* 1995). Therefore, to maintain production of viable phage, vector systems are used which ensure wild type pIII or pVIII genes are present in addition to the fusion protein. These can be either phage or phagemid vector-based. With the phage vector system, the recombinant gene (coding the fusion protein) is inserted directly into the phage genome and these are termed “33” or “88”, dependent on whether pIII or pVIII is used. Alternatively, they can be phagemid-based and termed “3+3” or “8+8” (Tikunova and Morozova 2009). Phagemid systems are more common than phage systems, primarily for the ease of cloning facilitated by the multiple restriction sites within the phagemid genome (Qi *et al.* 2012).

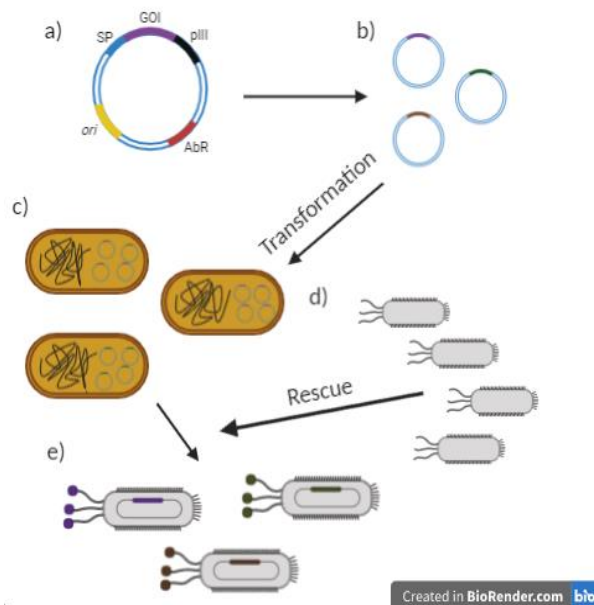


Figure 1-3. Phagemid rescue and phage particle production.

a) Example phagemid vector with SP – signal peptide, GOI – gene of interest, pIII – recombinant coat protein, AbR – selective marker, ori – origin of replication. b) library of phagemids with unique GOI's. c) Transformation of phagemids into *E. coli*. d) rescue of phagemids by superinfection of helper phage. e) production of infectious phage particles displaying protein of interest.

These phagemid vectors lack all typical Ff-phage genes, except for the display protein fused to the gene of interest and the packaging signal (Qi *et al.* 2012). To enable production of infectious phage particles, superinfection with a helper phage, such as M13KO7, occurs (Figure 1-3). Helper phage have the wild-type genes of an Ff-phage, but with a defective packaging signal to allow preferential packaging of the phagemid genome into the infectious particles (Fatemi *et al.* 2017). There is usually a separate high copy number origin of replication present in phagemids to facilitate high levels of the RF (Qi *et al.* 2012). High levels are needed to ensure there are enough fusion proteins to be delivered to the membrane, via a signal peptide, for assembly to outcompete the equivalent wild type coat protein derived from helper phage genome (Vieira and Messing 1987). However, this system (based on the defective packaging

signal and high production of fusion proteins) is not 100% efficient, leading to a heterogenous population with some wild-type particles (Chasteen *et al.* 2006).

Phage display relies on the production of very high diversity libraries that display millions to hundreds of billions of distinct ligands (Bazan, Calkosiński, and Gamian 2012). Creating a phage peptide library using these phagemids can be achieved through multiple routes. Usually, this involves annealing complementary oligonucleotides, one of which encodes a randomised sequence. The oligonucleotide not coding for amino acid diversity is then elongated to provide complementary bases and the final product is cut with restriction enzymes and cloned into the vector (Ryvkin *et al.* 2018). This introduces diversity into a series of codons usually coding for between 7 and 16 amino acids, and these can be displayed on either pIII or pVIII, and can be linear or constrained between two cysteine residues. The region of diversity can be introduced with codons that are either completely randomised or refined to introduce only specific amino acids as seen in the NNK codon usage to reduce the introduction of premature stop codons (Nov 2012). This is where N is an equimolar concentration of all nucleotides whereas K is equimolar of only G and T. Alternative ways include mutator strains of *E. coli* or error-prone PCR (Huang *et al.* 2015; Irving, Kortt, and Hudson 1996). These methods produce libraries of phagemids with virtually all containing a unique peptide gene sequence which are then transformed into *E. coli* to be rescued with a helper phage. The phage libraries produced will ideally contain between 10^9 to 10^{11} sequences, and this is largely dependent on the efficiency of transformations into *E. coli* (Ponsel *et al.* 2011). It is this diversity which makes phage display an attractive method for identifying novel sequences for AMPs. Specifically, there is a lack of understanding between an AMP's sequence and its

function and the use of vast libraries to select for AMP activity can provide a method that is not based on prior knowledge of AMP characteristics.

1.5.4 Biopanning and sequencing of phage peptide libraries

Once a phage display library has been obtained, a process called panning (or biopanning) is performed. This is where the phage library displaying the peptides is mixed with the binding target. Those peptides which do not bind are removed by washing steps, and those which do bind are eluted through a shift in pH and then propagated in *E. coli* to produce a sub-library of phage that is enriched for peptides that bind the target. This sub-library is then rescued and used as the input phage for the next round of panning. Subtraction steps can be employed as a way to remove phage which might be binding to non-targets (Siebke *et al.* 2012), this approach can also be used to increase specificity of AMPs against a particular pathogen (Bishop-Hurley *et al.*, 2005; Bishop-Hurley, Rea and McSweeney, 2010).

Once the iterative panning rounds are complete (usually three to five rounds), a selected number of phages are isolated and DNA extracted for sequencing to determine the most abundant sequences, and therefore which sequences bound preferentially to the target. A limitation of this method is the cost and time constraints to send multiple samples for Sanger sequencing. Another major limitation of panning with phage libraries to identify AMPs is that the method screens for binding, not antimicrobial activity. One recent publication by Tucker *et al.* (2018) proposed a different method to screen for antimicrobial activity. Tucker *et al.* (2018) transformed a library encoding 800,000 sequences into an *E. coli* system where the expressed peptide would be tethered on a cell-surface anchor, confining any antimicrobial effects

to the host cell. The *E. coli* library was sequenced using next generation sequencing (NGS) before and after growth and peptide expression to determine which sequences were lost which indicated they were killing their host. The lost sequences could then be selected for synthesis based on this potential antimicrobial activity. Although, this method can only screen against bacteria amenable to genetic modification and, due to NGS restrictions, library sizes were smaller than those used in phage display.

While NGS reads are currently limited to $\sim 10^7$ sequences, below the initial library sizes for phage display, it can still be used to estimate naïve library diversity and any bias contained within the library (Glanville *et al.* 2015). NGS has also obviated Sanger sequencing and improved phage display analysis through allowing a deeper read of the potentially enriched peptides. False positives arise from target-unrelated peptides (TUPs) which can be enriched in a phage display system, either through a biological advantage (often conferred through amino acid sequence) leading to faster propagation, or by non-target binding (Zade *et al.* 2017). NGS can also help prevent false-positive hits from TUPs by allowing extensive sequence comparisons between controls and panning outputs (Naqid *et al.* 2016). However, the number of peptide sequences returned after NGS analysis, which have been enriched to some degree, is usually far too high to have them all synthesised (Naqid *et al.* 2016). Therefore, the number of peptides that can be screened for AMP activity is still limited.

Guralp *et al.* (2013) developed an alternative technique to screen for AMP activity from a high diversity library. A strain of *E. coli*, which has a mutation in its outer membrane causing leakage from the periplasm, was transformed with a library encoding 12,000 mutants of the AMP plantaricin-423. By plating the transformed *E. coli* on a petri dish and inducing expression in overlays with agar containing target

bacteria, between 3,000 and 5,000 mutants could be screened for activity. This is still a comparatively tiny portion of the peptide sequences that can be encoded within a peptide library, and has only been characterised with one target bacteria and variants of one peptide. For all of the screening methods, once putative AMPs have been identified, they then require synthesis for further testing. For small-scale peptide production, solid-phase peptide synthesis (SPPS) is often used. However, to aid AMP production in becoming commercially viable, research into recombinant production methods would be beneficial to improve their cost-effectiveness as treatments.

1.6 AMP production methods

1.6.1 Chemical synthesis

Chemical synthesis methods have become more advanced recently with the use of SPPS. This is due to the single purification step required as opposed to after every amino acid addition; reaction completion is also more favourable due to larger reagent volumes (Bédard and Biron 2018). This chemical approach also allows incorporation of non-natural or D-amino acids as well as other modifications which can be hard to replicate in recombinant models. In older methods of peptide synthesis, sequences containing long hydrophobic stretches, common in an amphiphilic AMP, can collapse on the SPPS scaffold causing truncated peptide production (Adams *et al.* 2007). More recently, there have been a variety of methods developed to overcome this problem, including PEG-based resins and backbone protecting groups (Bédard and Biron 2018). Unfortunately, SPPS does require the use of toxic reagents within the process, such as N,N-dimethylformamide or dichloromethane, which have environmental impacts and

require special disposal. The chemical synthesis of peptides can also be complex and laborious, with low yields a persistent problem (Wibowo and Zhao 2019).

1.6.2 Recombinant production

Recombinant techniques are still seen as the best route for large-scale production of AMPs due to their sustainability, although this is disputed for those AMPs under 50 residues (Bédard and Biron 2018; Li 2011). *E. coli* is often seen as the best organism for production due to its fast growth, high cell density, cheap growth media, and thoroughly researched genome (Rosano and Ceccarelli 2014). While there are advantages to prokaryotic systems, when using them to produce AMPs care must be taken to ensure the peptides do not negatively affect the host, as this may lead to limited yields (Singh *et al.* 2015). One strategy that has been successful in producing a soluble, recombinant AMP was *in vivo* biotinylation of scygonadin (Li and Sousa 2012). By utilising the Avi-tag sequence, a site for biotinylation, production of biotinylated scygonadin was achieved. However, due to the use of a His-tag for purification, there needed to be a chromatographic purification step after cleavage from the recombinant AMP. Chromatography is often the most expensive part of the downstream processing, and obtaining high yields without it would reduce costs considerably (Azevedo *et al.* 2009).

1.7 Engineering AMPs

1.7.1 AMP modifications

While AMPs do have advantages as a pharmaceutical, they often have various characteristics which would inhibit their use, even if a streamlined production method was found. They can display sensitivity to exogenous factors such as salt, pH, and/or proteases, and *in vivo* they can also be rapidly cleared via the kidneys due to poor pharmacokinetic stability, which can subsequently result in nephrotoxicity to the host (Aoki and Ueda 2013). Instability and toxicity are two of the main inhibitors of creating a therapeutic AMP. Peptides often display similar host-cell toxicity at concentrations close to the MIC against their bacterial targets, and has also been noted when investigating the antiviral and anticancer activities of these peptides (Arias *et al.* 2020; Sun *et al.* 2016; Wang *et al.* 2010). Off-target toxicity can be attributed to the membrane-targeting effects of the AMPs, with unknown targets causing affinity to certain cell types. Despite this, due to their small size they are amenable to design and engineering efforts to improve their biochemical or pharmacological characteristics. There are two general approaches to engineering an AMP: modifying the AMP itself (Table 1-2) and conjugating compounds to the AMP (Table 1-3).

Table 1-2. Examples of research available, detailing AMP modification.

Antimicrobial peptide(s)	Origin of peptide	Method of engineering	Effect	Reference
Sushi-1 peptide	Horseshoe crab	Amino acid substitution	Improved antimicrobial activity, lower haemolytic activity	(Yang <i>et al.</i> 2018)
Dermaseptin S3	Tree frog	Amidation	Improved antimicrobial activity, increased helix stability	(Shalev, Mor, and Kustanovich 2002)
MerP	<i>E. coli</i> mercury transporter fragment	Pattern analysis	Improved AMP structure	(Cardoso <i>et al.</i> 2018)
Pg-AMP1	Guava	Genetic algorithm-led amino acid substitution	Improved antimicrobial activity, lower haemolytic activity	(Porto, Fensterseifer, <i>et al.</i> 2018)
Drosocin and pyrrhocoricin	Fruit fly and European sap-sucking bug	C-terminal 2, 4-diamino-butyric acid scaffold	Improved antimicrobial activity, wider range of activity	(Otvos <i>et al.</i> 2005)
CM18	Invertebrates	Shortened peptide sequence	Cheaper SPSS synthesis	(Maccari <i>et al.</i> 2013)
EBNA-3	Human herpesvirus 4	Linguistic Algorithm	Enabled antimicrobial activity	(Porto, Irazazabal, <i>et al.</i> 2018)

Modifications can include alanine scanning, which creates a library of mutants where each residue is changed in turn to an alanine. If the residue is of importance it will be exhibited in subsequent assays and this can then be used to inform optimal sites for further amino acid substitutions (Cantisani *et al.* 2014). Computer-aided processes have also proven useful with designing improved AMPs with techniques such as QSAR and machine learning algorithms at the forefront. However, these are still limited by either a lack of known relationships between sequence data and activity or

limited datasets. An often cited disadvantage to AMPs is their vulnerability to proteases, by modifying the AMPs using D-amino acids (Kindrachuk *et al.* 2011; Zhao *et al.* 2016), unnatural amino acids (Berthold *et al.* 2013), or cyclisation (Liu *et al.* 2017; Molhoek *et al.* 2011), this problem is commonly resolved often with no effect on activity. Although these strategies are possible, they do increase the cost of synthesis through increased complexity. Careful consideration should also be applied, as these modifications can reduce the helicity of the peptide which, although is mostly associated with cytotoxicity, can reduce antimicrobial activity (Huang *et al.* 2014a; Zelezetsky and Tossi 2006; Zhang *et al.* 2016).

Table 1-3. List of research available, detailing examples of AMP conjugation.

Antimicrobial peptide(s)	Origin of peptide	Method of engineering	Effect	Reference
VG16KRKP	Synthetic	Dimerization via disulphide bond	Improved antimicrobial activity	(Datta, Kundu, and Bhunia 2016)
UBI ₂₉₋₄₁	Humans	Conjugated to Chloramphenicol	Improved antimicrobial activity, decreased cytotoxicity	(Chen <i>et al.</i> 2015)
Lactoferrin	Mammals	Lipidation	Improved antimicrobial activity, improved endotoxin binding	(Majerle, Kidriq, and Jerala 2003)
Magainin 2	African Clawed frog	C-terminal Lysine dimerization	Improved antimicrobial activity, reduced time-kill period	(Lorenzón <i>et al.</i> 2016)
Unnamed	Synthetic	Lipidated histidine dimers	Enabled antimicrobial activity	(Arnusch <i>et al.</i> 2012)
Oncocin and Apideacin derivatives	Milkweed bug and honeybees	PEGylation	Enhanced serum half-life	(Nollmann <i>et al.</i> 2013)
Novispirin G-10	Ovispirin-1 derivative	Linked to bacterial pheromone	Targeted killing	(Eckert <i>et al.</i> 2006)
JH8194	Histatin derivative	Linked to titanium binding motif	Anti-biofilm activity	(Liu <i>et al.</i> 2016)
Magainin 2	African Clawed frog	Multimerization via 3,5-di-(2-aminoethoxy) benzoic acid	Dimer displayed increased antimicrobial activity	(Arnusch <i>et al.</i> 2007)
Unnamed	Synthetic	Multimerization via polymer PMA	Increased antimicrobial activity	(Liu <i>et al.</i> 2006)

1.7.2 AMP conjugation

1.7.2.1 Non-AMP conjugates

Due to the synergistic effects that have been noted between particular AMPs and antibiotics, thought to be due to the permeabilising activity of AMPs thereby improving antibiotic uptake, there have been multiple studies investigating AMP activity while conjugated to antibiotics (Sheard *et al.* 2019). However, while synergistic behaviour tends to remain once conjugated, in some cases linkage can interfere with the active site of the antimicrobial effect (Sheard *et al.* 2019). Therefore, a combination therapy where the antibiotic and AMP are administered individually and shown positive results in both *in vivo* and *in vitro* experiments, might be the most efficient route (Rishi *et al.* 2011; Ruden *et al.* 2019). Other conjugates attached to AMPs have included lipid tails, photosensitizers, or cell-penetrating peptides (Chu-Kung *et al.* 2010; Johnson, Muthukrishnan, and Pellois 2013; H. Lee *et al.* 2019). Lipidation is thought to improve interactions with the bacterial membrane, evidenced by greatly contributing to the antibacterial activity displayed by colistin, a membrane-targeting antibiotic (Chihara *et al.* 1974; Duwe *et al.* 1986; Reinhardt and Neundorff 2016). Research into the use of photosensitizers and cell-penetrating peptides, while promising, is in very early stages with little data on their use therapeutically.

Producing AMPs targeted to specific pathogenic bacteria have the potential to remove one of the problems with the antibiotics currently used as therapeutics, namely the indiscriminate killing of beneficial gut microbiota. One approach to produce targeted AMP therapies involves fusing a broad-spectrum AMP with a targeting peptide that binds specific pathogens. This has been shown to be possible with Kim *et al.* (2019)

developing an AMP conjugate with *Pseudomonas* specificity, and Choudhury *et al.* (2019) developing an AMP with *Staphylococcus* specificity using targeting peptides identified with phage display. Both strategies managed to retain antimicrobial activity against a specific pathogen while minimising activity against other bacteria (Choudhury *et al.*, 2019; Kim *et al.*, 2020). One concern regarding targeting peptides is whether there is a possibility of resistance development through mutations of the targeting peptide's binding site, as there is a lack of research into this aspect.

1.7.2.2 Dimerization via disulphide bridges

Dimerization can be beneficial for the antimicrobial activity of AMPs, with some peptides naturally occurring as homodimeric and even as heterodimers (Liu *et al.* 2010; Tencza *et al.* 1999; Verly *et al.* 2017). Oxidation reactions leading to disulphide bridges account for the majority of homodimeric peptides described (Tencza *et al.* 1999). Dimerization via cysteines is thought to improve an AMP's activity through an increased hydrophobicity and net surface charge, and can result in fewer peptides needed to reach permeabilization thresholds (Lorenzon *et al.* 2019). Disulphide bond dimerization can also improve resistance to proteolysis, and higher concentrations of salt (Campopiano *et al.* 2004; Raimondo *et al.* 2005). As a technique for improving synthetic AMPs, dimerization through disulphide bridges was found to only dramatically improve AMPs that are not particularly active by themselves, with potent AMPs barely improving (Liu *et al.* 2010). Disadvantages of using disulphide bonds comes from the side products produced during oxidation in synthesis and the possibility of multiple bond patterns for AMPs with more than one cysteine (Liu *et al.* 2010).

1.7.2.3 Dimerization via linkers

Another method of dimerising AMPs uses linker molecules with or without spacers. While the linker joins two monomers, the latter ensures that the monomers have the space required after linkage for biological activity. For example, lysine can be used as a linker due to its ϵ -amino group which can create isopeptide bonds to carboxyl groups in other amino acids. This lysine would then be the linker and is often attached to further amino acids, or other organic compounds, acting as spacers to avoid steric hindrance (Onaizi and Leong 2011). For brevity, this study will use “linker” as a term also encompassing any spacers used unless where otherwise specified.

Using lysine as a linker, it is possible to create branching structures based on the multiple antigenic peptides (MAPs) developed in the 1980's by Tam (1988). These MAPs were created by using lysine as a scaffold with a peptide attached to both the α - and ϵ -amine groups to improve the titre of antibodies elicited to a peptide antigen. AMPs can be attached onto these branching amine groups and this can result in improved antimicrobial activity, greater protease resistance, and lower cytotoxic effects (Pini *et al.* 2005). Linker molecules can be described as either flexible or inflexible, a characteristic determined by their primary structure and the residue's R groups. Generally, an inflexible linker is used to prevent interactions of the two monomers. In the case of AMPs, a flexible linker is often used as their mode of action commonly includes aggregation to form pores or membrane perturbation. It has also been suggested that flexibility promotes selectivity and increased activity against negatively charged membranes as they enable folding into more compact structures, thereby reducing their hydrophobicity and relying more on electrostatic interactions (Kai *et al.* 2018). The linker molecules themselves can have effects on the activity of

AMPs and so the primary structure must be chosen carefully and preferentially optimised for each use. For example, Lorenzón *et al.* (2012) found that, while dimerization of the AMP Ctx-Ha decreased the antibacterial activity, using a polar linker affected activity less than an apolar linker of the same size. In addition, Lee *et al.* (2013) showed that the structure of the linker is important, with a helical linker improving activity while a non-helical linker reduced it. Although, the size of the two linkers Lee *et al.* (2013) used was different, possibly affecting interactions.

A common flexible linker for polypeptides is a repeating motif of glycine and serine (Cao *et al.* 2018; Júnior *et al.* 2018; Van Rosmalen, Krom, and Merckx 2017). This formed the basis for Xi *et al.* (2013) to create a novel dimeric AMP from two individual peptides. LH28 had potent antimicrobial activity but host cytotoxic effects, and plectasin had low cytotoxic effects but low activity against Gram-negative bacteria. The hybrid peptide was termed LHP7, it combined the low cytotoxic effects of plectasin with the increased activity against Gram-positive bacteria of LH28.

1.8 Aims and objectives of the study

AMPs have the potential to serve as an alternative, or supplement, to antibiotic therapy. However, in the literature there is a lack of a standard pipeline for identifying, characterising, and improving novel AMPs. The aim of this study was to build on previous work that used next generation phage display to identify candidate peptides that bind pathogenic bacteria to establish a framework for testing and improving AMPs. It was hypothesised that AMPs can be identified by phage display and

improved to the extent that they could offer an economical, viable alternative to therapeutic antibiotics. The objectives of this study to test this were fourfold:

Firstly, due to the differences in testing methods used in the literature, to assess established and novel AMPs' activities in different assay formats and to define the important AMP characteristics: inhibitory activity, haemolytic behaviour, and synergistic properties (Characterisation and comparison of AMPs derived from phage display with known AMPs).

Secondly, to determine if dimerization of AMPs can alter specificity and improve potency (Dimerization of peptides for improved activity).

Thirdly, to apply the most effective AMPs within mixed culture assays to investigate the potential of the peptides to be highly selective to a specific target pathogenic bacteria (Function of AMPs in mixed culture models).

Fourthly, to investigate more effective multimerization and high-throughput screening methods. To produce and characterise five random 16-mer peptide libraries produced as dimeric AMPs by conjugation to five separate AMPs. To carry out high-throughput screens of libraries directly for AMP activity. In addition, to investigate the potential of alternative multimerization strategies for AMPs (Engineering dimeric peptides for improved efficacy and ease of production).

2 Methods

2.1 General media

All media for bacterial growth was prepared according to manufacturers' instructions using reverse osmosis (RO) water and then sterilised at 134°C and 15 psi for 15 minutes unless stated otherwise. Any broth produced using this method was stored in the Duran bottle at room temperature for no longer than three months before use. Any agar produced in this method was either cooled to approximately 50°C and poured into triple vented Petri dishes (2694785; Fisher Scientific), or the Duran bottle containing the medium was left at room temperature for no more than three months before use. This was then melted in a microwave on medium power until molten, left until it was cooled to approximately 50°C, and then poured into triple vented Petri dishes.

2.1.1 Mueller Hinton broth & agar

Mueller Hinton broth (MHB; 70192; Sigma-Aldrich) was prepared with 250 mL of RO water, with the addition of 3.75 g (1.5% w/v) bacteriological agar powder (20767.298; VWR) for Mueller-Hinton agar (MHA). MHB which has been supplemented with 0.4% (w/v) Bovine Serum Albumin (BSA) and 0.02% (v/v) glacial acetic acid (>99%) is termed adjusted-MHB (aMHB). Soft MHA (sMHA) was produced by using 70% of the total weights of ingredients used to produce MHA, but in the same volume of RO water.

2.1.2 2×YT broth & agar

2×YT broth was produced by adding 2.5 g of Yeast Extract (103753; Merck), 1.25 g Sodium Chloride (10428420; Fisher Scientific), and 4 g Tryptone (11385982; Fisher Scientific) to 250 mL RO water. To produce 2×YT agar, 3.75 g (1.5% w/v) bacteriological agar powder was added (20767.298; VWR).

2.1.3 MacConkey agar

MacConkey agar was produced by adding 12.5g MacConkey agar powder (M7408; Sigma-Aldrich) to 250 mL RO water.

2.1.4 Brilliant Green agar

Brilliant Green agar was produced by adding 12.5g Brilliant Green agar powder (CM0263; Fisher Scientific) to 250 mL RO water.

2.1.5 SOC broth

First, a 25% w/v glucose solution was prepared by adding 125 g D-Glucose Anhydrous (G/0500/53; Fisher Scientific) to 500 mL RO water. This was then filter sterilised through a 0.22 µM pore filter (SLGP033RS; Millipore) and stored at room temperature for no longer than three months. Next, SOB broth was produced to the following specifications:

- 0.5% (w/v) Yeast Extract (103753; Merck)
- 2% Tryptone (w/v) (11385982; Fisher Scientific)

- 10 mM NaCl (10428420; Fisher Scientific)
- 2.5 mM KCl (26764.260; VWR)
- 20 mM MgSO₄ (11596; Alfa Aesar)

The pH of the SOB medium was adjusted to pH 7.5 with 1 M NaOH and sterilised by autoclaving. SOC broth was produced by adding 1 mL of the 25% w/v Glucose solution to 19 mL aliquots of SOB as needed.

2.1.6 Bactericidal test buffer

Bactericidal test buffer was produced by adding 1.21 g of Tris base (BP152-1, Fisher Scientific) and 1.46 g of NaCl (10428420; Fisher Scientific) to 500 mL of RO water. The pH was then adjusted with 1 M HCl to 7.4 and then the buffer was sterilised by autoclaving and subsequently stored at room temperature until use, for a maximum of three months.

2.1.7 Peptide dilution solution

First, a 10% (w/v) BSA (4215015; VWR) stock solution was made with RO water and filter sterilised through 0.22 µm syringe filter (SLGP033RS; Millipore). A volume of 1 mL was then aliquoted into individual 1.5 mL sterile Eppendorf tubes and stored at -20°C until needed. To prepare the peptide dilution solution (PDS), an aliquot of 10% (w/v) BSA was thawed for 15 minutes in a 37°C incubator. Next, 400 µL of 10% (w/v) BSA was added to 9.6 mL of autoclaved RO water in a 50 mL Falcon tube. To this, 3 µL of glacial acetic acid (>99% (v/v)) was added.

2.1 Bacterial cultures

All bacterial cultures were stored at -80°C in 20% (v/v) glycerol stocks in either MHB or 2×YT broth (Table 2-1). To revive the Gram-negative bacteria used in this study, a loopful of frozen stock was streaked onto MHA and incubated overnight at 37°C. Each MHA plate was kept for no more than two weeks before discarding and re-culturing from -80°C frozen stocks. To revive the Gram-positive bacteria used in this study, a loopful of frozen stock was added directly to 10 mL of MHB and incubated overnight at 37°C. For contamination checks, stocks were plated on selective agar where available (MacConkey agar for Gram-negatives) or onto non-selective agar for colony morphology checks (MHA). Where overnight incubation was stipulated, this was kept between 16 and 18 hours. All bacterial culturing was performed under aerobic conditions.

Table 2-1. Origins and genetic information of the bacterial strains used in this study.

Bacterial strain	Sourced from	Reference
<i>E. coli</i> serotype O2	Serotyped field isolate gifted from P. Barrow	Not available
<i>E. coli</i> serotype O78:H9	Serotyped field isolate gifted from P. Barrow	Not available
<i>S. Typhimurium</i> 4/74	Sequenced field isolate gifted from P. Barrow Accession number: NC_016857	(Rankin and Taylor 1966)
<i>S. Enteritidis</i> P125109	Sequenced field isolate gifted from P. Barrow Accession number: NC_011294	(Thomson <i>et al.</i> 2008)
<i>B. subtilis</i>	Non-sequenced field isolate gifted from R. Atterbury	Not available
<i>L. innocua</i>	Non-sequenced field isolate gifted from R. Atterbury	Not available
<i>E. coli</i> TG1	Commercial lab strain from Lucigen Genotype: [F' traD36 proAB lacIqZ Δ M15] supE thi-1 Δ (lac-proAB) Δ (mcrB-hsdSM)5(rK - mK -)	(Chai <i>et al.</i> 2020)
<i>E. coli</i> BL21	Commercial expression strain from Amid Biosciences Genotype: fhuA2 [lon] ompT gal (λ DE3) [dcm] Δ hsdS pBirA(StrR) [λ DE3 = λ sBamHIo Δ EcoRI-B int:: (lacI::PlacUV5::T7 gene1) i21 Δ nin5]	Not available
<i>E. coli</i> BL21	Commercial expression strain from ThermoFisher Genotype: F-ompT hsdSB (rB-, mB-) gal dcmrne131 (DE3) pLysS (CamR)	(Feng <i>et al.</i> 2002)
<i>E. coli</i> JE5505	Commercial expression strain from Yale University Genotype: Δ (gpt-proA)62 lacY1 tsx-29 glnX44(AS) galK2(Oc) λ - Δ lpp-254 pps-6 hisG4(Oc) xylA5 mtl-1 argE3(Oc) thiE1	(Hellman <i>et al.</i> 2000)

2.2 Peptides

Monomeric peptides were synthesised by Biomatik (USA) at crude concentrations (Table 2-2); dimeric peptides were synthesised by Genscript (Netherlands), at a purity of $\geq 70\%$. The lyophilised peptides were resuspended in 100% DMSO (BP231-1; Fisher Scientific) at 10 mg mL⁻¹ and stored at -20°C.

Table 2-2. Peptide sequence information

Peptide	Sequence
1	EQIWLHLFRLINQSLC
2	WRGTALKFICYPVLLL
3	RFISEAqDQICIPLRA
4	PVWVFVQSLRPKNTCVE
5	LWNWPGVLTCLVCLIP
6	VLCSSLQSVPLFERVW
7	GTSFLVCGLSIFVIFK
8	WSSGFRWLTCLLTWH
9	LLGWYTPLECFRKL
10	GPTVMSMWKPFPSLA
11	LTTVCAIWHVLTSPH
12	IGYWLSDCVLPWLLQT
13	WGSKLLQVFSTICNMV
14	YFLHHWVLPWLCTLHM
15	GPTVMSMWKPFSLFSLA
16	PVGACVYILSSFAPIA
17	LLGVVVYSVGFFVSWV
18	AGDYCLFLCQLSRLA
19	RqTVLSWILCYSDRWW
20	GTSFFGLWSKYFCNLK
21	LGTHFSELFQHWPLR
22	SHVVLNWLERWLYCSL
23	WFQRIDVFFQTPFLLF
24	STYHWHNFIYFVCIL
25	GPTVMSMWKPFSLLVFA
26	LLGVGGILRWSVFSWV
27	WGSKVNFPSRIWCLLH
28	GNqFFGLWSKYFCNLK
29	LGTHFRSFFVSIGPLR
30	APGWWRIpAFLqGPFC
31	GMFGGLCNPWIFAHP
32	EVSWLGWGIWPSWqVE
33	VWEIGPRWWTqLTCPS
34	FHWPFRPFAWLDHGYC
35	WRERLSAPGCFLAYLL
36	VVSSWPWLLAHVSCWI
37	SFGVRQLLCMMTGFWH
38	PNQIYRSRQWVECLLF
39	SLKLVPSPFGNSICq
40	LGKLRTFICqLDYILV
41	FADFMlqMACRANLFV
42	LLHRMVCLSAqAADWL
43	TYWADWWRLGGREPC
44	WDLAWCMLRANAFSF
45	GWGKWGLRQLRDFAVC
46	AGSLFRSVIDLVCVWG
47	PFWGGEALKWLGFRAC
48	VREVVCWAVWAVqGV
49	RVPDLFDVLCCHHKGSq

50	FGQSLRTWVLSLWCYD
51	FMGWFVNWLSCGYHGL
52	WDEAVVGYLRQFWCWA
53	qILGKVWKSIDWFLCD
54	SLNSVLSLLDNEYHPN
55	SVqDFVCRYMSLFFST
56	FWDDWLFWARAGLCAL
57	SVPLRWLCSDNFYqKN
58	STEAWRVPLMRHVCSL
59	AFVPPRPAFLHKLFLCL
60	VSGLRIWLRDTACAVL
E-50	TTKNYGNVCNSVNWQCQGNVWASCNLATGCAAWLCKLA
C-BF	KFFRKLKKS VKKRAKEFFKKPRVIGVSIPF
Plectasin	GFGCNGPWDEDDMQCHNHCKSIKGYKGGYCAKGGFVCKCY
Plantaricin-423	KYYGNGVTGCGKHSCSVNWGQAFSCSVSHLANFGHGKC

2.3 Peptide inhibitory activity

To determine which peptides had inhibitory activity against the panel of bacteria utilised in this study, two assays were performed. Both techniques were modified versions of the broth microdilution and agar dilution assays as outlined by Wiegand, Hilpert and Hancock (2008) and Guralp *et al.* (2013), respectively.

2.3.1 Broth Microdilution

This protocol was based on various Clinical Laboratory Standards Institute sources (CLSI 2015, 2018; Humphries *et al.* 2018) and Troxler *et al.* (2000) to obtain the most appropriate guidance for AMP testing and the various bacterial incubation requirements (Appendix 9.1).

First, peptides were diluted to 256 mg L⁻¹ in aMHB. Then 50 µL of each peptide dilution was added in triplicate to a 96-well polypropylene plate (CLS3879-50EA, Sigma Aldrich). MHB 10 mL was then inoculated with the test bacterium by either adding two or three colonies grown overnight on MHA (for Gram-negatives) or 100 µL of culture grown overnight in MHB (for Gram-positives). This was then incubated

at 37°C, shaking at 200 rpm. When the culture reached 1×10^8 CFU mL⁻¹, as determined by an experimentally determined OD₆₀₀, the culture was diluted 1:100 in pre-warmed MHB. Of the diluted culture, 50 µL was mixed with the peptide dilutions in the 96-well plate to reach a final concentration of 5×10^5 CFU mL⁻¹ of bacteria and ~60 µM of peptide. Controls comprised 100 µL of MHB added to a row of wells to determine media sterility, and 50 µL bacteria at 10^6 CFU mL⁻¹ added to aMHB in another row of wells to determine the value for 100% growth. From the growth control, 10 µL was added to 990 µL of bactericidal test buffer and mixed by aspiration, 100 µL of this dilution was then added to a further 900 µL of bactericidal test buffer and mixed by aspiration. Finally, 100 µL of the final dilution was spread-plated onto MHA, in duplicate. The growth control MHA plates were then incubated alongside the 96-well plates for a time and temperature as outlined in Appendix 9.1. After incubation, 65 µL was then aspirated from the wells and transferred to a flat-bottomed 96-well plate (442404; SLS) for visualisation in a spectrophotometer (VL0000D0; Fisher Scientific). The mean OD₆₀₀ value of the sterility wells was then subtracted from all wells to account for media influence, and the mean OD₆₀₀ value of the growth wells was divided by two to obtain the IC₅₀.

A further assay was performed to determine the minimal inhibitory concentration (MIC) for those peptides which were active against the target pathogen. This was performed as with the screening assay, but with halving concentrations from 128 mg L⁻¹ to 4 mg L⁻¹. For C-BF, this was extended down to 0.125 mg L⁻¹. An exception was for the testing of *L. innocua*, for this bacterium the 96-well plate used in the incubation period was photographed and visually appraised for wells with inhibited growth. This was due to the spectrophotometer values not correlating with visual inspection. To

determine the MIC, the assay was performed on three times, independent of each other. To account for experimental variability the mode of the values from each of the three independent assays (nine data points from three assays with technical triplicates) was used (Humphries *et al.* 2018; Shagaghi *et al.* 2020).

2.3.2 Agar dilution

Peptide inhibitory activity in agar was tested by following a modified protocol outlined by Guralp *et al.* (2013). First, the test bacterium was either streaked onto MHA or a loopful of bacteria was directly added to 10 mL MHB and then incubated overnight at 37°C. The next day, 10 mL MHB was inoculated with either two or three colonies or 100 µL overnight culture. This culture was then incubated at 37°C, 200 rpm, until reaching an OD₆₀₀ of ~0.6. Meanwhile, peptides were diluted to 512 mg L⁻¹, approximately 240 µM for the phage display-derived peptides and approximately 130 µM for the peptides derived from the Database of Antimicrobial Activity and Structure of Peptides (DBAASP). A two-fold serial dilution was then performed down to 4 mg L⁻¹. When the culture of the test bacterium was in the mid-log phase of growth, it was diluted to the OD₆₀₀ equivalent of 1×10⁸ CFU mL⁻¹ in pre-warmed MHB. Once diluted, 1 mL was then added to 25 mL of molten sMHA, kept at 50°C. The molten sMHA was then poured into a sterile 100×100 mm square Petri dish (82.9923.422; Sarstedt) and left to set. Then, 5 µL of the peptide dilutions was spotted onto the surface in triplicate and left to dry, after which the petri dish was inverted and incubated at 37°C overnight. Following incubation, the Petri dishes were photographed using the ChemiDoc MP Imaging System (17001402; BioRad). Peptides were deemed to be active at concentrations which produced a clear zone of

inhibition. To reduce subjective bias in the analysis of the assays, the “clear zone” determinations were independently corroborated by a colleague. If the colleague disputed the MIC stated, the image would go onto a second colleague to achieve a consensus.

2.4 Peptide bactericidal activity

The bactericidal screen was taken from Tucker *et al.* (2018) and was performed similarly to the protocol in 2.3.1. For the bactericidal screen, peptides were diluted in PDS rather than MHB. The bacterial culture was incubated at 37°C at 200 rpm until visibly turbid and then centrifuged at 3,000 $\times g$ for five min. The cell pellet was then washed with bactericidal test buffer and centrifuged at 3,000 $\times g$ for five min. This was repeated, the pellet re-suspended in bactericidal test buffer, and OD₆₀₀ modified via dilution with bactericidal test buffer until it reached the equivalent of 1×10^8 CFU mL⁻¹. This was then diluted further by 1:100 in bactericidal test buffer. This was then treated the same as the inhibitory screen with 50 μ L of both bacterial suspension and peptide inoculated into 96 well plates with the same controls and in triplicate. Instead of monitoring growth, adding 50 μ L of inoculum to 50 μ L of PDS allowed a ‘survival’ control to ensure any loss in viability was due to peptide interference. After 24 hours incubation at 37°C, 3 $\times$ 10 μ L of each peptide/bacterial suspension was spotted onto MHA, left to dry for 20 minutes, then inverted and incubated overnight at 37°C. Bactericidal activity was determined by 3-log₁₀ reduction in CFU mL⁻¹, determined by counting colonies present on the plate (Tucker *et al.* 2018).

As with 2.3.1, a further assay was performed to determine the minimal concentration for those peptides which were active against the target pathogen. This was performed

similar to the screening assay, but with halving concentrations from ~60 μM to 1.9 μM . The minimum bactericidal concentration (MBC) was the lowest concentration causing a 3- $\log_{10}$ reduction in CFU mL^{-1} when compared with the growth control after the 24 hour incubation (Tucker *et al.* 2018). To determine the MBC, the mode average was taken after two independent technical repeats. If no modal value was obtained after two repeats, a third repeat was performed. If there was still no modal value, but the results in each assay were within 8-fold of each other, then a mean average was taken from across the repeats. If there was no modal value, and the results from each assay were not within 8-fold of each other, then the median value was taken. When peptide results were out of range of the assay, above or below the highest/lowest concentration, the closest concentration used was taken as the MBC value for that result.

2.5 Haemolysis assay

The haemolysis assay was performed according to Oddo and Hansen (2017). In a 96-well plate, 150 μL of a 5 μM Melittin solution in PBS was added to six wells as the positive control. After overnight incubation at room temperature ($18^{\circ}\text{C} \pm 2$), the Melittin solution was aspirated and the wells washed with two to three volumes of PBS. A 150 μL dilution of AMP was then added to row A at either 256 mg L^{-1} or 512 mg L^{-1} , in either duplicate or triplicate technical repeats. Then, 75 μL of PBS was added to the empty wells down to row G. A total volume of 150 μL PBS in six wells were used as negative controls. From row A, 75 μL of the peptide dilution was added to row B and mixed. This was repeated to row G and then 75 μL was taken and discarded, so rows A-G contained 75 μL . For a four-fold dilution series, initial volume in row A was adjusted to 100 μL with 25 μL aspirated and used in subsequent

dilutions. Then, 1 mL of fresh chicken blood (FB010AP; TCSbiosciences) was added to 3 mL PBS. This was mixed gently and then centrifuged at 700 $\times g$ for eight minutes. The supernatant was discarded, and 4 mL PBS added. This was then centrifuged again at 700 $\times g$ for eight minutes. The supernatant was discarded and 4 mL PBS added. A final centrifugation step at 1,000 $\times g$ was used for eight minutes. The supernatant was then discarded and 1 mL of PBS added. From this, an 8% (v/v) blood suspension was made in PBS and 75 μ L added to the microtitre plates. This was then incubated for one hour at 37°C and then centrifuged at 1,000 $\times g$ for 10 minutes. Of the supernatant, 65 μ L was taken and added to its corresponding well in a 96-well flat bottom plate. Absorbance readings were taken at 414 nm as outlined by Oddo and Hansen (2017). To obtain percentage haemolysis of the red blood cells (RBCs), the following formula was used:

$$\%Haemolysis = \frac{Abs(sample) - Abs(NegAvg)}{Abs(PosAvg) - Abs(NegAvg)} \times 100$$

2.6 Synergy

2.6.1 Checkerboard assay

Checkerboard assays were performed by adding 25 μ L of each peptide, for a starting concentration of 256 mg L⁻¹ for peptide #59 and 128 mg L⁻¹ for peptide #45, into the relevant wells of a 96-well polypropylene plate. In the plate, a serial two-fold dilution of both peptide #45 and #59 were made. In row A, peptide #45 was added at 128 mg L⁻¹ and then in two-fold dilutions down to 2 mg L⁻¹ in row G (0 mg L⁻¹ in row H); in column 8, peptide #59 was added at 256 mg L⁻¹ and then in two-fold dilutions down to 4 mg L⁻¹ in column 2 (0 mg L⁻¹ in column 1). A bacterial culture of *E. coli* O78 was

prepared as detailed in 2.3.1, and 50 μ L added to each well. One column of the 96-well plate was used as a negative control of 100 μ L MHB only. After overnight incubation at 37°C, 65 μ L of the culture in each well was transferred to a flat-bottomed 96-well plate for visualisation in a spectrophotometer. The mean OD₆₀₀ value of the sterility wells was then subtracted from all wells to account for media influence, and the mean OD₆₀₀ value of the growth well (no peptide added) was divided by two to obtain the IC₅₀. To quantify the level of synergy a fractional inhibitory (or bactericidal) concentration (FIC) was calculated. The FIC is determined by the following equation where A and B represent each peptide:

$$FIC (AB) = \frac{MIC(A)Combo}{MIC(A)Alone} + \frac{MIC(B)Combo}{MIC(B)Alone}$$

An FIC value of ≥ 2 means the combination of peptides have an antagonistic effect on antibacterial activity, a value between 0.5 and 2 would indicate that the peptides have only an additive effect on antibacterial activity, and a value of ≤ 0.5 would show synergy between the two peptides (He, Starr, and Wimley 2015).

2.6.2 Alternative synergy assay format

Unless otherwise stated as a checkerboard assay, synergy assays were performed as a modified version of the protocol outlined by He, Starr and Wimley (2015). Each two-peptide combination was tested in a 96-well plate, with 25 μ L of each pair of peptides added, at their MBC, to individual columns of row A. Rows B and C contained 75 μ L of PDS. To dilute the peptide combinations, 25 μ L of each dual peptide combination from row A was aspirated and mixed with wells containing 75 μ L PDS in row B. This was repeated from row B to row C. From row C, 50 μ L was then discarded and 25 μ L

from row B. Rows D-F of the 96-well plate contained a duplicate of rows A-C. Rows G and F were survival and sterility controls, respectively, as outlined in 2.4. To prepare the inoculum, steps outlined in 2.4 were taken to obtain a concentration of test pathogen of approximately 5×10^5 CFU mL⁻¹. To determine synergism within an inhibitory assay similar steps were taken, except the preparation of inoculum would follow the inhibitory assay protocol in 2.3.1.

2.7 Streptavidin multimerization

The streptavidin multimerization assay was adapted from (Kwon *et al.* 2013). The target peptide was synthesised at crude concentration (~40-60%) from Biomatik with N-terminal biotinylation and C-terminus amidation to avoid any change in charge. In row A of a 96-well plate, 50 µl streptavidin (S4762; Sigma-Aldrich) in aMHB was added to 50 µL target peptide in aMHB in molar ratios of 1:1, 1:2, 1:3, and 1:4, with three technical repeats for each ratio. The peptide was added to all columns at a concentration of 128 mg L⁻¹ with an adjusted streptavidin concentration as required. In rows B-F, 50 µL of aMHB was added for a two-fold dilution series. Before dilution, the plate was incubated at 37°C for 30 minutes to allow conjugation of the peptides to streptavidin. After incubation, 50 µL was aspirated from row A and mixed with row B, then 50 µL from row B was aspirated and mixed with row C. This was repeated to row F, where 50 µL was then discarded. Wells within row G contained 50 µL of aMHB and 50 µL of bacterial suspension to obtain the value representing 100% growth, and wells in row H contained only 100 µL of MHB to ensure sterility. A separate 96-well plate contained 100 µL of two-fold serial dilutions of other controls for the experiment: columns 1-3 contained streptavidin only wells to ensure no adverse effects were caused by this molecule, columns 4-6 contained biotinylated peptide only

wells to obtain an experiment-specific MIC value, and columns 7-9 contained non-biotinylated peptide only wells to eliminate any effects from the biotinylation. Then, a bacterial suspension of *E. coli* O78 at approximately 1×10^8 CFU mL⁻¹, as determined by OD₆₀₀, was diluted 1:100. Of this diluted suspension, 50 µL was then added to the wells. From the growth control, 10 µL was then diluted 1:1000 and spread-plated in duplicate to determine the viable count. After incubation for the recommended duration as outlined in Appendix 9.1, the plate was read at 600 nm for optical density measurements.

2.8 Time-kill assay

This assay was adapted from the MBC assay in 2.4. Peptides were added to a 96-well plate in a volume of 120 µL at 2× MBC concentration. Bacterial cultures *E. coli* O2, *E. coli* O78, *S. Typhimurium* and *S. Enteritidis* were incubated at 37°C at 200 rpm until mid-log growth was reached, and were then centrifuged at 3,000 ×*g* for five minutes. The cell pellet was then washed with 5 mL of bactericidal test buffer and centrifuged at 3,000 ×*g* for five minutes. This was repeated once more and the pellet then re-suspended and OD₆₀₀ modified via dilution until it reached equivalent to 1×10^6 CFU mL⁻¹. The application of inoculum to wells followed the protocol in 2.4, but with 120 µL of the bacterial suspension added to the peptides. Volumes of 3 × 10 µL were taken at the time points 1, 2, 4, 8, 18 and 24 hour post-inoculation. Samples were spotted onto MHA, dried for 20 minutes, inverted and incubated overnight at 37 °C. The colonies on the plates were then counted to determine by which time point there was a 3-log₁₀ reduction in CFU mL⁻¹.

2.9 Recombinant peptide production

2.9.1 Electroporation

Custom plasmids were created using the Genscript (Netherlands) express cloning service, the constructs were cloned into a pET11a vector via the Nde1 and Bln1 restriction sites (Appendix 9.4). In one mm cuvettes (1652089; Bio-Rad), a total of 100 ng of plasmid was electroporated (1.8 kV, 25 μ F, 200 Ω ; GenePulser Xcell; Bio-Rad) into separate 50 μ L aliquots of two *E. coli* BL21 strains, pBirA (BLB-201; Amid Biosciences) and pLys (C602003; ThermoFisher). A total of 100 μ L of pBirA BL21 electroporation mixture, after one hour recovery in 1 mL SOC media at 37°C 220 rpm, was spread-plated on 2 $\times$ YT agar, supplemented with 50 μ g mL⁻¹ Streptomycin sulfate (15424871; Fisher Scientific), 100 μ g mL⁻¹ Carbenicillin (10396833; Fisher Scientific) and 1% glucose (v/v), and incubated overnight at 37°C. Following incubation, three colonies were individually picked to extract plasmid DNA from (27104; Qiagen). This followed the manufacturer's instructions, with 3 $\times$ 5 mL of 2 $\times$ YT broth, supplemented with 50 μ g mL⁻¹ Streptomycin sulfate, 100 μ g mL⁻¹ Carbenicillin and 1% glucose (v/v), inoculated with an individual colony and incubated overnight at 37°C and 200 rpm. After incubation, 2 mL of each culture was used for separate DNA extractions and the remaining 3 mL used to make 20% (v/v) glycerol stocks, stored at -20°C. The plasmid DNA was then submitted for Sanger sequencing to determine if the correct sequence was present. The pLys BL21 strain was treated similarly, but without addition of Streptomycin sulfate in the media used.

2.9.2 Induction

In all induction experiments using pBirA BL21 *E. coli*, growth media was supplemented with 50 $\mu\text{g mL}^{-1}$ Streptomycin sulfate and 100 $\mu\text{g mL}^{-1}$ Carbenicillin. In induction experiments using pLys BL21, *E. coli* growth media was only supplemented with 100 $\mu\text{g mL}^{-1}$ Carbenicillin. Before induction 1% glucose (v/v) was added to all media. All induction experiments were initiated using a loop containing approximately 2 μL of the stock culture streaked onto 2 $\times$ YT agar and incubated overnight at 37°C, unless otherwise stated.

For induction protocol #2 - #3, two to three colonies of pBirA BL21 *E. coli* were used to inoculate 2 $\times$ 20 mL 2 $\times$ YT broth per clone, induction protocol #4 required only 1 $\times$ 20 mL per clone, after overnight incubation. For induction protocol #5, colonies from both pBirA and pLys *E. coli* were used to create separate 20 mL cultures. These cultures were then incubated at 37°C, 200 rpm until OD₆₀₀ 0.4-0.6 was reached. The cultures were then split pre-induction from the individual 20 mL cultures into two 10 mL cultures each; one 10 mL culture was unaltered and kept in the 2 $\times$ YT broth with 1% glucose (v/v). The second 10 mL culture was centrifuged at 3,000 $\times g$ for five minutes to remove the 1% glucose media and then the pellet was resuspended in 10 mL of fresh, pre-warmed 2 $\times$ YT broth with 1 mM IPTG and 50 μM d-biotin.

2.9.2.1 Induction experimental protocol #1

After overnight incubation on 2 $\times$ YT at 37°C, two to three colonies of pBirA BL21 *E. coli* were used to inoculate 10 mL 2 $\times$ YT broth. This culture was then incubated at 37°C, 200 rpm until OD₆₀₀ 0.4-0.6 was reached. Then, 20 μL of each culture was taken

and mixed with 20 μ L Novex Tricine SDS sample buffer (LC1676, ThermoFisher), and heated to 98°C for five minutes and subsequently frozen at -20°C until use. Each remaining culture was centrifuged at 3,000 $\times g$ for five minutes to remove the glucose-containing media. The pellet was then resuspended in 10 mL of fresh, pre-warmed 2 $\times$ YT broth containing 1 mM IPTG (46-102-RF; SLS) and 50 μ M d-biotin (A14207.03; VWR). The cultures were then further incubated at 37°C for a total of six hours, at 200 rpm, with a 20 μ L sample was taken at time points 2, 4, 6 hours post-induction. Each 20 μ L sample was mixed with 20 μ L Novex Tricine SDS sample buffer and heated to 98°C for five minutes and subsequently frozen at -20°C until use.

2.9.2.2 Induction experimental protocol #2

After induction, the cultures were then incubated overnight with one set of 2 $\times$ 10 mL cultures at 30°C at 200 rpm and the other 2 $\times$ 10 mL cultures at 18°C ($\pm$ 2) on a rotating platform. After overnight incubation, 50 μ L of each 10 mL culture was mixed with 50 μ L of Novex Tricine SDS sample buffer and then heated to 98°C for five minutes and used for SDS-PAGE immediately.

2.9.2.3 Induction experimental protocol #3

After induction, the cultures were then incubated at 200 rpm overnight with one set of 2 $\times$ 10 mL cultures at 30°C and the other 2 $\times$ 10 mL cultures at 37°C. After overnight incubation, each culture was centrifuged at 3,000 $\times g$ for 10 minutes. The supernatant was discarded and the pellet resuspended in 1 mL fresh, pre-warmed 2 $\times$ YT broth. Then, 50 μ L of each 1 mL culture was mixed with 50 μ L of Novex Tricine SDS sample buffer and then heated to 98°C for five minutes and used for SDS-PAGE immediately.

2.9.2.4 Induction experimental protocol #4

After induction, the cultures were then incubated at 30°C, 200 rpm overnight during induction. After overnight incubation, each culture was centrifuged at 3,000 $\times g$ for 10 minutes. The supernatant was discarded and the pellet resuspended in 1 mL fresh, pre-warmed 2 $\times$ YT broth. Then, 50 μ L of each 1 mL culture was mixed with 50 μ L of Novex Tricine SDS sample buffer and then heated to 98°C for five minutes and used for SDS-PAGE immediately.

2.9.2.5 Induction experimental protocol #5

After induction, the cultures were incubated at 200 rpm for five hours at 30°C, then centrifuged at 3,500 $\times g$ for five minutes. The supernatant was discarded and then the pellet was resuspended in 1 mL BugBuster (70584; Millipore) with 1 $\times$ protease inhibitor (B14001; Stratech Scientific) and 1 μ L benzonase (70746; VWR) added. The suspension was rocked for 30 minutes at room temperature, then 100 μ L was aliquoted into a separate 1.5 mL Eppendorf tube and both suspensions centrifuged at 16,000 $\times g$ for 10 minutes and the supernatant discarded. To the 100 μ L volume pellet, 100 μ L of Novex Tricine SDS sample buffer was added and then the sample was heated to 98°C for five minutes and used for SDS-PAGE immediately.

2.9.3 Visualisation of expression levels

For frozen samples, a heat block was used to thaw them at 50°C for 15 minutes and either 10 μ L was pipetted into the wells of a 16% Novex Tricine gel (EC66955BOX; Life Technologies), or 20 μ L pipetted into a 10-12% NuPAGE Tris-Bis gel (NP0342BOX; Invitrogen). For Coomassie Blue staining, 5 μ L of PageRuler

unstained low range protein ladder (26632; Fisher Scientific) was added and either 1× MES SDS running buffer (K856; VWR International), or 1× Novex Tricine SDS running buffer (12020146; Fisher Scientific) added at 1× concentration into the gel tank reservoir. For western blotting, 5 µL of SeeBlue Plus2 prestained protein standard (LC5925; ThermoFisher) was added instead and Novex Tricine SDS running buffer added at 1× concentration to the gel tank reservoir. A voltage of 125 V was then applied to the gel for either 1.5 hours for 16% Tricine gels, 1.25 hours for 12% Bis-Tris gels, or until the dye reached the bottom of the gel, by an XCell Surelock Mini-Cell (Thermofisher). Coomassie staining used Coomassie Blue R-250 (45% methanol, 45% RO water, 10% acetic acid, 2 g Coomassie R-250) for five minutes after being heated to roughly 60°C as suggested by Kurien and Scofield (2012) and then destained until clear using destaining solution (40% methanol, 10% acetic acid, 50% RO water), unless stated otherwise.

The western blotting procedure used the semidry electroblotting technique as advised by (Schägger 2006). Briefly, a 6.5 × 8 cm PVDF membrane was activated by soaking in methanol for 10 minutes. The gel was then placed onto the membrane, stacked between Chromatography papers, and proteins transferred onto the membrane using transfer buffer (300 mM Tris and 100 mM acetic acid) for 16 hours at 50 mA. After transfer, the membrane was placed into blocking buffer. This was then slowly rocked at room temperature for one hour. The membrane was then washed 6× with PBST (1× PBS, 0.2% Tween-20) for five minutes per wash. Next, blocking buffer containing a 1:2000 dilution of Streptavidin-AP conjugate (11 089 161 001; Sigma-Aldrich) was added to the membrane. The membrane was then incubated for one hour at room temperature, with slow rocking. Then, the membrane was washed 6×, with five

minutes per wash, in PBST and one final wash for five minutes in RO water. Finally, 20 mL of Western Blue (S3841; Promega) was added to the membrane, which was incubated for 2.5 hours, and then viewed using the ChemiDoc MP Imaging System (17001402; BioRad).

2.10 Multiple antigenic peptide synthesis

The target peptide was linked via the α - and ε - amine groups of lysine residues resulting in a dimer and a tetramer (Figure 2-1). The MAPs (Biomatik, USA) had a free carboxyl residue on the initial lysine and free amine groups on each conjugated peptide.

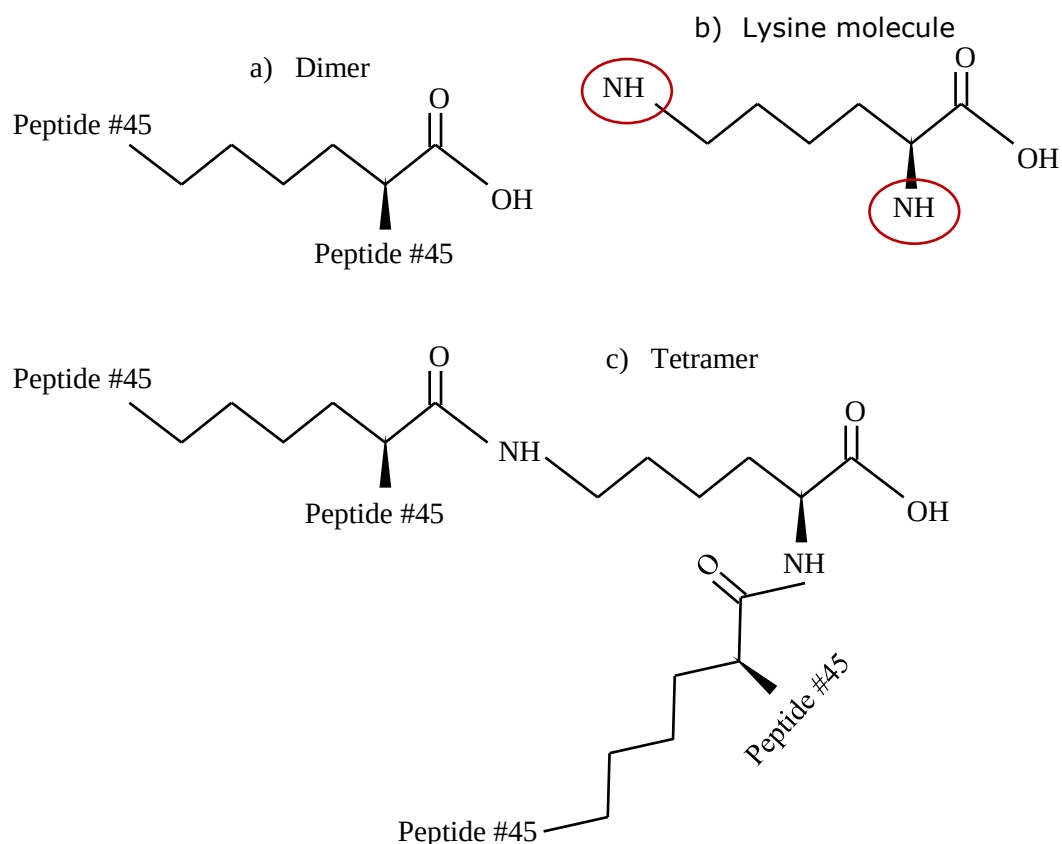


Figure 2-1. MAP design

Displayed here are the three MAP constructs synthesised by Biomatik a) structure of the peptide b) the linking unit is the amino acid lysine, which uses the two amine groups circled to form isopeptide and peptide bonds with either AMPs or other lysines, depending on the final number of peptides to be displayed #45 dimer c) structure of the peptide #45 tetramer.

2.11 Addition of DNA sequences by inverse PCR

To insert AMP sequences, each PCR used the pSD3 phagemid vector as described by Li *et al.* (2010), without a phage coat protein gene, as the template DNA. For the creation of the homo- and hetero-dimers, the template DNA was the pSD3 vector described above but with the relevant monomeric AMP and linker sequence inserted by inverse PCR. Each PCR used an optimised set of reagents (Table 2-3).

Table 2-3 Optimised PCR reagent protocol

Volume (µl)	Reagent	Catalogue number; Source
10	Q5 buffer	M0491S; New England Biolabs
10	GC enhancer	M0491S; New England Biolabs
2.5	Forward primer (9.2)	Sigma-Aldrich
2.5	Reverse primer (9.2)	Sigma-Aldrich
Variable	Template DNA	Li <i>et al.</i> , (2010)
0.5	Q5 polymerase	M0491S; New England Biolabs
1	10mM dNTPs	N0447; New England Biolabs
Up to 50	Molecular biology grade water	W4502; Sigma-Aldrich

The PCR reaction was mixed in a 0.2 mL PCR tubes (CLS6571; Sigma-Aldrich) and placed into a Life ECO thermal cycler (Bioer) to undergo the following protocol:

94°C five minutes

94°C 30 seconds	}	Repeat ×20
55°C 60 seconds		
72°C 330 seconds		

94°C 30 seconds	}	Repeat ×10
58°C 60 seconds		
72°C 330 seconds		

94°C 30 seconds	}	Repeat ×10
60°C 60 seconds		
72°C 330 seconds		

72°C five minutes

Store at 4°C

2.11.1 PCR clean-up

Once the thermal cycler protocol was finished, 5 μL of loading dye (SM1334; Fisher Scientific) was added to each PCR tube. Next, a 1% agarose gel was made using 1 $\times$ TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and 15 μL of sample added to each well alongside Generuler 1 Kb plus ladder (SM1334; Fisher Scientific). A voltage of 100 V was then applied to the gel for one hour, after which the bands were excised and cleaned by a NucleoSpin Gel and PCR clean-up kit according to manufacturer's instructions (740609.5; Fisher Scientific). DNA was ligated at 18°C for 48 hours using 1 μL DNA ligase (D2886; Sigma-Aldrich), 2 μL ligase buffer, and 50 ng DNA from the clean-up kit in a total reaction volume of 20 μL . Next, the ligated DNA was microdialysed against molecular biology grade water using Millipore membranes (VSWp02500; Sigma-Aldrich) for approximately 30 minutes.

2.11.2 Transformation

In separate 1 mm cuvettes (1652089; Bio-Rad), 2 μL of dialysed DNA was electroporated (1.8 kV, 25 μF , 200 Ω ; GenePulser Xcell, Bio-Rad) into 50 μL of electrocompetent JE5505 cells and electroporated. The cells were transferred to 1 mL SOC broth to recover for one hour before spread-plating onto 2 $\times$ YT agar supplemented with 100 $\mu\text{g mL}^{-1}$ Carbenicillin and 1% (v/v) glucose. The 2 $\times$ YT plates were then incubated overnight at 37°C. After incubation, five colonies were picked and used to separately inoculate 5 $\times$ 5 mL 2 $\times$ YT broth supplemented with 100 $\mu\text{g mL}^{-1}$ Carbenicillin and 1% (v/v) glucose, which was then incubated overnight at 37°C at 200 rpm. Following incubation, 2 mL of each bacterial suspension was used to extract plasmid DNA following the manufacturer's instructions (27104; Qiagen). The

extracted DNA was then submitted for Sanger sequencing and the remainder stored in 20% glycerol stocks at -20°C.

2.12 Randomised peptide library creation

2.12.1 Inverse PCR addition of NNK degenerate sequence

Cloning of the NNK degenerate sequences used, as the template DNA, pSD3 phagemid clones with the coat protein removed and the addition of the relevant AMP sequence produced by the reactions described in 2.11. Each PCR used an optimised set of reagents (Table 2-3).

The PCR mixture was mixed in a 0.2 mL PCR tubes (CLS6571; Sigma-Aldrich) and placed into a Life ECO thermal cycler (Bioer) to undergo the following protocol:

94°C five minutes

94°C 30 seconds	}	Repeat ×30
63°C 60 seconds		
72°C 330 seconds		

72°C five minutes

Store at 4°C

2.12.2 PCR Cleanup

After the PCR amplification, 5 µL of loading dye (SM1334; Fisher Scientific) was added to each PCR tube. Next, a 1% agarose gel was made using 1×TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) and 15 µL of sample added to each well

alongside Generuler 1 kb plus ladder (SM1334; Fisher Scientific). A voltage of 100 V was then applied to the gel for one hour, after which the bands were excised and cleaned by a NucleoSpin Gel and PCR clean-up kit according to manufacturer's instructions (740609.5; Fisher Scientific). Then, 1 µg of DNA was digested with 1 µL of both DnpI (R0176; NEB) and SapI (R0569; NEB), in a total volume of 50 µL. A total reaction volume of 200 µL at 18°C for 48 hours was then used to ligate 500 ng DNA using 10 µL DNA ligase (D2886; Sigma-Aldrich) and 20 µL ligase buffer. Next, the ligated DNA was microdialysed against molecular biology grade water using Millipore membranes (VSWp02500; Sigma-Aldrich) for approximately 30 minutes.

2.12.3 Transformation

In separate 1 mm cuvettes (1652089; Bio-Rad), 5 µg dialysed DNA was electroporated (1.8 kV, 25 µF, 200 Ω; GenePulser Xcell; Bio-Rad) into 50 µL of electrocompetent TG1 *E. coli* cells (60502-1; Lucigen). The cells were transferred to 5 mL SOC broth to recover for one hour before spread-plating onto 2× bioassay dishes (CLS431111; Sigma-Aldrich) containing 2×YT agar supplemented with 100 µg mL⁻¹ Carbenicillin and 1% (v/v) glucose and incubated overnight at 37°C. After incubation, 5 mL of 2×YT was then used to detach the bacterial growth from each bioassay dish. Of this 10 mL pooled sample, 1 mL was then used to extract plasmid DNA following the manufacturer's instructions (27104; Qiagen) with the remainder being supplemented with 50% glycerol and frozen at -80°C. DNA was eluted from 4× repeats of plasmid extraction in a total of 200 µL. This protocol was then repeated with 2 µg of the eluted plasmid DNA from the TG1 extraction being used to transform JE5505 *E. coli* cells. Single clones of JE5505 transformed cells were also used to extract plasmid DNA

from, which was then sent for Sanger sequencing (Source Bioscience, UK) to determine if the correct sequences were present.

2.12.4 Next-generation sequencing of peptide libraries

To facilitate next-generation sequencing, plasmid DNA from clones produced in 2.12.1 was amplified to add linker sequences and barcodes. Both primers and barcode sequences can be found in Appendix 9.2. An optimised set of reagents was used for the PCR cycles with the modification of 1 μ l each of primers (Table 2-3).

To add the linker onto the relevant AMP sequence, the PCR reagents were mixed in 0.2 mL PCR tubes (CLS6571; Sigma-Aldrich) and placed into a Life ECO thermal cycler (Bioer) to undergo the following protocol:

95°C five minutes

95°C 30 seconds	} ×15 cycles
60°C 30 seconds	
68°C 30 seconds	
68°C five minutes	

Store at 4°C

After the PCR cycles were completed, 10 μ L of each reaction was mixed with 2 μ L of loading dye (SM1334; Fisher Scientific), and pipetted into the well of a 3% agarose gel to confirm amplification. The 3% agarose gel was electrophoresed at 100 V for 60 minutes before visualisation with the ChemiDoc MP Imaging System (17001402; BioRad). The remaining 40 μ L of each reaction was cleaned using NucleoSpin Gel

and PCR clean-up kit according to manufacturer's instructions (740609.5; Fisher Scientific). To remove contaminating primer-dimers, the NTI buffer was diluted to a ratio of 1:5 with sterile RO water. Elution of the DNA from the column used 20 μ L of buffer NE.

Each reaction was barcoded with a second round of PCR, using the same conditions and volumes of reagents as round one with two exceptions: 1 μ L of the reaction volume was used as template DNA and only 10 cycles were used. The reaction was then analysed as after round one, with 10 μ L of each reaction mixed with 2 μ L loading dye (SM1334; Fisher Scientific) and electrophoresed on a 3% agarose gel to confirm size increase. Then, 200 ng of each barcoded reaction was pooled into a single 1.5 mL Eppendorf in a total volume of 200 μ L. The pooled reactions were then mixed with 40 μ L of loading dye (SM1334; Fisher Scientific) and electrophoresed on a 3% agarose gel at 100 V for 70 minutes. The resultant DNA bands were then excised and cleaned up using NucleoSpin Gel and PCR clean-up kit according to manufacturer's instructions (740609.5; Fisher Scientific). The DNA was then eluted in a total of 50 μ L NE buffer. The elution was then mixed with 90 μ L of AMPure XP beads (A63880; Beckman Coulter), and purified following manufacturer's instructions. The DNA was subsequently eluted in 30 μ L of NE buffer. A total of 200 ng of the eluted DNA was then sent to the University of Pennsylvania for NGS using the IonTorrent system.

2.12.5 IonTorrent data analysis

NGS data were interrogated with custom Perl scripts as described elsewhere (Naqid *et al.* 2016). Custom Perl scripts were kindly provided by Dr. Owen (ADAS, Nottingham) Briefly, FASTQ files were converted to FASTA files and the barcode

used in each library identified by use of the “FASTQ/A Barcode splitter” (part of the FASTX-toolkit from http://hannonlab.cshl.edu/fastx_toolkit/index.html). Further quality control analyses on the sequences were not performed, with the presence of each barcode and both sequence motifs used to ensure sequence quality. The DNA sequences in each of the barcode-binned FASTA files were translated in all three frames and concatenated to a single file (translate.pl). FASTA files were then processed to identify matching flanking sequence motifs (AQPAMA and KESGSV) to capture insert (peptide) sequence. For an upper limit of diversity estimation, DNA sequences were also analysed across all three frames for a single insertion/deletion, the subsequent frameshift, and the presence of the correct nucleotides for both flanking motifs and a 16-mer insert. The parameters used as cut-offs for analysis of the NNK-randomised library data were as follows: a maximum of two amino acid mismatches were allowed per barcode and a minimum of one between the flanking motifs. Translated files were then interrogated for the total number and positioning of amino acid residues within each sequence.

2.13 Overlay assays

The overlay assay protocol was a modified version of that described by Guralp *et al.* (2013). Briefly, 10 mL of MHB supplemented with 100 $\mu\text{g mL}^{-1}$ Carbenicillin was inoculated with two or three colonies of recombinant JE5505 *E. coli* grown overnight on MHA at 37°C. This culture was then incubated overnight at 200 rpm at 37°C. After incubation, 10 mL of MHB, supplemented with 100 $\mu\text{g mL}^{-1}$ Carbenicillin, was inoculated with 100 μL of the overnight culture and further incubated at 37°C at 200 rpm. Once the culture reached mid exponential phase as determined from OD_{600} , ~0.6, it was diluted to approximately 170 CFU mL^{-1} , and 330 μL was spread-plated on

sMHA in 150 mm petri dishes. These plates were then dried for 30 minutes before overlaying with a further 10 mL sMHA and incubating at 37°C overnight. Plates were then overlaid with 10 mL of sMHA inoculated with 1 mL of the test bacterial species, at a MacFarland standard of 0.5, and 1 mM IPTG (46-102-RF; Scientific Laboratory Supplies). The plates were incubated overnight at 30°C. Following incubation, the petri dishes were then photographed using the ChemiDoc MP Imaging System (17001402; BioRad).

2.14 Inhibition of mixed bacterial cultures

Two different bacteria were streaked onto individual MHA plates and incubated overnight at 37°C. Then, three colonies from each plate were used to inoculate separate 10 mL of pre-warmed MHB and were incubated at 37°C at 200 rpm. Then, the MIC values of each test peptide against both test bacteria were compared. The test peptide was then diluted to 4×, 1×, and $\frac{1}{4}$ the lowest MIC value out of the pair of bacteria in aMHB. Then 50 µL of each peptide dilution was added to three wells of a 96-well polypropylene plate (CLS3879-50EA, Sigma Aldrich). When each of the 10 mL of bacterial cultures reached approximately 1×10^8 CFU mL⁻¹, as determined by an experimentally determined OD₆₀₀, the cultures were diluted 1:100 in pre-warmed MHB. Of the diluted cultures, 25 µL was mixed with the peptide dilutions in the 96-well plate to reach a final concentration of 5×10^5 CFU mL⁻¹ of bacteria and 128 mg L⁻¹ of peptide. An MIC assay was also performed alongside. Controls comprised 100 µL of MHB added to a row of wells to determine media sterility, and 50 µL bacteria at 1×10^6 CFU mL⁻¹ added to aMHB in another row of wells to determine the value for 100% growth. From the growth control, a two-step dilution to reach 1:1000 was performed in bactericidal test buffer and 100 µL was then spread-plated onto MHA,

in duplicate. The growth control MHA plates were then incubated alongside the 96-well plates for the standardised time and temperature (Appendix 9.1). After incubation, 65 μL was then aspirated from the wells and transferred to a flat-bottomed 96-well plate (442404, SLS) for visualisation in a spectrophotometer (VL0000D0; Fisher Scientific). The mean OD_{600} value of the sterility wells was then subtracted from all wells to account for media influence, and the mean OD_{600} value of the growth wells was divided by two to obtain the IC_{50} .

2.14.1 Mixed culture assay validation

Bacterial strains were grown using the same method as in 2.14, with the absence of any AMP, and in both monoculture and mixed culture. To selectively quantify *Salmonella* isolates, MHA supplemented with 30 $\mu\text{g mL}^{-1}$ Nalidixic acid was used. To quantify *E. coli* O78, MHA supplemented with 100 $\mu\text{g mL}^{-1}$ Carbenicillin was used. Viable counts of *E. coli* O2 were obtained by subtracting *Salmonella* counts (taken from Nalidixic acid plates) from total viable counts on MHA. Samples of 2×10^6 μL were taken from each mixed culture and monoculture at the point of dilution and after overnight incubation at 37°C, 200 rpm. Samples were then serially diluted by 100-fold and 100 μL of each sample spread-plated onto the appropriate agar until the number of colonies present were countable. Sample plates were incubated overnight at 37°C. Viable counts were tested for statistical significance by performing an unpaired t-test.

2.15 Viability-PCR differentiation of AMP-treated cultures

2.15.1 PCR amplification of APEC-associated genes

A loop of stock *E. coli* O78 was streaked onto MHA and incubated overnight at 37°C. From this culture, two to three colonies were used to inoculate 10 mL of MHB which was then incubated at 37°C at 200 rpm overnight. This culture was then centrifuged at 6,800 $\times g$ for three minutes and then the plasmid DNA extracted according to the manufacturer's instructions (27104; Qiagen). The DNA was eluted in 50 μ L and was assessed for concentration using a Qubit 3.0 Fluorometer (Invitrogen). Reaction volumes were mixed in a 0.2 mL PCR tubes (CLS6571; Sigma-Aldrich) and for each PCR was as follows:

10 μ L Q5 buffer (M0491S; New England Biolabs)

1 μ L 10 mM dNTPs (N0447; New England Biolabs)

2.5 μ L Forward primer (Appendix 9.2; Sigma-Aldrich)

2.5 μ L Reverse primer (Appendix 9.2; Sigma-Aldrich)

1 μ L DNA (10 ng μ L⁻¹)

0.5 μ L Q5 DNA polymerase (M0491S; New England Biolabs)

Up to 50 μ L Molecular grade water (10787944; Fisher Scientific)

The PCR protocol was performed in a Life ECO thermal cycler (Bioer) as follows:

94°C five minutes

94°C	five seconds	}	30× cycles
60°C	20 seconds		
72°C	30 seconds		
72°C one minute			

Store at 4°C

From the reaction volume, 25 µL was mixed with 5 µL loading dye (27104; Qiagen) and electrophoresed on a 3% agarose gel for one hour at 100 W. Then, the gel was imaged using the ChemiDoc MP Imaging System (17001402; BioRad).

2.15.2 Plasmid DNA preparation for qPCR amplification

2.15.2.1 Limit of detection

A loop of stock *E. coli* O78 was streaked onto MHA and incubated overnight at 37°C. From this culture, two to three colonies were used to inoculate 10 mL of MHB which was then incubated at 37°C at 200 rpm until an OD₆₀₀ of 1 was reached. Then, the culture was diluted to concentrations ranging from 10⁵ to 10 CFU mL⁻¹. For each concentration, there were three replicates consisting of 1 mL each. The diluted cultures were then incubated for one hour at 37°C at 200 rpm. After incubation, the cultures were centrifuged at 6,800 ×g for three minutes and the supernatant discarded. Plasmid DNA was then extracted according to the manufacturer's instructions (27104; Qiagen) and eluted in 30 µL.

2.15.2.2 Monoculture assays

A loop of stock culture of *E. coli* O78 was streaked onto MHA and incubated overnight at 37°C. From this overnight culture, four to five colonies were selected and used to inoculate 10 mL of MHB. The culture was then incubated at 37°C at 200 rpm until an OD₆₀₀ of 1 was reached, then it was either diluted 1:100 with pre-warmed MHB to reach $\sim 10^6$ CFU mL⁻¹ or left undiluted for $\sim 10^8$ CFU mL⁻¹. From this culture, 1 mL was pipetted into separate 1.5 mL Eppendorfs. The 1 mL cultures were then split into treatment groups which comprised two sets of triplicates in which AMPs were added at 4× MIC. Controls comprised DMSO at an equivalent volume to the AMP used, and heat-treatment at 95°C for five minutes. After treatment, the cultures were incubated for one hour at 37°C, 200 rpm. After incubation, cultures were treated with PMAxx (2.15.2.4).

2.15.2.3 Bacterial spike assays

A loop of stock culture, both *E. coli* O78 and *S. Enteritidis*, was streaked onto MHA and incubated overnight at 37°C. From these overnight cultures, four to five colonies were selected and used to inoculate 20 mL of MHB. The cultures were then incubated at 37°C at 200 rpm until an OD₆₀₀ of 1 was reached, then the “spike” bacteria was either diluted 1:100 with pre-warmed MHB to reach $\sim 10^6$ CFU mL⁻¹ or left undiluted for $\sim 10^8$ CFU mL⁻¹. The “background” bacteria was also left at OD 1, for $\sim 10^8$ CFU mL⁻¹. For 10^4 CFU mL⁻¹ of “spike” bacteria in the final assay, 100 µL of diluted culture was pipetted into 9.9 mL of “background” bacteria. For 10^6 CFU mL⁻¹ of “spike” bacteria, 100 µL of undiluted culture was pipetted into 9.9 mL “background” bacteria. From this mixed culture, 500 µL was pipetted into separate 1.5 mL Eppendorfs. The

500 µL cultures were then split into treatment groups which comprised two sets of triplicates in which AMPs were added at 4× MIC. Controls consisted of DMSO at an equivalent volume to the AMP used, and heat-treatment at 95°C for five minutes. After treatment, the cultures were incubated for one hour at 37°C, 200 rpm. After incubation, cultures were treated with PMAxx (2.15.2.4).

2.15.2.4 PMAxx treatment

Each treatment group had two sets of triplicates, within one set 1.6 µL of PMAxx dye (40069, Cambridge Bioscience) and 250 µL of PMA enhancer (31038, Cambridge Bioscience) was added in the dark. No treatment was added to the second set. Cultures were then wrapped in foil and set on a rocker for 10 minutes. Next, cultures were exposed to blue light for 15 minutes using a DarkReader Transilluminator (DR88X, LabTech) for photoactivation of the PMAxx dye. After this, the cultures were centrifuged at 6,800 ×*g* for three minutes and the supernatant discarded. The pellet was then frozen at -20°C until use when plasmid DNA was extracted according to the manufacturer's instructions (27104; Qiagen) and eluted in 30 µL.

2.15.3 qPCR amplification of plasmid DNA

Reaction volumes for v-PCR were as follows:

25 µL KiCqStart SYBR Green qPCR ReadyMix (KSQS00; Merck Life Science)

Forward primer 6 µM (Appendix 9.2; Sigma-Aldrich)

Reverse primer 8 µM (Appendix 9.2; Sigma-Aldrich)

20 μ L DNA

Amplification was performed in 96-well plates (LW2216; Alpha Laboratories) using a CFX96 Connect Real-Time PCR system (Bio-Rad) with the following protocol:

95°C three minutes

95°C	20 seconds	}	40× cycles
60°C	30 seconds		

2.16 Viable counting of bacterial spike assays

A loop of stock culture, both *E. coli* O78 and *S. Enteritidis*, was streaked onto MHA and incubated overnight at 37°C. From these overnight cultures, four to five colonies were selected and used to inoculate 20 mL of MHB. The cultures were then incubated at 37°C at 200 rpm until an OD₆₀₀ of 1 was reached, then the “spike” bacteria was either diluted 1:100 with pre-warmed MHB to reach $\sim 10^6$ CFU mL⁻¹ or left undiluted for $\sim 10^8$ CFU mL⁻¹. The “background” bacteria was also left at OD 1, for $\sim 10^8$ CFU mL⁻¹. For 10^4 CFU mL⁻¹ of “spike” bacteria in the final assay, 100 μ L of diluted culture was pipetted into 9.9 mL of “background” bacteria. For 10^6 CFU mL⁻¹ of “spike” bacteria, 100 μ L of undiluted culture was pipetted into 9.9 mL “background” bacteria. From this mixed culture, 500 μ L was pipetted into separate 1.5 mL Eppendorfs. The 500 μ L cultures were then split into treatment groups containing five replicates each and AMPs were added at 4× MIC. Controls consisted of DMSO at an equivalent volume to the AMP used, and where a positive control was needed Streptomycin sulfate (15424871; Fisher Scientific) was used at 50 μ g mL⁻¹.

Cultures were then incubated for one hour, unless otherwise stated, at 37°C at 200 rpm. After incubation, 10 μ L from each replicate in each treatment group was diluted

in MHB to obtain an appropriate dilution factor for viable counts. From each replicate, 100 μL of the diluted sample was spread-plated onto two plates of MHA so a total of 10 counts could be registered to each treatment. After spread-plating, the dilutions were incubated at 37°C overnight and then the number of colonies counted and CFU mL^{-1} calculated.

2.17 JE5505 growth curve

A loop of stock culture for each JE5505 clone was streaked onto 2×YT agar supplemented with 100 $\mu\text{g mL}^{-1}$ Carbenicillin and 1% glucose and then incubated overnight at 37°C. From these overnight cultures, two colonies were selected and used to inoculate 2×10 mL of 2×YT for overnight incubation at 37°C at 180 rpm. After incubation, the cultures were centrifuged at 3,000 $\times g$ for five minutes and then re-suspended in 10 mL pre-warmed 2×YT supplemented with 100 $\mu\text{g mL}^{-1}$ Carbenicillin. The OD₆₀₀ reading of each culture was taken and used to calculate the dilution to reach an OD₆₀₀ of 0.02. Each culture was diluted in pre-warmed 2×YT supplemented with 100 $\mu\text{g mL}^{-1}$ Carbenicillin and verified by spectrophotometer. Then, 8 μL of 25% glucose (v/v) and 8 μL of 25 mM IPTG (46-102-RF; SLS) were added to three wells each per culture of a 96-well plate before adding 192 μL of each culture into the total 6× wells. The plate was then transferred into a TECAN Genios Pro 96 Microplate reader for shaking incubation at 37°C and absorbance readings at 595 nm every 30 minutes for 20 hours.

3 Characterisation and comparison of AMPs derived from phage display with known AMPs

3.1 Introduction

Globally, antibiotics have been used not just to treat infection, but due to their growth-promoting or prophylactic effects as well (Cully 2014). Antibiotic treatment in food animals is thought to be driving resistance gene development and spread, potentially leading to complications in human clinical therapeutics (Cohen and Tauxe 1986; Garcia-Migura *et al.* 2014). This is especially concerning in poultry farming as therapeutic treatments are often given to the entire flock via feed or water due to the impractical nature of individual application. Therefore, it is imperative that alternatives to antibiotics are found, as resistance genes to even last-resort drugs such as colistin are now widely disseminated (Gelbířová *et al.* 2019). One alternative could be AMPs. These small, amphipathic, cationic peptides could replace or supplement antibiotics within agriculture. This could then help preserve antibiotics for human clinical interventions. Most novel AMPs are the result of interrogating genetic data from AMP producing-organisms, but there are also novel AMPs which are the result of improved (engineered) natural AMPs. Alternatively, phage display is becoming a prominent method for the detection of new small AMPs due to its ability to screen vast libraries without the need for combinatorial chemistry. Here, it was attempted to identify, characterise and compare novel AMPs with known AMPs found in the literature. Of note, one phage display-derived peptide (#45) showed greater activity than other AMPs identified by similar techniques and provides an ideal starting sequence for modifications. While an AMP identified from the literature proved to be the most efficacious, other putative AMPs from literature failed to display any activity.

3.2 Introduction - origin of AMPs used in this study

For one set of peptides, termed “phage-derived”, a peptide library was created in-house using inverse PCR (Personal communication with Dr. A. Gray, University of Nottingham). Briefly, degenerate primers were designed to add a randomised 16-mer sequence to the pIII coat protein of Ff-phage encoded within the pSD3 vector (Li *et al.* 2010). Next, the library was transformed into TG1 *E. coli* and was estimated to have a diversity of $\sim 1 \times 10^9$. The displayed peptides were panned separately against *E. coli* O2 and *S. Typhimurium*, with a subtraction step against *Lactobacillus acidophilus* (Personal communication with Ms. C. Baker; ADAS, Nottingham). In the final round of panning, the phage sublibrary was bound to both target bacteria and against the *L. acidophilus* control in multiple replicates. The output phage DNA was sent for NGS using the IonTorrent platform (at University of Pennsylvania). The resulting data were analysed and peptides were identified that were highly enriched specifically against the target bacteria, and not the control bacteria replicates. A further set of peptides, which were enriched to a lower degree, were also identified by analysing their sequence information to identify peptides that conformed to online AMP algorithms. Across all panned bacteria, a total of 60 peptides were synthesised based on these criteria (Personal communication with Dr. B. Maddison and Dr. J. Owen; ADAS, Nottingham). Peptides 1-29 were identified from *E. coli* O2 serotype panning, while peptide 30-60 were identified from *S. Typhimurium* panning. For detailed instructions on this pipeline, please refer to Appendix 0. Three of the four remaining peptides screened in this study were identified from the DBAASP. This database holds a manually-curated resource of known AMPs alongside their reported activities. The database was searched for AMPs which represented a range of reported MIC values below 1 μ M. A total of three known AMPs were chosen from DBAASP: C-BF, E-50,

and Plectasin (Liu *et al.* 2015; Mygind *et al.* 2005; Svetoch *et al.* 2008). Another AMP, Plantaricin-423, was identified through the publication of Guralp *et al.* (2013) and investigated due to its use in a successful high-throughput screening method.

3.3 Characterisation of established AMPs described within the scientific literature

3.3.1 MIC microbroth dilution assays

The synthesised peptides were screened in microbroth dilution assays against a panel of bacteria to determine antimicrobial efficacy and specificity. To understand how the phage-derived peptides may compare with AMPs previously reported in the scientific literature, a range of established AMPs were characterised. To test antimicrobial efficacy, two serotypes of *E. coli* (O78 and O2) and two serovars of *Salmonella* (Typhimurium and Enteritidis) were used as two Gram-negative pathogens of note, for both poultry and human health. These were either isolates obtained from clinical cases of infection (*E. coli* O78 and O2) or widely used isolates within the literature (*Salmonella*). There is also widespread evidence of multidrug resistance within *E. coli* and *Salmonella* populations when poultry farms are sampled, highlighting the importance of finding new therapeutics for these two pathogens (Assoumy *et al.* 2021; Hassan 2014; Kakooza *et al.* 2021). There were two Gram-positive, non-pathogenic bacteria included in the panel: *B. subtilis* and *L. innocua*. These two bacteria were used as markers for testing the specificity of the established AMPs. All bacterial isolates were characterised by colony morphology, plating on selective agar (where possible), and Gram-staining. Microbroth dilution assays were performed as recommended by CLSI (2015) and Wiegand, Hilpert and Hancock (2008), with bacterium-specific

requirements described in Appendix 9.1. During initial screening assays, peptides were tested alongside two positive control peptides and a negative control peptide SHA (sequence – AEGEFYEDRYYYRENDPAKAA), which was known in our lab to have no antimicrobial activity, and two positive control peptides. When testing peptides against Gram-negative bacteria, positive control peptide EC5 was used (sequence – RLLFRKIRRLKR) as the testing methodology within its originating paper was similar (Sainath Rao *et al.* 2013). When testing peptides against Gram-positive bacteria, positive control peptide BS was used as it was purported to be antimicrobial against *B. subtilis* in buffer, and therefore useful in further assays (sequence – KPQQHNRPLRHK; Tanaka, Kokuryu and Matsunaga, 2008). Peptide EC5 showed activity against both serotypes of *E. coli* and both serovars of *Salmonella* at 128 mg L⁻¹ but BS showed no activity in aMHB when tested alongside putative phage-derived AMPs (data not shown). After initial testing indicated its activity, phage-derived peptide #45 was used as a positive control. In addition to the control peptides, a carrier solvent control was also included. As peptides were reconstituted in 100% DMSO, due to their hydrophobicity, DMSO was added to wells without peptide to ensure bacterial growth ensued. Once all bacteria had been screened with the appropriate controls, only growth and sterility controls were used in subsequent assays. Peptides that were identified as inhibitory by a 50% decrease in OD₆₀₀ measurement compared to growth controls were then subjected to a two-fold dilution assay to determine their MIC against the panel of bacteria. Starting concentrations were 128 mg L⁻¹, which is equivalent to between ~30 and ~35 µM (dependent on peptide), and were diluted step-wise to 4 mg L⁻¹. An exception to this methodology occurred when testing peptides against *L. innocua*: after incubation the bacteria could

not be re-suspended for OD measurement and so the MIC of an AMP was defined as the lowest concentration with no visible growth.

Of the DBAASP-derived peptides, C-BF was determined to have potent inhibitory activity. C-BF was active against all of the bacteria tested, with a relatively high MIC against *L. innocua* at 64 mg L⁻¹, when compared against the other bacteria tested which produced MIC values at either 1 mg L⁻¹ or 0.5 mg L⁻¹ (Figure 3-1, Figure 3-2). E50-52, Plectasin and Plantaricin-423 were determined to have no AMP activity (Table 3-1).

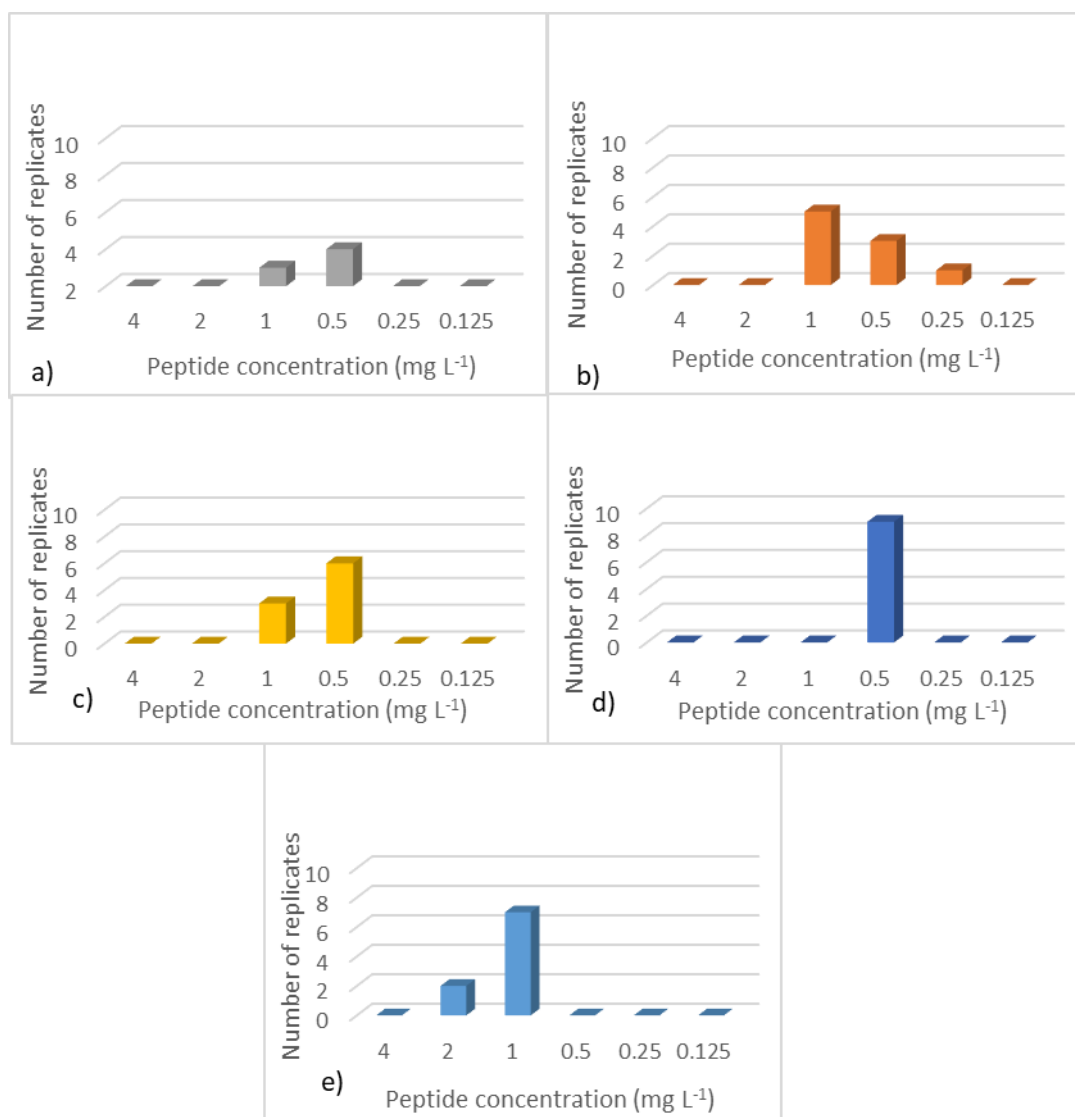


Figure 3-1. Inhibitory activity of peptide C-BF against a panel of bacteria.

Three MIC assays, independent of each other, were carried out on a panel of bacteria which consisted of *E. coli* serotypes O78 (a) and O2 (b), *Salmonella enterica* serovars Enteritidis (c) and Typhimurium (d), and *B. subtilis* (e). For each MIC assay, a two-fold serial dilution of peptide C-BF was performed from a starting concentration of 128 mg L⁻¹ down to 4 mg L⁻¹. Each dilution was performed in triplicate assays. After inoculation with the target bacteria, the 96-well plate was incubated overnight when the OD₆₀₀ readings of each well containing bacteria and peptide were calculated as a percentage of the uninhibited growth control (bacteria only). The lowest concentration of each replicate from all three MIC assays (nine data points in total) which caused >50% inhibition of growth was then documented and plotted with the modal value used as the MIC for that peptide-bacteria combination.

Table 3-1. Full list of MIC data from the synthetically synthesised peptide monomers.

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S.</i> Typhimurium	<i>S.</i> Enteritidis	<i>L. innocua</i>	<i>B.</i> <i>subtilis</i>
E50-52	>128 ¹	>128	>128	>128	>128	>128
Plantaricin-423	>128	>128	>128	>128	>128	>128
Plectasin	>128	>128	>128	>128	>128	>128
C-BF	1 mg L ⁻¹ 0.3 µM ²	0.5 mg L ⁻¹ 0.14 µM	0.5 mg L ⁻¹ 0.14 µM	0.5 mg L ⁻¹ 0.3 µM	64 mg L ⁻¹ 17.6 µM	1 mg L ⁻¹ 0.3 µM

¹ All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM

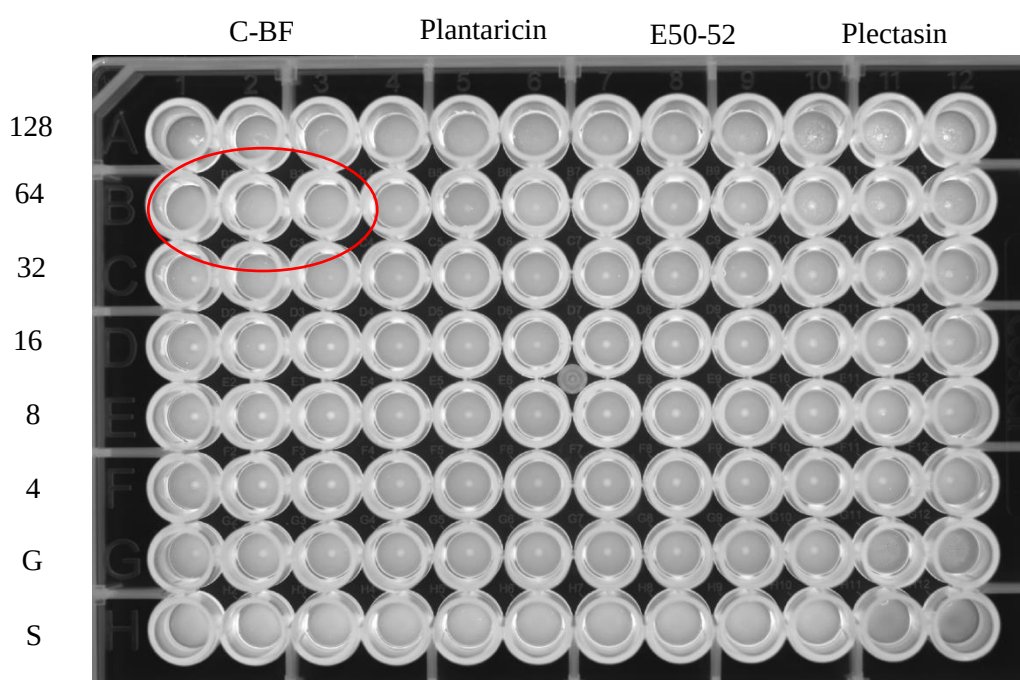


Figure 3-2. Representative example of a MIC activity assay against *L. innocua*.

The four established AMPs were tested in an MIC assay against *L. innocua* with concentrations starting at 128 mg L⁻¹ and ending at 4 mg L⁻¹, as indicated. Rows G and H, entitled “G” and “S”, were used as a growth and sterility control, respectively. As *L. innocua* could not be re-suspended after incubation, a positive result was defined as a lack of visible growth in a well after overnight incubation and the MIC is the lowest concentration a positive result is seen in. This is a photograph of the MIC assay plate, with C-BF circled at its MIC, 64 mg L⁻¹.

3.3.2 MIC agar dilution assays

To determine if peptide inhibitory activity is consistent when using different test media, peptides were also tested in sMHA. Using a modified protocol based on Guralp *et al.* (2013), a lawn of bacteria in sMHA was treated with 5 μ L of each test peptide. Peptides were spotted onto the agar at concentrations ranging from 4 to 512 mg L⁻¹, for DBAASP-derived peptides this is equivalent to approximately ~1 to 120-140 μ M. Higher concentrations were used in the Agar MIC assays as, instead of being directly mixed with the test bacteria, the peptide needed to diffuse through the agar to cause a clear zone of inhibition. Peptide MICs were the lowest concentration which produced a clear zone of inhibition as determined by two independent observers, including the author.

Table 3-2. Agar MIC values of established AMPs.

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S.</i> Typhimurium	<i>S.</i> Enteritidis	<i>L.</i> <i>innocua</i>	<i>B.</i> <i>subtilis</i>
E50-52	>512 ¹	>512	>512	>512	>512	>128
Plantaricin-423	>512	>512	>512	>512	16 mg L ⁻¹ 4.1 μ M	>128
Plectasin	>512	>512	>512	>512	>512	>128
C-BF	4 mg L ⁻¹ 1.1 μ M ²	8 mg L ⁻¹ 2.2 μ M	32 mg L ⁻¹ 8.8 μ M	32 mg L ⁻¹ 8.8 μ M	>512	32 mg L ⁻¹ 8.8 μ M

¹ All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in μ M.

C-BF was active against all bacteria tested, except for *L. innocua*, with a range of values from 4 mg L⁻¹ to 32 mg L⁻¹ (Table 3-2). Plantaricin-423 was also active in this assay, against *L. innocua* down to 16 mg L⁻¹ (Figure 3-3).

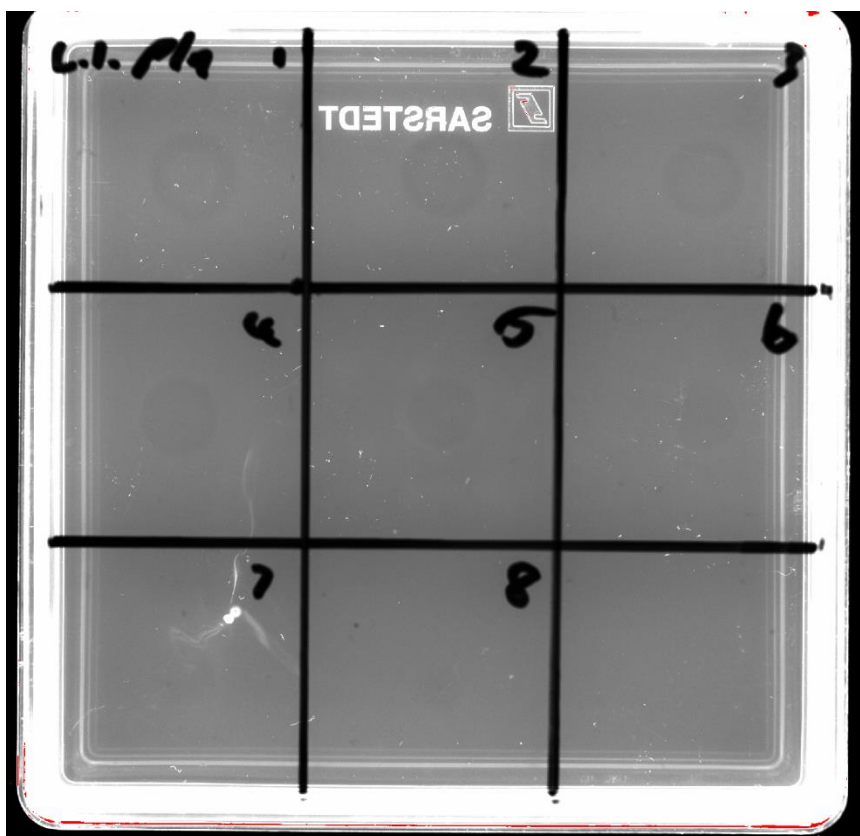


Figure 3-3. Representative example of the agar MIC assay.

Peptide Plantaricin-423 was serially diluted from 512 mg L⁻¹ to 4 mg L⁻¹ and 5 µL of each dilution spotted onto an agar plate inoculated with *L. innocua*. The agar plate was split into nine sections, with eight of those numbered 1-8. Section 1 was spotted with 5 µL Plantaricin-423 at 512 mg L⁻¹ and the serial dilutions followed numerical order, with 256 mg L⁻¹ spotted on section 2. After overnight incubation, the agar plate was photographed and the image analysed by two independent observers to determine that 16 mg L⁻¹ (section 6) was the lowest concentration to produce a clear zone of inhibition.

3.3.3 MBC assays

Next, the DBAASP peptides were screened in microbroth dilution assays against the Gram-negative pathogens for bactericidal activity in an alternative assay. When determining the suitability of the Gram-positive bacteria screened in both inhibitory assays for use in this assay, it was determined their survival was too poor (data not shown). By using the method as outlined in Tucker *et al.* (2018), it is possible to identify peptides that are not active in broth but might possess antimicrobial potential.

Briefly, peptides were diluted in PDS and screened at 128 mg L⁻¹, as detailed previously for DBAASP-derived peptides this is equivalent to between ~30 and ~35 μM. Assay conditions were the same as the inhibitory assay for broth microdilution, except the bacterial inoculum was suspended in bactericidal test buffer and all assays were incubated for 24 h. Bactericidal activity was determined by the peptide reducing the viable count by 3-log₁₀. Peptides identified by the bactericidal screen were then investigated further to determine a MBC value, using 2-fold dilutions starting at 128 mg L⁻¹ and ending at 4 mg L⁻¹. Only one peptide, C-BF, was shown to be effective in the assay. However, due to variable results, the MBC values for C-BF were interpreted in two different ways. MBC values which were consistent (within 8-fold of each other) across technical replicates were evaluated using the mode. MBC values outside of this threshold were evaluated using the median (Table 3-3). C-BF only exhibited consistent antimicrobial activity against *E. coli* O2 with an MBC of 32 mg L⁻¹ (Figure 3-4).

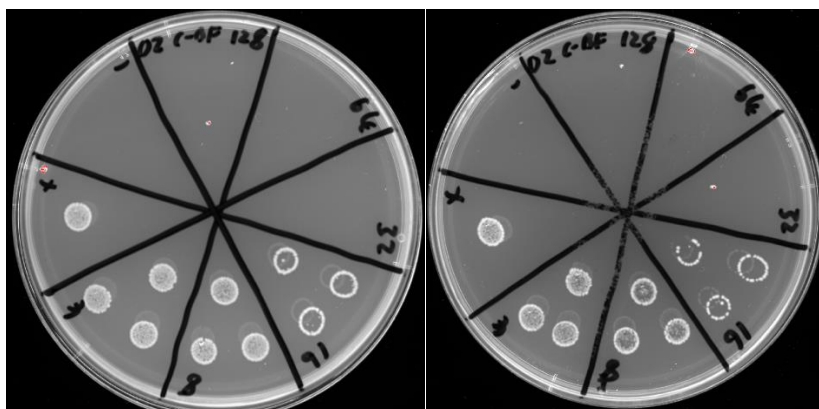


Figure 3-4. Representative images of an MBC assay determining the bactericidal activity of peptide C-BF against *E. coli* O2.

For determination of the MBC value, a Petri dish was split into eight sections, six sections from “128” to “4” and a “+” and “-” section. The 128 section contains 3× 5 µL spots from a well containing 128 mg L⁻¹ of peptide C-BF and *E. coli* O2 and this is repeated to 4 mg L⁻¹ of C-BF. The “+” section contains a 5 µL spot of *E. coli* O2 without peptide, and the “-” section contains a 5 µL spot of PDS only. These two plates indicate a consensus MBC value of 32 mg L⁻¹, as the 16 mg L⁻¹ section displays too many colonies to meet a 3-log₁₀ reduction criteria, as determined by viable count of *E. coli* O2 pre-incubation. Further MBC images for remaining AMPs can be found in Appendix 9.7.

Table 3-3. MBC values for each of the monomeric peptides synthesised.

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S. Typhimurium</i>	<i>S. Enteritidis</i>
E50-52	>128 ¹	>128	>128	>128
Plantaricin-423	>128	>128	>128	>128
Plectasin	>128	>128	>128	>128
C-BF	32 mg L ⁻¹ 8.8 µM ³	8 mg L ⁻¹ * 2.2 µM	8 mg L ⁻¹ * 2.2 µM	>128 mg L ⁻¹

¹ All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM; *MBC value determined by median

3.4 Putative AMPs identified through phage-peptide panning

3.4.1 MIC assay

With C-BF verified as a potent, broad-spectrum AMP, the 60 peptides identified through phage display as putative AMPs were then tested within the broth MIC and

MBC assays. The Gram-positive organisms *B. subtilis* and *L. innocua* were included in the screening panel in the MIC assay for the purpose of testing the specificity of the peptides, and therefore the success of the subtraction round within the panning process. Peptides were tested at concentrations ranging from 4 to 128 mg L⁻¹. The phage-derived peptides #27, #45 and #59 were determined to have inhibitory activity in aMHB (Table 3-4).

Table 3-4. MIC values for phage-derived peptides

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S.</i> Typhimurium	<i>S.</i> Enteritidis	<i>L.</i> <i>innocua</i>	<i>B.</i> <i>subtilis</i>
#27	>128 ¹	>128	>128	>128	>128	32 mg L ⁻¹ 16.5 µM
#35	>128	>128	>128	>128	>128	>128
#36	>128	>128	>128	>128	>128	>128
#45	16 mg L ⁻¹ 8.5 µM ²	16 mg L ⁻¹ 8.5 µM	64 mg L ⁻¹ 33.8 µM	128 mg L ⁻¹ 67.7 µM	>128	8 mg L ⁻¹ 4.2 µM
#59	>128	64 mg L ⁻¹ 34.5 µM	>128	>128	>128	16 mg L ⁻¹ 8.6 µM

¹ All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM.

Peptide #27 was the most specific peptide, inhibitory only against *B. subtilis*, with an MIC of 32 mg L⁻¹ (Figure 3-5). Peptide #59 was slightly more broad-spectrum, effective against *E. coli* O78 (64 mg L⁻¹) as well *B. subtilis* (16 mg L⁻¹; Figure 3-6). Peptide #45 was the most broad-acting, with antimicrobial activity against all Gram-negatives tested (Figure 3-7).

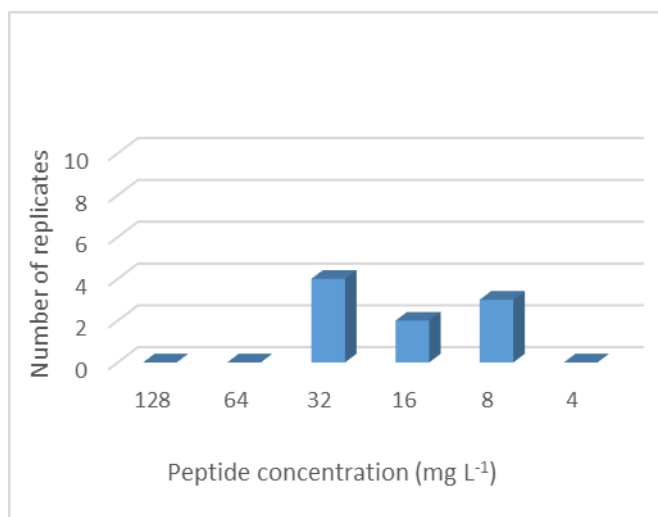


Figure 3-5. Inhibitory activity of peptide #27 against *B. subtilis*.

Three MIC assays, independent of each other, were carried out on *B. subtilis*. For each MIC assay, a two-fold serial dilution of peptide #27 was performed from a starting concentration of 128 mg L⁻¹ down to 4 mg L⁻¹. Each dilution was performed in triplicate. After inoculation with the target bacteria, the 96-well plate was incubated overnight and the OD₆₀₀ readings of each well containing bacteria and peptide were calculated as a percentage of the uninhibited growth control (bacteria only). The lowest concentration of each replicate from all three MIC assays (nine data points in total) which caused >50% inhibition of growth was then documented and plotted with the modal value used as the MIC for that peptide-bacteria combination.

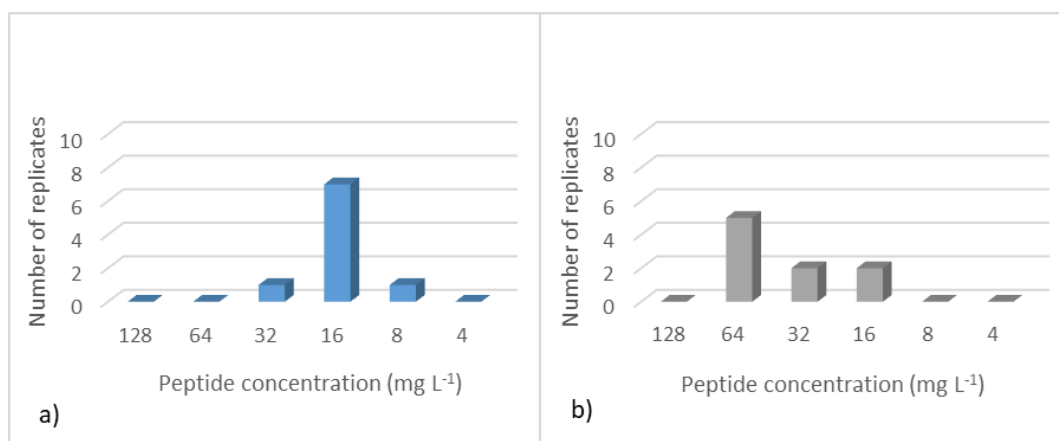


Figure 3-6. Inhibitory activity of peptide #59 against *B. subtilis* (a) and *E. coli* O78 (b).

Three MIC assays, independent of each other, were carried out on *B. subtilis* (a) and *E. coli* O78 (b). For each MIC assay, a two-fold serial dilution of peptide #59 was performed from a starting concentration of 128 mg L⁻¹ down to 4 mg L⁻¹. Each dilution was performed in triplicate assays. After inoculation with the target bacteria, the 96-well plate was incubated overnight and the OD₆₀₀ readings of each well containing bacteria and peptide were calculated as a percentage of the uninhibited growth control (bacteria only). The lowest concentration of each replicate from all three MIC assays (nine data points in total) which caused >50% inhibition of growth was then documented and plotted with the modal value used as the MIC for that peptide-bacteria combination.

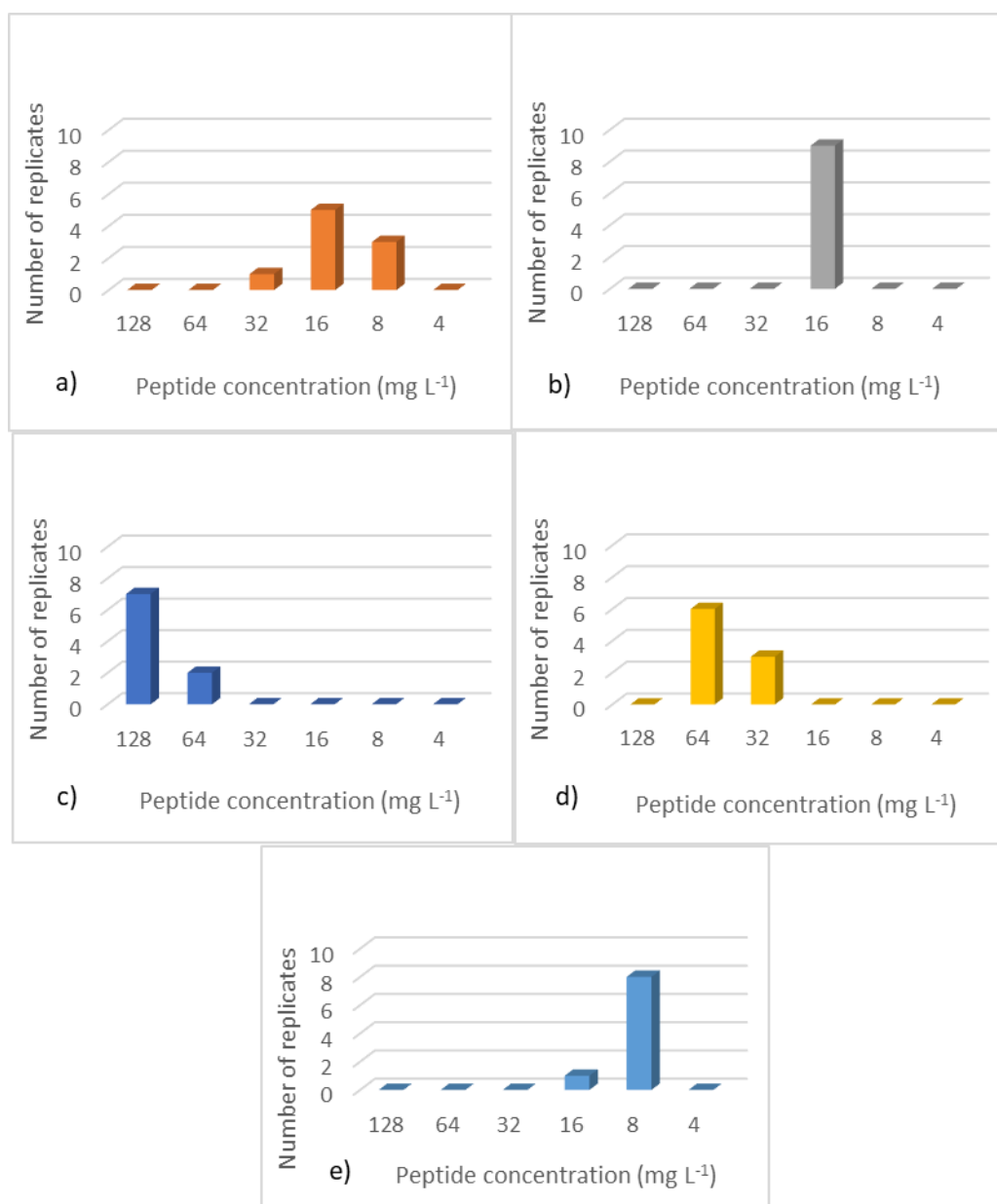


Figure 3-7. Inhibitory activity of peptide #45 against a panel of bacteria.

Three MIC assays, independent of each other, were carried out on a panel of bacteria which consisted of *E. coli* serotypes O2 (a) and O78 (b), *Salmonella enterica* serovars Enteritidis (c) and Typhimurium (d), and *B. subtilis* (e). For each MIC assay, a two-fold serial dilution of peptide #45 was performed from a starting concentration of 128 mg L⁻¹ down to 4 mg L⁻¹. Each dilution was performed in triplicate assays. After inoculation with the target bacteria, the 96-well plate was incubated overnight and then the OD₆₀₀ readings of each well containing bacteria and peptide were calculated as a percentage of the uninhibited growth control (bacteria only). The lowest concentration of each replicate from all three MIC assays (nine data points in total) which caused >50% inhibition of growth was then documented and plotted with the modal value used as the MIC for that peptide-bacteria combination.

3.4.2 MBC assay

Next, the 60 putative AMPs were assayed to determine if they display antimicrobial activity in a solution containing no nutrients for growth (10 mM Tris, 25 mM NaCl). Peptides displaying bactericidal activity in this alternative media did not always correlate with those identified from the previous inhibitory assay (Table 3-5). Peptide #45 displayed a similar bactericidal range to its inhibitory activity with MBC values of 16 mg L⁻¹ for both *E. coli* serotypes O78 and O2, respectively, and 64 mg L⁻¹ for *S. Typhimurium*. While not active against *E. coli*, peptide #59 was able to kill *S. Typhimurium* in the bactericidal assay at 64 mg L⁻¹ showing similar specificity as peptide #35. Peptides #36 was another peptide which displayed activity in this assay, but not in the broth MIC. Peptide #36 had a broad range with an MBC of 16 and 8 mg L⁻¹ against the *E. coli* serotypes O78 and O2, respectively, as well as activity against *S. Typhimurium* (32 mg L⁻¹). Peptide #27 displayed bactericidal activity against only *S. Enteritidis* at 128 mg L⁻¹.

Table 3-5. Bactericidal activities of phage-derived peptides

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S. Typhimurium</i>	<i>S. Enteritidis</i>
#27	>128 ¹	>128	>128	128 mg L ⁻¹ 65.9 µM ²
#35	>128	>128	32 mg L ⁻¹ 17 µM	>128
#36	8 mg L ⁻¹ 4.2 µM	16 mg L ⁻¹ 8.5 µM	32 mg L ⁻¹ 17 µM	>128
#45	16 mg L ⁻¹ 8.5 µM*	16 mg L ⁻¹ 8.5 µM	64 mg L ⁻¹ 33.8 µM	>128
#59	>128	>128	64 mg L ⁻¹ 34.5 µM	>128

¹ All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM.

3.4.3 Agar MIC assay

For peptides displaying activity in either the MIC or MBC assays, they were further analysed by the Agar MIC assay. Peptides were spotted onto the agar at concentrations ranging from 4 to 512 mg L⁻¹, for phage-derived peptides this is equivalent to approximately ~2 to ~230-240 µM. The results resembled those found when the peptides were assayed in broth. Peptide #45 was still active against both serotypes of *E. coli* and *S. Typhimurium* (Figure 3-8), and peptides #27, #45, and #59 were all active against *B. subtilis* (Figure 3-9), but the lowest MIC value for the agar dilution method was 64 mg L⁻¹ (Table 3-6).

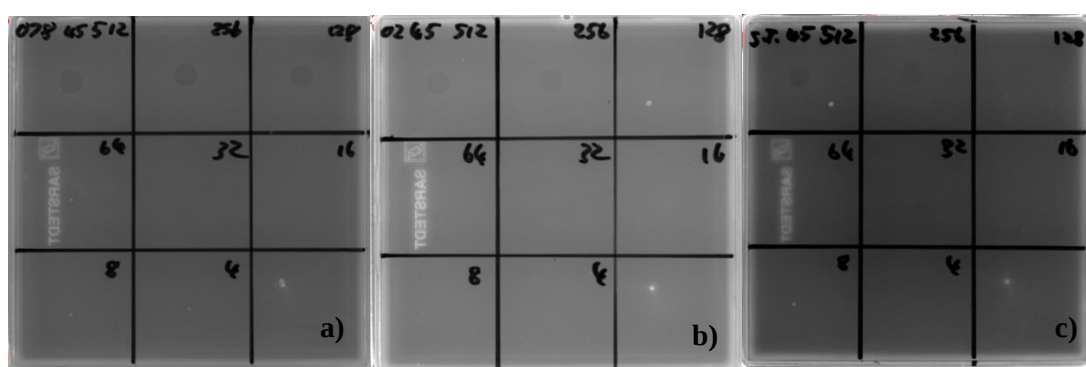


Figure 3-8. Inhibitory activity of peptide #45 in agar.

An MIC assay was performed on the bacterial pathogens, *E. coli* serotypes O78 and O2, and *S. Typhimurium*. Peptide #45 was diluted in a two-fold serial manner from 512 mg L⁻¹ to 4 mg L⁻¹ and 5 µL spotted onto sMHA that had been inoculated with **a)** O78, **b)** O2 or **c)** *S. Typhimurium*. After overnight incubation at 37°C the plates were photographed, and the lowest concentration resulting in a clear zone of inhibition was classified as its MIC.

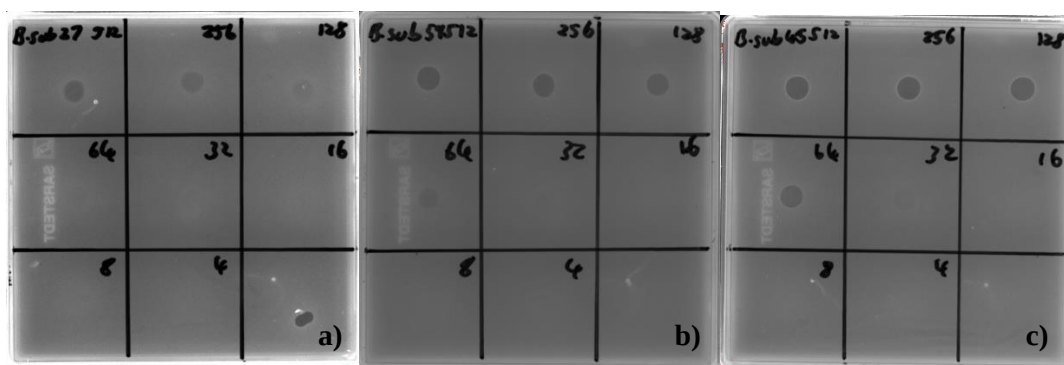


Figure 3-9. Inhibitory activity of peptides #27, #45, and #59 against *B. subtilis*.

An MIC assay was performed on *B. subtilis*. Peptides **a)** #27, **b)** #59, and **c)** #45 were diluted in a two-fold serial manner from 512 mg L⁻¹ to 4 mg L⁻¹ and 5 µL spotted onto sMHA that had been inoculated with *B. subtilis*. After overnight incubation at 37°C the plates were photographed, and the lowest concentration resulting in a clear zone of inhibition was classified as its MIC.

Table 3-6. Inhibitory activities of phage-derived peptides in agar

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S.</i> Typhimurium	<i>S.</i> Enteritidis	<i>L.</i> <i>innocua</i>	<i>B.</i> <i>subtilis</i>
#27	>512 ¹	>512	>512	>512	>512	128 mg L ⁻¹ 65.9 µM ²
#35	>512	>512	>512	>512	>512	>512
#36	>512	>512	>512	>512	>512	>512
#45	256 mg L ⁻¹ 135.3 µM	128 mg L ⁻¹ 67.7 µM	256 mg L ⁻¹ 135.3 µM	>512 mg L ⁻¹	>512 mg L ⁻¹	64 mg L ⁻¹ 33.8 µM
#59	>512	>512	>512	>512	>512	64 mg L ⁻¹ 34.5 µM

¹ All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM.

3.4.4 Synergistic activities

Phage-derived peptides displaying either inhibitory or bactericidal activity were investigated for synergistic behaviour. Firstly, a checkerboard assay was used to investigate whether peptides #45 and #59 against *E. coli* O78: by sequentially increasing concentration of one peptide and concomitantly decreasing concentrations

of the other within a 96-well plate, synergistic interactions could be found. However, the FIC remained below two and was therefore not classed as synergistic.

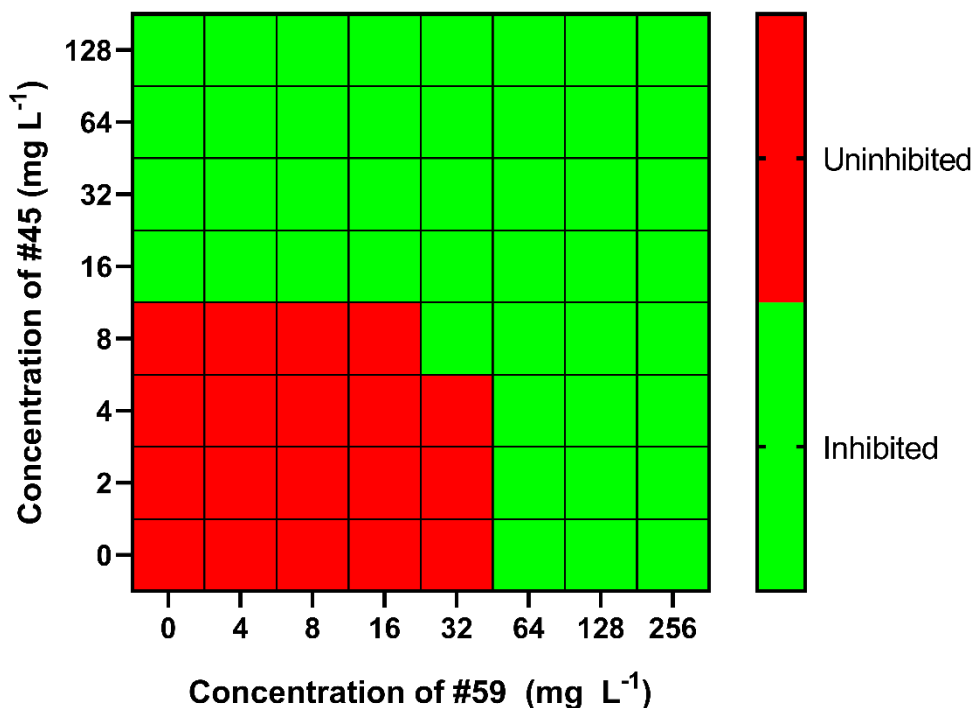


Figure 3-10. Checkerboard assay of peptides #45 and #59 against *E. coli* O78.

In a 96-well plate serial two-fold dilutions of both peptide #45 and #59 were made. In row A peptide #45 was added at 128 mg L⁻¹ and diluted down to 2 mg L⁻¹ in row G (0 mg L⁻¹ in row H), in column 8 peptide #59 was added at 256 mg L⁻¹ and diluted down to 4 mg L⁻¹ in column 2 (0 mg L⁻¹ in column 1). A culture of *E. coli* O78 at 5×10^5 CFU mL⁻¹ was added to each well and then the plate was incubated overnight at 37°C. Then the OD₆₀₀ readings of each well containing bacteria and peptide were calculated as a percentage of the uninhibited growth control (bacteria only). Values below 50% of the growth control were plotted as a green square and values above this plotted as a red square.

Investigations using a different experimental format was then used due to the cost of the checkerboard assay, caused by large amounts of peptide needed, and the low power of the assay, caused by a very small number of wells which provide the data necessary for determining synergy. Briefly, peptides were tested, in pairs, starting at ¼ of their MIC or MBC values. This concentration ensured that any activity seen would result

in at least a FIC of 0.5, thereby indicating synergy. Peptides were then diluted twice, by four-fold each time. Peptides active against a particular pathogen were tested with each other to cover all possible combinations. For example, when testing for bactericidal synergy against *E. coli* O78 the following peptide combinations were used: 36 + 45, 36 + 59, and 45 + 59. An MBC or MIC assay was also performed alongside the assay to ensure the FIC, or FBC, calculation used the correct values. No synergistic or antagonistic interactions were found (data not shown).

3.4.5 Time-kill assays

The phage-derived AMPs were investigated to determine the rapidity of their bactericidal action. This was to determine if there was any difference between the time taken to kill by the peptides able to inhibit growth in broth and those only able to display antimicrobial action in bactericidal test buffer. Peptides at concentrations 2-fold higher than their MBC, to account for experimental variation, were tested against *E. coli* O2, *E. coli* O78, *S. Enteritidis*, or *S. Typhimurium* (Table 3-7). At time points 1, 2, 4, 8, 18, and 24 hour post-inoculation, samples were assessed for bacterial growth. Intriguingly, all peptides caused a 3-log₁₀ reduction in bacterial viability in less than one hour except for #27 (Figure 3-11).

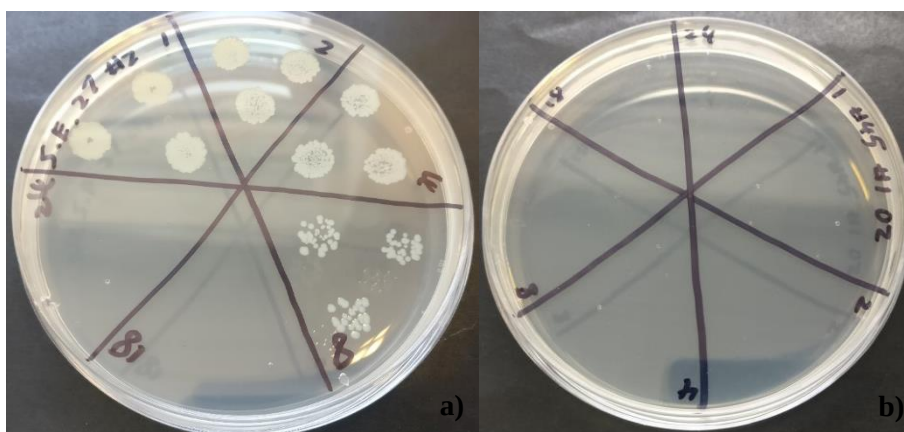


Figure 3-11. Comparative plates from time-kill assays of both peptide #27 and #45.

Peptides are diluted to $2\times$ MBC values and mixed with a pathogen, with samples being taken at 1, 2, 4, 8, 18, and 24 hour and plated onto corresponding sections of a Petri dish. The CFU mL^{-1} is then compared with counts pre-incubation and the time taken to reduce the bacterial load by 3-log_{10} recorded. **a)** Peptide #27 reaching criteria after 8 hours **b)** peptide #45 exhibiting activity before one hour.

Table 3-7. Time kill assays results for phage-derived peptides with an MBC value.

Peptide	<i>E. coli</i> O78	<i>E. coli</i> O2	<i>S. Typhimurium</i>	<i>S. Enteritidis</i>
#27	Not tested ¹	Not tested	Not tested	<18 h
#35	Not tested	Not tested	<1 h	Not tested
#36	<1 h	<1 h	<1 h	Not tested
#45	<1 h	<1 h	<1 h	Not tested
#59	Not tested	Not tested	<1 h	Not tested

¹Data from MBC assays was used to select which peptides to test against which bacterial isolates. Peptides were not tested against bacterial isolates they had no displayed activity against.

3.5 Further characterisation of phage-derived AMPs

3.5.1 Haemolytic activities and therapeutic index calculation

Next, the set of phage-derived AMPs were tested for haemolytic behaviour as outlined in Oddo and Hansen (2017). Each peptide was tested in duplicate at 128 mg L^{-1} in 2-fold dilutions down to 4 mg L^{-1} . PBS was a negative control and the haemolytic AMP melittin was used as a positive control (Figure 3-12).

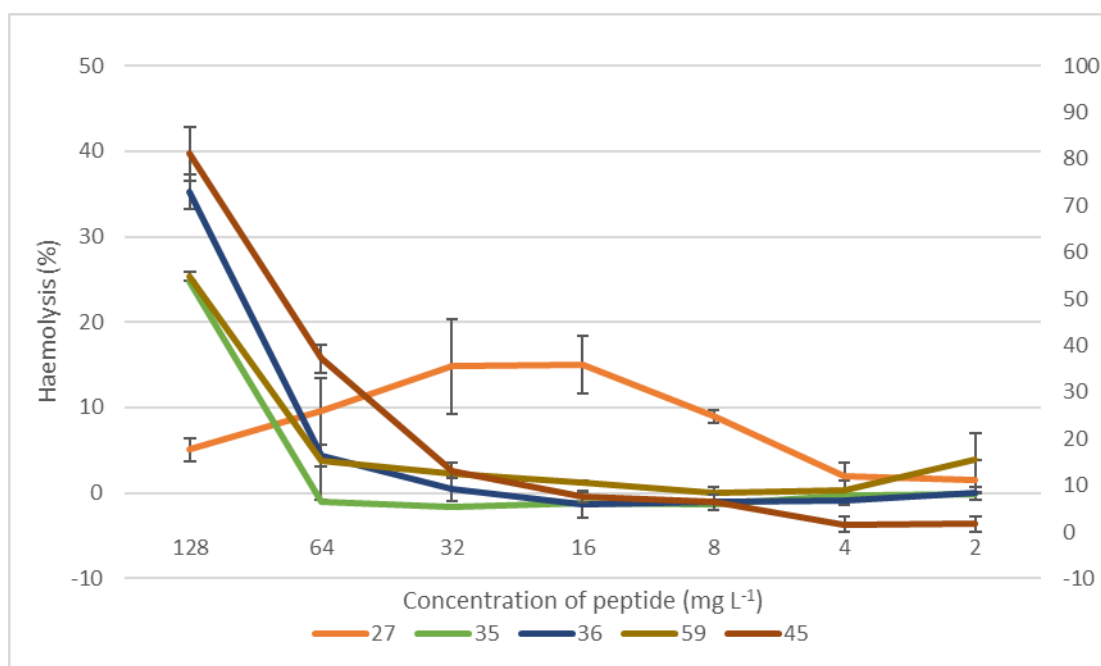


Figure 3-12. Haemolytic activity of phage-derived peptides.

Peptides were tested in duplicate, starting at 128 mg L⁻¹ with subsequent two-fold dilutions, against chicken RBCs. The AMP melittin was used as a positive control, which was used to calculate 100% lysis. RBCs suspended in 1× PBS was used as a negative control to calculate 0% lysis. After incubation for one hour with the peptides, the RBC suspension was centrifuged and supernatant transferred to be measured via OD₄₁₄. All samples were analysed in technical duplicates, rather than triplicates, so only one 96-well plate was used to eliminate inter-plate variation. The mean average of the values was plotted as a percentage compared to melittin and PBS, with SD shown for each peptide at each concentration as indicated. Peptide #45 is plotted on the secondary axis, to enable visualisation of the highest haemolysis percentage (81%).

Only one peptide, #45, lysed more than 50% of RBCs allowing a therapeutic index to be calculated. The therapeutic index, in this context, is the ratio between the concentration of the peptide which lysed 50% of RBCs and the concentration of the peptide which inhibited bacterial growth by 50%. For peptide #45 this was calculated to be six. A therapeutic index of greater than 10 is considered a good safety margin, but drugs with indexes as low as two can still be used (Tamargo, Le Heuzey, and Mabo 2015).

3.6 Discussion

An ideal therapeutic AMP can be defined by a few characteristics: non-toxic to the receiving host, high bioavailability, limited clearance via the kidneys, resistant to proteolytic degradation, potent antimicrobial activity, and retains activity within different environments and pH (J *et al.* 2013; M *et al.* 2015; Nguyen, Haney, and Vogel 2011; Slaninová *et al.* 2012; Wang *et al.* 2019). Current AMPs in clinical research, much like antibiotics, have mostly been isolated from natural sources with a large body of AMP research devoted to improving existing AMPs rather than *de novo* designing without a template (Borah, Deb, and Chakraborty 2021; De Castro and Franco 2015; Chen *et al.* 2020; Kumar *et al.* 2018). An existing problem with AMP research is that, unlike antibiotics, there is no standardised method for testing them. Methods developed for antibiotic testing, such as microbroth dilution, have to be adapted for use with AMPs. While there have been attempts to provide this standardisation, there are still many publications adhering to their own experimental design. Therefore, to try to allow comparisons of AMPs investigated within this study with those detailed in the scientific literature, multiple assays with different conditions were performed. These assays included two methods for determining bacteriostatic behaviour, one in broth and one in agar to represent variations on CLSI's microbroth dilution and agar dilution methods, and one method using only a buffered solution, as some peptides have been reported to be active only when the growth media has been removed.

Also, to allow a direct comparison between AMPs noted within the literature and the novel AMPs discovered within this study, selected AMPs from published reports, aided by DBAASP, were produced as synthetic peptides (Biomatik, NL). The selected

AMPs had published MICs and also represented a variety of antimicrobial assays. For example, the MIC for Plantaricin-423 was defined by spotting the peptide onto TSB soft agar which had been inoculated with bacteria. C-BF and Plectasin were assayed by the microbroth dilution method in aMHB, and E50-52 was tested by spotting onto Brucella agar which had been inoculated with bacteria (Guralp *et al.* 2013; Liu *et al.* 2011; Svetoch *et al.* 2008; Xi *et al.* 2013). The MIC of Plantaricin-423 was defined as 0.075 μM against *L. innocua*, equivalent to $\sim 0.3 \text{ mg L}^{-1}$, this is in comparison with 16 mg L^{-1} as defined in this study. C-BF was determined to have an MIC against various serotypes and lab strains of *E. coli* of between 1 mg L^{-1} and $>256 \text{ mg L}^{-1}$, but notably for an O78 serotype it was 16 mg L^{-1} . For *S. Typhimurium* and *S. Enteritidis* the MIC value reported was 4 mg L^{-1} . In this instance, the MIC reported in the literature was higher than the ones found in this study (0.5 mg L^{-1} for O78 and *S. Typhimurium* and 1 mg L^{-1} for *S. Enteritidis*), although the margins of difference are much smaller than for Plantaricin-423.

The larger discrepancy for the MIC value obtained for Plantaricin-423 in the current study could be due to the media used; in Guralp *et al.* (2013), tryptone soya broth (TSB) was used to culture *L. innocua* and was then used to inoculate TSB soft agar for the MIC assay. The impact of different medias on antimicrobial susceptibility testing has been assessed in various publications, with the general consensus being the same: in different media AMPs can perform significantly differently (Kumaraswamy *et al.* 2016; Schwab *et al.* 1999; Tucker *et al.* 2018). The differences could also be caused by variations in several stages of the experimental protocols, for example the plasticware used can affect the result, or even the additives used in the peptide solvent (Sharafi and Ardebili 2019). In the literature, plectasin was reported to have a MIC

against *S. Enteritidis* of 14.55 μM ($\sim 64 \text{ mg L}^{-1}$), and E50-52 was defined as having a MIC of below 1.6 mg L^{-1} against both *E. coli* and four undefined *Salmonella spp.* However, both plectasin and E50-52 showed no activity in any of the assays performed in the present study. A further experiment to investigate this may be to use a phenotypic microarray, to identify if the presence of those AMPs altered the metabolic state of the bacteria, even if no inhibition was found. This would elucidate if the activity is wholly media-specific or if some medias allow better resilience/recovery against AMP attack as well as determining any potential intracellular targets.

Randomised phage peptide libraries are an established method for identifying novel AMP sequences (Pini *et al.*, 2005; Bishop-Hurley, Rea and McSweeney, 2010; Sainath Rao, Mohan and Atreya, 2013). With a lack of structure-function relationships regarding antimicrobial peptides to underpin rational design, creating large, randomised libraries may be the best way to identify new or improved sequences. From a total of 60 peptides chosen from a combination of phage panning and bioinformatic analysis to identify peptides enriched against the target bacteria, five peptides were identified as antimicrobial using three different antimicrobial assays. While all peptides were identified as potential binders to Gram-negative bacteria, peptides 1-29 were identified from panning against the *E. coli* serotype O2 while the remaining peptides were identified from panning against *S. Typhimurium*. The five active peptides that were found did not display a correlation between which bacteria they were panned against and which bacteria they were active against, despite panning being used previously to identify specifically-targeting AMPs (Bishop-Hurley *et al.* 2010).

For example, peptides such as #27, which was only inhibitory against the Gram-positive bacterium *B. subtilis* and bactericidal against the pathogen *S. Enteritidis*, shows no activity towards *E. coli* serotypes. Furthermore, only one peptide, #45, was inhibitory against *S. Typhimurium*. This is despite the majority (4/5) of active peptides being identified from panning rounds against *S. Typhimurium*. Of the AMPs that were isolated from panning against *S. Typhimurium*, only #35 showed specificity towards the *S. Typhimurium* isolate in the MBC assay. Peptides #36, #45 and #59 displayed a wide target spectrum across the panel of bacteria tested. A reason for this could be that eluting with low pH during phage panning may not remove the strongest, and most specific, binders (Lunder *et al.* 2008). Whether the binding affinities for whole cell panning can surpass the eluting properties of low pH should be investigated and its impact on novel AMP discovery determined. The data clearly indicate that while phage display can be used to identify binding peptides, this does not translate into antimicrobial activity and large panels of putative binders need to be produced in order to isolate AMPs. Furthermore, directing binding activity in panning does not always lead to similar specificities in AMP activity. However, the data also clearly shows that from 60 putative peptide binders from phage display experiments, five novel peptides with AMP activity were isolated.

By using a Gram-positive bacterium, *L. acidophilus*, as a subtractive binding tool in the panning rounds, the aim was to improve specificity towards Gram-negative bacteria. This was due to the impact Gram-negatives have on the health and economic efficiencies of poultry farming (Landman and van Eck 2015). Interestingly, there were still peptides which were active against the Gram-positive *B. subtilis*, but not *L. innocua*. One peptide, #27, was inhibitory in both broth and agar against *B. subtilis*

and bactericidal in a buffered solution against *S. Enteritidis*, which is a seemingly unique combination. With the media-specific effects on antimicrobial effects already discussed, there could be differences in binding ability also based on the media used. During the panning rounds, the phage peptide library was mixed with bacteria in 1× PBS. The salt concentration within the PBS solution may hinder AMP binding, as the effect of higher salt concentrations on antimicrobial activity is already well documented (Chu *et al.* 2020; Goldman *et al.* 1997; Lee, Cho, and Lehrer 1997). This is thought to be due to the disruption of the initial binding step, which is an ionic interaction between the positively charged AMP and negatively charged bacterial membrane. The link between binding and antimicrobial activity is a critique of using phage display, as the former does not always lead to the latter, indicated by only 5/60 peptides from the panel displaying antimicrobial activity. Perhaps if the panning rounds could be completed in the same media as the antimicrobial assays are performed, this may in turn offer more success to the selection process and to the hit rate. However, *L. acidophilus* was not included in the bacterial panel due to difficulties in culturing so it could be only the peptides specific to *L. acidophilus* were excluded and that excluding all binders to an entire Gram-stain category is unlikely.

AMP synergism has been noted in various natural AMPs (Pino-Angeles, Leveritt, and Lazaridis 2016). Conventionally, checkerboard assays are used for determining synergy but are susceptible to experimental variation and low statistical power (He *et al.* 2015; Zharkova *et al.* 2019). In this study, a modified version of the protocol used in He, Starr and Wimley (2015) was utilised. The lack of synergism exhibited by these peptide was not unexpected, most cases of synergism between AMPs has been documented between peptides which have co-evolved. For example, magainin and

PGLa are two AMPs found on frog skin and which have noted synergistic activities (Bechinger *et al.* 2020). Whether or not these peptides would synergise with particular antibiotics, as has been shown to be possible with co-administration, was not determined and could be investigated further (Ruden *et al.* 2019).

In terms of toxicity, 4/5 of the AMPs discovered would allow high dosages to be used without causing unwanted side effects. However, the therapeutic index of peptide #45 is low for a peptide to be of use clinically, but with careful titration and monitoring it could still be of use as is seen with other narrow therapeutic index drugs (Blix *et al.* 2010). However, haemolysis cannot be used as a specificity determinant on its own. There are other toxicity issues that also need to be investigated which are not always related to haemolytic activity. For example, colistin sulfate is a cationic, membrane-active, peptide antibiotic that displays no haemolysis but can cause nephro- and neurotoxicity (Almutairy *et al.* 2020). However, haemolysis assays are useful as a screening tool, due to their rapid completion.

As antibiotic resistance, and the risks and costs associated with it, is increasing, research into AMPs is a steadily improving area. However, the reported success rates of AMPs in clinical trials is worryingly low and this could be linked to a sub-optimal testing routine within the *in vitro* assays used to determine potential. Not only are current antimicrobial susceptibility tests not optimised for AMPs, there is also considerable inconsistency within the scientific literature with the experimental protocols used. Steps should be taken to improve and standardise the current antimicrobial assays, and to promote a greater use of these across studies. Within the current study, attempts were made to use a range of assays currently available to not only highlight the differences in AMP activity found, but to enable comparisons across

multiple studies. Phage display was also shown to be a useful tool for isolating novel AMPs from a randomised peptide library, although there are limits to the panning and selection process with regards to exclusion criteria (Gram-positives may be too broad), and inclusion criteria (the majority of peptides synthesised were not antimicrobial in the assays performed). Despite its limitations, phage display has proven to be an effective method to isolate novel, short AMPs. Next, this study investigated whether the antimicrobial activity of the peptides, and their specificity, could be improved by engineering them as bivalent peptides.

4 Dimerization of peptides for improved activity

4.1 Introduction

Methods such as phage display have led to the discovery of multiple AMPs. However, a common characteristic shared between AMPs found using phage display is that they tend to have less efficient antimicrobial activity than antibiotics and selectively designed AMPs (Ebbensgaard *et al.* 2015). The process of dimerization can confer a greater antimicrobial activity than their monomeric counterpart (Campopiano *et al.* 2004; Verly *et al.* 2017). In research, heterodimers are more common as they have promising potential to combine the effects of two separate AMPs. Linking different AMPs to each other has been used to mitigate the negative effects of either AMP while retaining the desired characteristics from each. This can also be used to improve specificity of broad-spectrum AMPs, to reduce off-target killing which is often cited as a disadvantage to current antibiotic therapeutic courses.

Dimerization is not always successful, with multiple cases of dimeric AMPs having similar, or lower, antimicrobial activity than their monomer counterparts (Lorenzón *et al.* 2012, 2013; Yang, Kim, and Shin 2009). However, the lack of knowledge surrounding AMP's structure-function relationship contributes uncertainty to all modifications that have been used to improve AMP activity. Here, it was attempted to alter the specificity and efficacy of the AMPs identified in "Characterisation and comparison of AMPs derived from phage display with known AMPs". By creating heterodimers with the potent literature peptide C-BF, both efficacy and specificity were altered with a lower molar concentration needed for antimicrobial action. However, haemolysis was often increased. Further modifications to the structure of the heterodimers produced is likely to be beneficial.

4.2 Bacteriostatic activity of dipeptides

The monomeric phage-derived peptides #35, #36, #45 and the DBAASP-derived peptide C-BF were chosen to be synthesised as dimeric constructs based on their activities across the antimicrobial assays used. The AMPs were linked as various homodimers and heterodimers by a flexible linker adapted from Chen, Zaro and Shen (2013). The linker was modified to remove the net charge, changing the sequence to KESGSVSSEQLAQFRSLA. Each monomeric peptide was synthesised as a homodimer and a heterodimer with C-BF. All dimeric peptides were then tested in an MIC assay against the same six bacterial isolates that the monomeric peptides were tested against. The homodimeric phage-derived peptides did not perform well in inhibitory assays. The dimers 35-35 and 45-45 were inactive against all bacterial isolates tested (data not shown), and 36-36 was solely active against *L. innocua* at 128 mg L⁻¹ (22.6 µM; Figure 4-1). Homodimeric C-BF displayed high levels of inhibitory activity against most of the isolates tested. For example, against *E. coli* O2 and *B. subtilis* homodimeric C-BF had an MIC of 8 mg L⁻¹ (0.9 µM) (Figure 4-2).

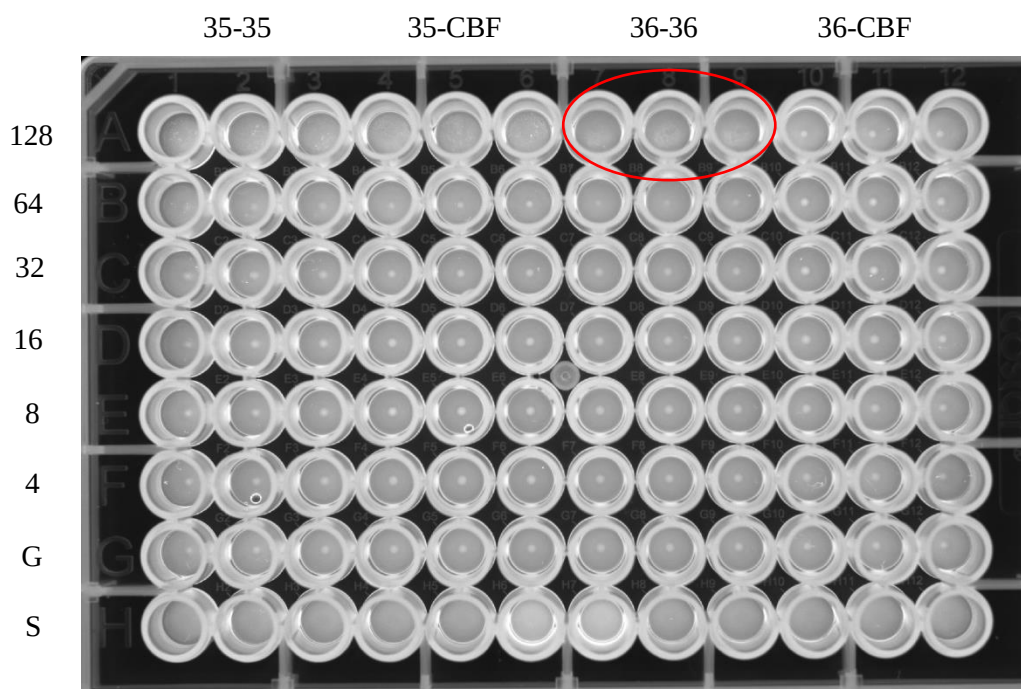


Figure 4-1. An MIC assay of dimeric peptides 35-35, 35-CBF, 36-36, and 36-CBF against *L. innocua*.

The four dimeric AMPs were tested against *L. innocua* with concentrations starting at 128 mg L⁻¹ and ending at 4 mg L⁻¹, as indicated. Rows entitled “G” and “S”, were used as a growth and sterility control, respectively. As *L. innocua* could not be resuspended after incubation, a positive result was defined as a lack of visible growth in a well after overnight incubation and the MIC is the lowest concentration a positive result is seen in. This is a photograph of the MIC assay plate, with 36-36 circled at its MIC, 128 mg L⁻¹.

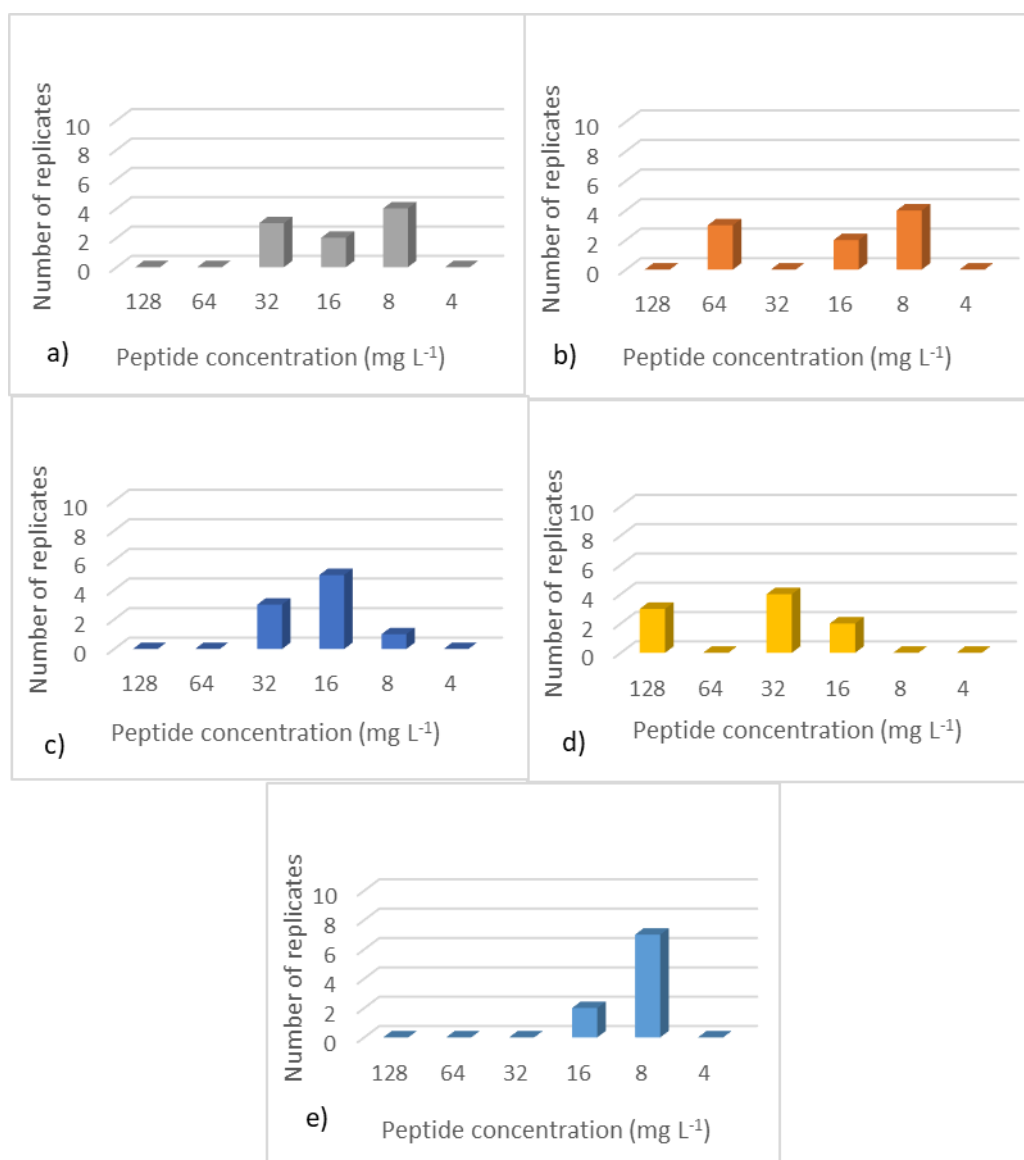


Figure 4-2. Inhibitory activity of peptide CBF-CBF against a panel of bacteria.

Three MIC assays, independent of each other, were carried out on a panel of bacteria, which consisted of *E. coli* serotypes O78 (a) and O2 (b), *Salmonella enterica* serovars Typhimurium (c) and Enteritidis (d), and *B. subtilis* (e). For each MIC assay, a two-fold serial dilution of peptide CBF-CBF was performed from a starting concentration of 128 mg L⁻¹ down to 4 mg L⁻¹. Each dilution was performed in triplicate. After inoculation with the target bacteria, the 96-well plate was incubated overnight and then the OD₆₀₀ readings of each well containing bacteria and peptide were calculated as a percentage of the uninhibited growth control (bacteria only). The lowest concentration of each replicate from all three MIC assays (nine data points in total) which caused >50% inhibition of growth was then documented and plotted with the modal value used as the MIC for that peptide-bacteria combination.

Heterodimeric peptides showed an increase of their inhibitory activity, and range of activity, when compared to the homodimeric phage-derived peptides. This is seen especially for peptide #35, as both the monomer and homodimer have no activity (Table 4-1), and #45 as 45-45 has no activity (Table 4-3) but was less obvious for dimeric versions of #36 (Table 4-2). Peptide 35-CBF has a relatively low MIC against all bacterial isolates, except *L. innocua* where it remains inactive (Figure 4-3). Peptide 36-CBF exhibited inhibitory activity against *E. coli* O78 at a concentration of 64 mg L⁻¹ (8.6 µM), and 45-CBF was active against all isolates tested (Figure 4-4, Figure 4-5).

Table 4-1. Comparison of MIC values for dimeric and monomeric #35

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S. Typhimurium</i>	<i>S. Enteritidis</i>	<i>L. innocua</i>	<i>B. subtilis</i>
35	>128 ¹	>128	>128	>128	>128	>128
35-35	>128	>128	>128	>128	>128	>128
35-CBF	16 mg L ⁻¹ 2.2 µM ²	16 mg L ⁻¹ 2.2 µM	64 mg L ⁻¹ 8.6 µM	>128	>128	32 mg L ⁻¹ 4.3 µM

¹All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM.

Table 4-2. Comparison of MIC values of dimeric and monomeric #36

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S. Typhimurium</i>	<i>S. Enteritidis</i>	<i>L. innocua</i>	<i>B. subtilis</i>
36	>128	>128	>128	>128	>128	>128
36-36	>128	>128	>128	>128	128 mg L ⁻¹ 22.6 µM ²	>128
36-CBF	>128	64 mg L ⁻¹ 8.6 µM	>128	>128	>128	>128

¹All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM.

Table 4-3. Comparison of MIC values of dimeric and monomeric #45

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S.</i> Typhimurium	<i>S.</i> Enteritidis	<i>L.</i> <i>innocua</i>	<i>B.</i> <i>subtilis</i>
45	16 mg L ⁻¹ 8.5 µM ²	16 mg L ⁻¹ 8.5 µM	64 mg L ⁻¹ 33.8 µM	128 mg L ⁻¹ 67.7 µM	>128	8 mg L ⁻¹ 4.2 µM
45-45	>128	>512	>512	>512	>128	>128
45-CBF	32 mg L ⁻¹ 4.3 µM	32 mg L ⁻¹ 4.3 µM	64 mg L ⁻¹ 8.6 µM	128 mg L ⁻¹ 17.3 µM	128 mg L ⁻¹ 17.3 µM ¹	64 mg L ⁻¹ 8.6 µM

¹All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM.

Table 4-4. Comparison of MIC values of dimeric and monomeric C-BF

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S.</i> Typhimurium	<i>S.</i> Enteritidis	<i>L.</i> <i>innocua</i>	<i>B.</i> <i>subtilis</i>
C-BF	1 mg L ⁻¹ 0.3 µM ²	0.5 mg L ⁻¹ 0.14 µM	0.5 mg L ⁻¹ 0.14 µM	1 mg L ⁻¹ 0.3 µM	64 mg L ⁻¹ 17.6 µM	0.5 mg L ⁻¹ 0.14 µM
CBF-CBF	8 mg L ⁻¹ 0.9 µM	4 mg L ⁻¹ 0.4 µM	16 mg L ⁻¹ 1.7 µM	32 mg L ⁻¹ 3.5 µM	64 mg L ⁻¹ 7 µM	8 mg L ⁻¹ 0.9 µM

¹All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM.

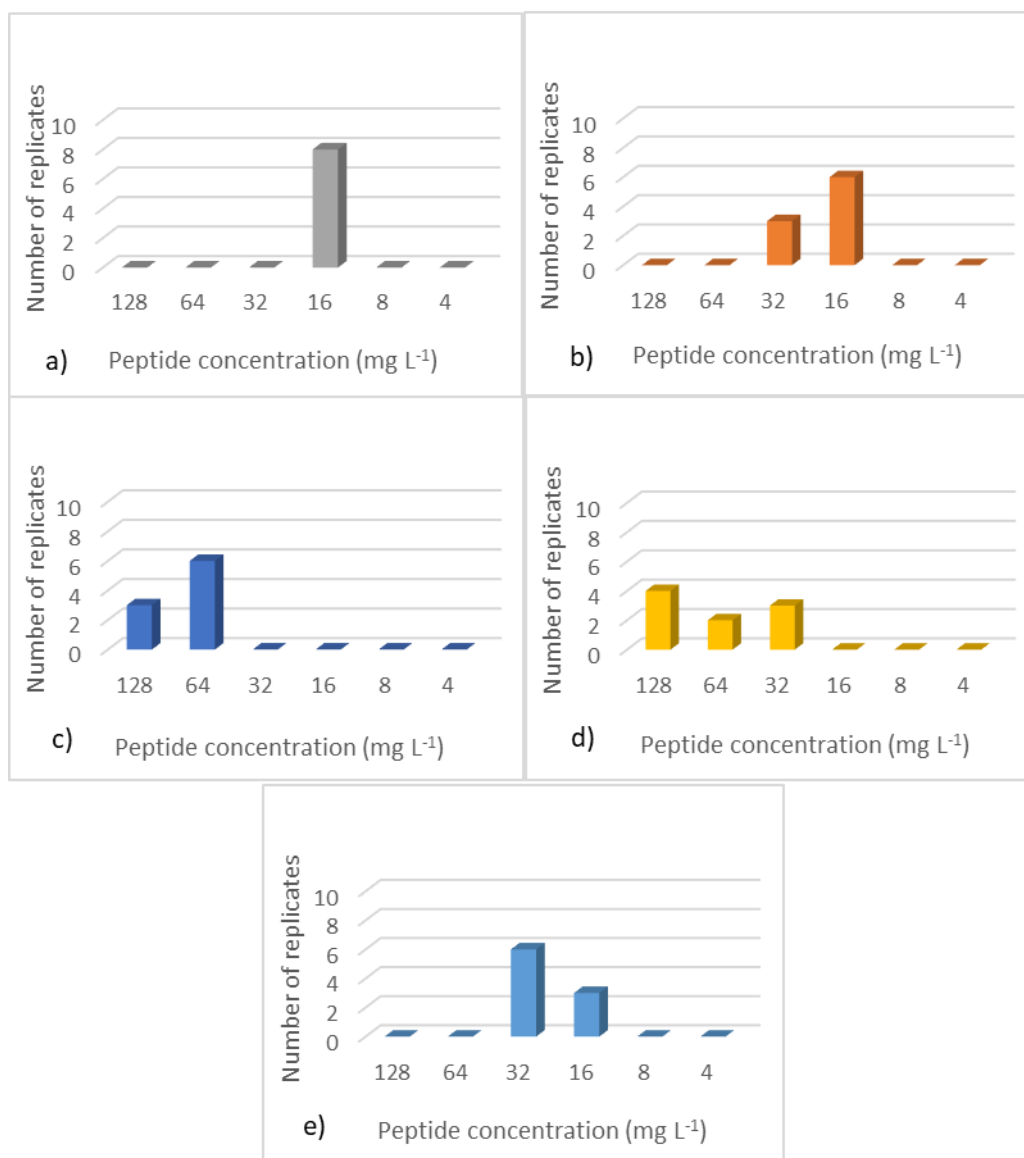


Figure 4-3. Inhibitory activity of peptide 35-CBF against a panel of bacteria.

Three MIC assays, independent of each other, were carried out on a panel of bacteria, which consisted of *E. coli* serotypes O78 (a) and O2 (b), *Salmonella enterica* serovars Typhimurium (c) and Enteritidis (d), and *B. subtilis* (e). For each MIC assay, a two-fold serial dilution of peptide 35-CBF was performed from a starting concentration of 128 mg L⁻¹ down to 4 mg L⁻¹. Each dilution was performed in triplicate. After inoculation with the target bacteria, the 96-well plate was incubated overnight and then the OD₆₀₀ readings of each well containing bacteria and peptide were calculated as a percentage of the uninhibited growth control (bacteria only). The lowest concentration of each replicate from all three MIC assays (nine data points in total) which caused >50% inhibition of growth was then documented and plotted with the modal value used as the MIC for that peptide-bacteria combination.

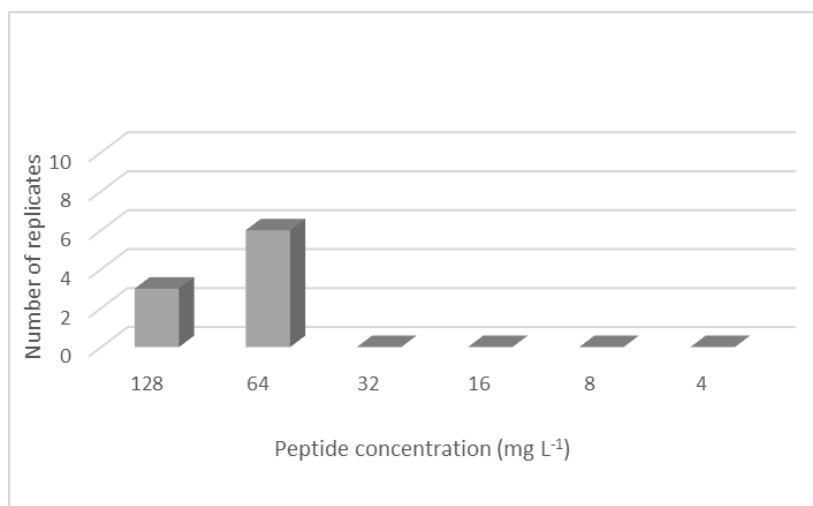


Figure 4-4. Inhibitory activity of peptide 36-CBF against *E. coli* O78.

Three MIC assays, independent of each other, were carried out on *E. coli* O78. For each MIC assay, a two-fold serial dilution of peptide 36-CBF was performed from a starting concentration of 128 mg L⁻¹ down to 4 mg L⁻¹. Each dilution was performed in triplicate. After inoculation with the target bacteria, the 96-well plate was incubated overnight and then the OD₆₀₀ readings of each well containing bacteria and peptide were calculated as a percentage of the uninhibited growth control (bacteria only). The lowest concentration of each replicate from all three MIC assays (nine data points in total) which caused >50% inhibition of growth was then documented and plotted with the modal value used as the MIC for that peptide-bacteria combination.

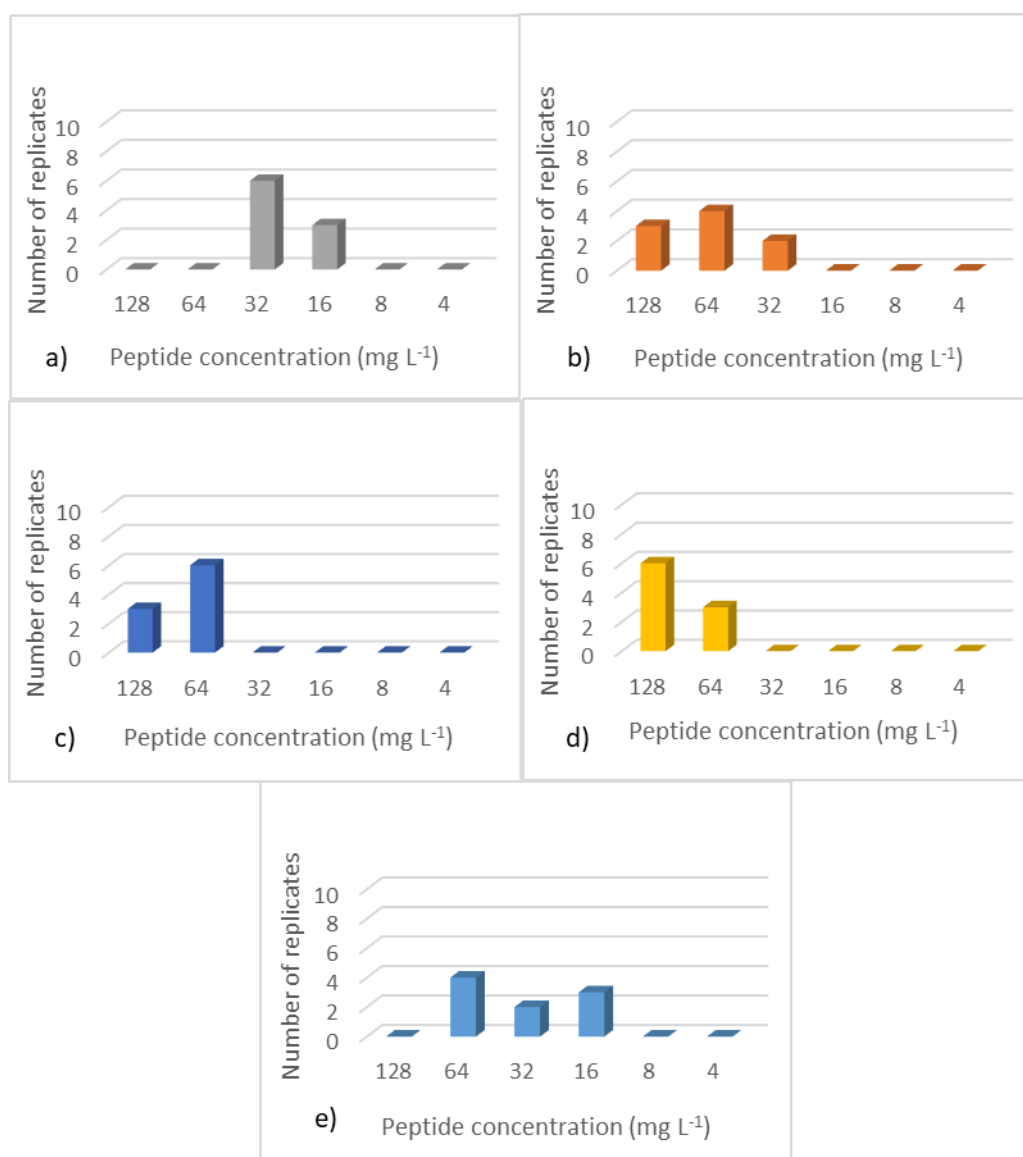


Figure 4-5. Inhibitory activity of peptide 45-CBF against a panel of bacteria.

Three MIC assays, independent of each other, were carried out on a panel of bacteria, which consisted of *E. coli* serotypes O78 (a) and O2 (b), *Salmonella enterica* serovars Typhimurium (c) and Enteritidis (d), and *B. subtilis* (e). For each MIC assay, a two-fold serial dilution of peptide 45-CBF was performed from a starting concentration of 128 mg L⁻¹ down to 4 mg L⁻¹. Each dilution was performed in triplicate. After inoculation with the target bacteria, the 96-well plate was incubated overnight and then the OD₆₀₀ readings of each well containing bacteria and peptide were calculated as a percentage of the uninhibited growth control (bacteria only). The lowest concentration of each replicate from all three MIC assays (nine data points in total) which caused >50% inhibition of growth was then documented and plotted with the modal value used as the MIC for that peptide-bacteria combination.

4.3 Bactericidal activity of dipeptides

To determine the effects of dimerization on the bactericidal activity, the dimeric peptides were tested in the MBC assay. Homodimers 35-35 and 36-36 were inactive against all bacterial pathogens tested, and 45-45 exhibited no activity against *E. coli* O78 at concentrations tested. However, the homodimer 45-45 did show activity against *E. coli* O2 with an MBC value of 64 mg L⁻¹ (11.3 µM) and *S. Typhimurium* with an MBC value of 16 mg L⁻¹ (2.8 µM; Table 4-3).

Heterodimeric forms of the peptides performed better than the phage-derived monomers (Table 4-5, Table 4-6, Table 4-7). Peptide 35-CBF was active against all bacterial pathogens tested in the bactericidal assay, with the lowest MBC value of 2 mg L⁻¹ (0.27 µM) against *E. coli* O78. Peptide 36-CBF displayed activity against all isolates, except *E. coli* O2, with an MBC of 8 mg L⁻¹ (1.1 µM) against *E. coli* O78 and *S. Typhimurium* and an MBC of 32 mg L⁻¹ (4.3 µM) against *S. Enteritidis*. The heterodimer 45-CBF exhibited bactericidal activity against all isolates except *S. Enteritidis*. MBC values of 8, 4 mg L⁻¹ were found against *E. coli* serotypes O78 and O2 while *S. Typhimurium* had an MBC value of 4 mg L⁻¹.

Table 4-5. Comparison of MBC values of dimeric and monomeric #36

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S. Typhimurium</i>	<i>S. Enteritidis</i>
#35	>128	>128	32 mg L ⁻¹ 17 µM	>128
35-35	>128	>128	>128	>128
35-CBF	4 mg L ⁻¹ 0.5 µM	2 mg L ⁻¹ 0.3 µM	4 mg L ⁻¹ 0.5 µM	16 mg L ⁻¹ 2,2 µM

¹ All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM.

Table 4-6. Comparison of MBC values of dimeric and monomeric #36

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S.</i> Typhimurium	<i>S.</i> Enteritidis
#36	8 mg L ⁻¹ 4.2 µM	16 mg L ⁻¹ 8.5 µM	32 mg L ⁻¹ 17 µM	>128
36-36	>128	>128	>128	>128
36-CBF	>128	8 mg L ⁻¹ 1.1 µM	8 mg L ⁻¹ 1.1 µM	32 mg L ⁻¹ 4.3 µM

¹ All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM.

Table 4-7. Comparison of MBC values of dimeric and monomeric #45

Peptide	<i>E. coli</i> O2	<i>E. coli</i> O78	<i>S.</i> Typhimurium	<i>S.</i> Enteritidis
#45	16 mg L ⁻¹ 8.5 µM*	16 mg L ⁻¹ 8.5 µM	64 mg L ⁻¹ 33.8 µM	>128
45-45	64 mg L ⁻¹ 11.3 µM	>128	16 mg L ⁻¹ 2.8 µM	>128
45-CBF	4 mg L ⁻¹ 0.6 µM	8 mg L ⁻¹ 1.1 µM	4 mg L ⁻¹ 0.6 µM	>128

¹ All values are mg L⁻¹; ² for active AMPs, MIC concentrations are also shown in µM.

4.4 Bacteriostatic activity of dipeptides in agar

The dimeric peptides were tested in the Agar MIC assay, against both serotypes of *E. coli*, both serovars of *Salmonella*, *B. subtilis* and *L. innocua*. No dimeric peptide displayed any antimicrobial activity up to the highest concentration tested, which was 512 mg L⁻¹ (data not shown).

4.5 Haemolysis

All dimeric peptides, and C-BF, were assessed for haemolytic activity in the same assay used for the monomeric peptides. Each peptide, except C-BF and CBF-CBF, was tested in triplicate at 256 mg L⁻¹ in two-fold dilutions down to 4 mg L⁻¹. Both C-BF and its homodimer were tested in triplicate, in four-fold dilutions from 256 mg L⁻¹ down to 0.0625 mg L⁻¹. PBS was a negative control and the haemolytic AMP melittin

was used as a positive control. Of the phage-derived homodimers, only 45-45 showed any haemolysis when incubated with chicken RBCs, but lysis reached less than 10% at concentrations below 32 mg L⁻¹ (Figure 4-6). All three heterodimeric peptides were haemolytic at the highest concentration tested: 155%, 40%, and 97% haemolysis for peptides 35-CBF, 36-CBF, and 45-CBF at 256 mg L⁻¹ (Figure 4-7). C-BF displayed negligible haemolytic activity up to 256 mg L⁻¹, where it reached 30% haemolysis, in comparison the dimeric peptide CBF-CBF only showed no significant haemolytic behaviour up to 16 mg L⁻¹ (Figure 4-8). To calculate the TI for C-BF, higher concentrations would need to be used against chicken RBCs. This is in comparison to CBF-CBF, which a TI range against pathogens of between 2 to 16. The TI of 35-CBF and 45-CBF ranged from 1 to 8 and 2 to 8, respectively.

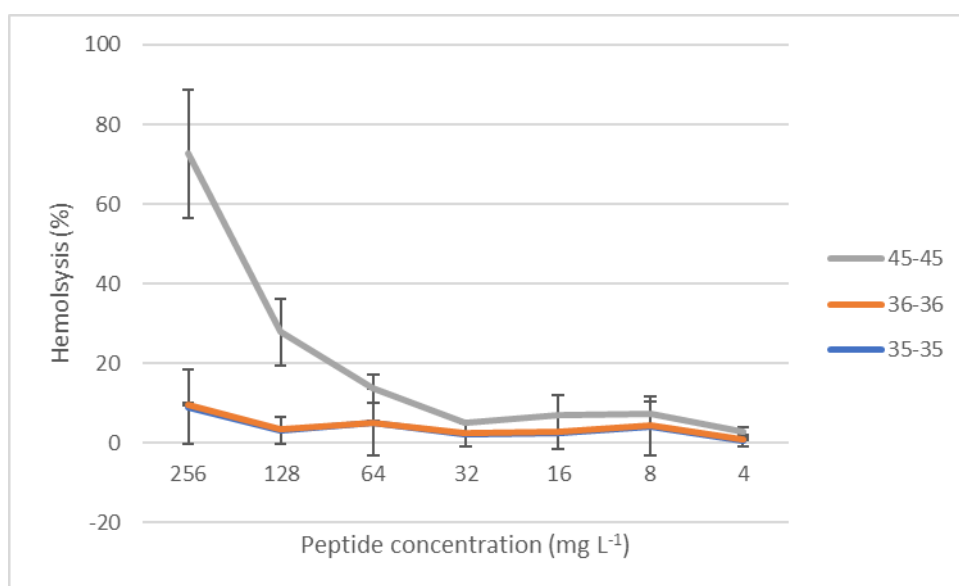


Figure 4-6. Haemolytic behaviour of phage-derived homodimers against chicken RBCs.

Peptides were tested in triplicate, starting at 256 mg L⁻¹ with subsequent two-fold dilutions, against chicken RBCs. The AMP melittin was used as a positive control, which was used to calculate 100% lysis. RBCs suspended in 1× PBS was used as a negative control to calculate 0% lysis. After incubation with the peptides, the RBC suspension was centrifuged and supernatant transferred to be measured via OD₄₁₄. All samples were analysed in triplicate, and the values were averaged and plotted as a percentage compared to melittin and PBS, with SD shown for each peptide at each concentration as indicated.

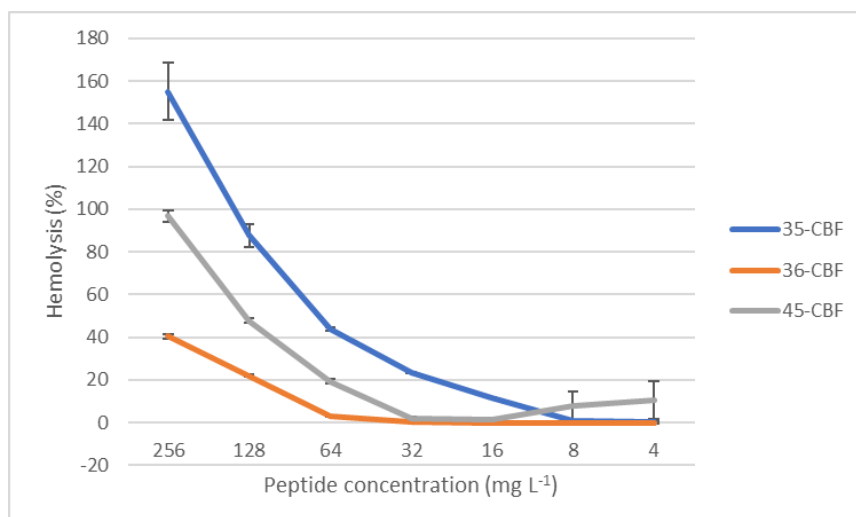


Figure 4-7. Haemolytic behaviour of heterodimeric peptides against chicken RBCs.

Peptides were tested in triplicate, starting at 256 mg L⁻¹ with subsequent two-fold dilutions, against chicken RBCs. The AMP melittin was used as a positive control, which was used to calculate 100% lysis. RBCs suspended in 1× PBS was used as a negative control to calculate 0% lysis. After incubation with the peptides, the RBC suspension was centrifuged and supernatant transferred to be measured via OD₄₁₄. All samples were analysed in triplicate, and the values were averaged and plotted as a percentage compared to melittin and PBS, with SD shown for each peptide at each concentration as indicated.

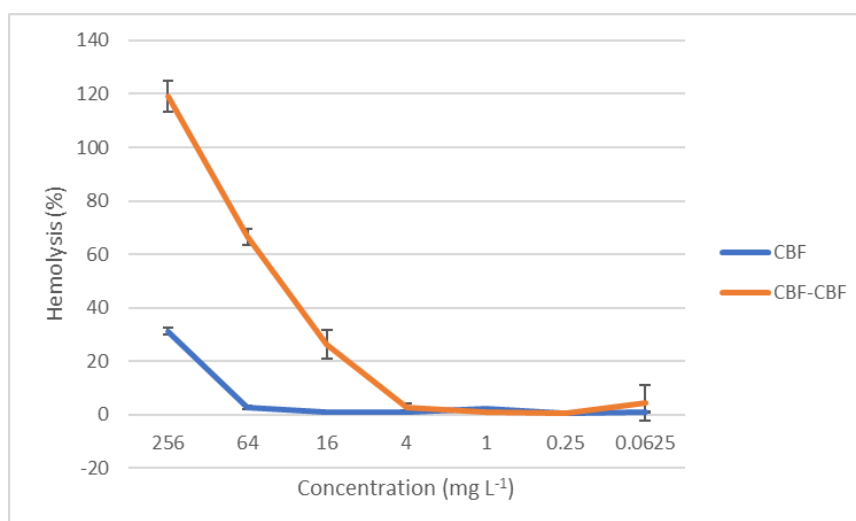


Figure 4-8. Haemolytic behaviour of C-BF and the homodimer CBF-CBF against chicken RBCs.

Peptides were tested in triplicate, starting at 256 mg L⁻¹ with subsequent four-fold dilutions, against chicken RBCs. The AMP melittin was used as a positive control, which was used to calculate 100% lysis. RBCs suspended in 1× PBS was used as a negative control to calculate 0% lysis. After incubation with the peptides, the RBC suspension was centrifuged and supernatant transferred to be measured via OD₄₁₄. All samples were analysed in triplicate, and the values were averaged and plotted as a percentage compared to melittin and PBS, with SD shown for each peptide at each concentration as indicated.

4.6 Prediction of secondary structure

Peptide helicity is believed to be strongly linked to toxicity against eukaryotic cells (Huang *et al.*, 2014). By utilising online tools such as PEP-FOLD3 (Lamiable, Thevenet, and Tufféry 2016; Shen *et al.* 2014; Thévenet *et al.* 2012) and PEP2D, predictions of secondary structure can be generated to determine if the AMP is likely to act via α -helices. A limitation of these tools is that the maximum permitted length of the peptides to interrogate with the associated algorithms is 50, therefore only monomers from this study which were synthesised as dimers were investigated. Using PEP2D, the proportion of each secondary structure (α -helix, β -sheet, and random coil) can be estimated (Table 4-8).

Table 4-8. Proportions of secondary structure within monomeric peptides

Peptide	Helix (%)	Sheet (%)	Coil (%)
35	18.75	0	81.25%
36	18.75	0	81.25
45	31.25	6.25	62.50
C-BF	50	6.67	43.33

Peptide #45's amino acid sequence was estimated to produce an α -helix secondary structure for approximately five of 16 residues (Figure 4-9). While on the lower end of helicity, disrupting this further by switching a non-polar residue within the α -helix, such as Leucine at position 7, with a positively charged amino acid could provide a decrease in cytotoxicity. However, modifications based on strategies to minimise haemolysis might be a less effective route to improve this studies' AMPs.

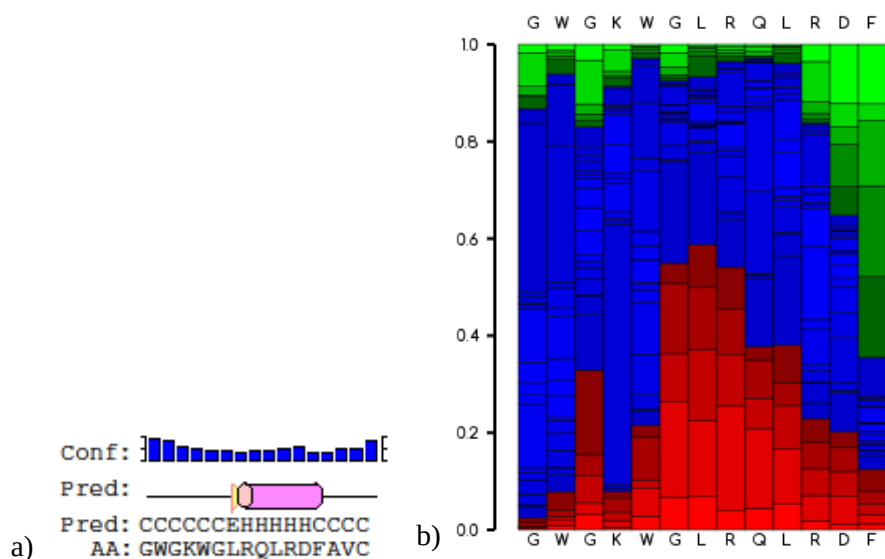


Figure 4-9. Structure prediction of AMP #45.

The amino acid sequence of peptide #45 was used as the input for both PEP2D (a) and PEP-FOLD3 (b) algorithms. The output from PEP2D shows the confidence level of its prediction that most of peptide #45 conforms to a random coil structure, (black line; “C”) with an α -helical section (pink cylinder; “H”). PEP-FOLD3 produces a graphical representation of the structural probabilities at each residue with blue representing random coil, red representing α -helix and green for β -sheet. The model uses a four residue window when sampling, which truncates the final three residues from the analysis. Both algorithms show moderate consensus that peptide #45 may have a slight α -helix structure in its centre.

4.7 Discussion

The dimerization strategy described here was used to determine if favourable characteristics from AMPs could be combined. By using next-generation phage display AMPs with a high specificity were created, as discussed in the previous chapter. Conjugation of these AMPs with a narrow spectrum of activity to a potent AMP, such as C-BF, was hypothesised to produce an AMP with both a narrow spectrum and high efficacy. The dimerization strategy was also utilised to test if potency would increase by conjugating monomers into dipeptides. These experiments yielded mixed results. The homodimers of #35 and #36 failed to show any improvement with the former being completely inactive and the latter only slightly active against *L. innocua*. The homodimer of #45 performed only slightly better, with

activity against two separate pathogens: *E. coli* and *Salmonella*. When comparing dimeric peptides to a monomeric peptide, using the molar concentration available can provide a better description of efficacy based on number of AMP molecules available rather than the mass of peptide available. For example, the homodimer 45-45's activity in MBC was 64 and 16 mg L⁻¹ against *E. coli* O2 and *S. Typhimurium*, respectively. Monomeric #45 killed *E. coli* O2 and *S. Typhimurium* at 16 and 64 mg L⁻¹, respectively. This is a four-fold difference between both values. However, when the molar concentration is used, 45-45 almost has the same activity as #45 against *E. coli* O2 at 11.3 μ M and 8.5 μ M, while the difference between 45-45's and #45's MBC value against *S. Typhimurium* widens greatly from four-fold to 12-fold. Therefore, for the purposes of comparisons between peptides of different molecular size, molar concentration was used.

Monomeric C-BF had a potent activity against every bacterial isolate tested, but with a noticeable decrease from MIC to MBC. Against *S. Enteritidis* this was most pronounced, with an MIC of 0.3 μ M and an MBC of >35.2 μ M. For peptide CBF-CBF, the difference between the MIC and MBC values was smaller, and the MBC value was lower than the MIC value for all isolates where the MBC was evaluated. C-BF had a lower MIC value for all isolates than CBF-CBF, except *L. innocua*, but in the *E. coli* serotypes the difference was only 3-fold. A consideration of note was that the MBC values for CBF-CBF were all calculated using the median as the inter-assay results displayed over an eight-fold difference (data not shown). This is in contrast to C-BF monomer where results with *E. coli* O78 and *S. Typhimurium* required this method of analysis, but results with *E. coli* O2 were within an eight-fold variation (data not shown). The MBC assay against *S. Enteritidis* was only performed once as

C-BF displayed no activity against this isolate. The difference in the MIC values for the *Salmonella* serovars was slightly higher (~10-fold) for the homodimer than the monomer. A complete lack of activity in the Agar MIC from all the dimers, not just CBF-CBF was ascribed to a failure to diffuse through the agar, and therefore to reach all the bacteria necessary to create a zone of inhibition. Overall, the peptide C-BF showed that dimerization is an excellent tool for altering peptide activity, but a robust tool for predicting how the dimerization will alter the activity is needed. By increasing the activity within the MBC assay, CBF-CBF shows potential to be more effective than C-BF in further studies where growth conditions may not be optimal for target bacteria.

Linking the phage-derived peptides to the potent DBAASP-derived peptide C-BF proved to be a more successful strategy than creating homodimers of the phage-derived peptides. In particular, 35-CBF showed a significant increase in its spectrum of activity compared to monomeric #35. Peptide #35 was only active against one isolate in one assay, *S. Typhimurium* in the MBC assay; 35-CBF was active against all isolates, except *L. innocua*, in both the MIC and MBC assay. However, in terms of the strategy to create a potent AMP with high specificity, 35-CBF compared poorly with those described previously (Choudhury *et al.* 2019; Xu *et al.* 2020). One consideration of note was that previous attempts to reduce the spectrum of activity of a potent AMP did not use another AMP as the targeting peptide, but used binding peptides with no inherent antibacterial activity. One example of this is by Kim *et al.* (2020) who used phage display to identify peptide binders to the OprF porin of *Pseudomonas aeruginosa* and then linked the candidates with a potent, broad-spectrum AMP which resulted in the novel PA2-GNU7 peptide. PA2-GNU7

selectively killed *P. aeruginosa* in mixed cultures while exhibiting minimal host toxicity, and was also efficacious in a mouse model of *P. aeruginosa* infection.

Linking a highly specific AMP to a potent AMP, such as #35 to C-BF, was hypothesised to produce an AMP combining the two favourable characteristics. However, the attributes of 35-CBF were dominated by the activity spectrum of C-BF. Despite this, there were limited indications that the phage-derived peptide retained some of its attributes. For example, 35-CBF showed over a 30-fold higher MIC value against *B. subtilis* than C-BF which could indicate that the addition of the Gram-negative targeting #35 reduced C-BF's non-selective activity. Another result of note was the activity of 35-CBF against *S. Enteritidis*, where both #35 and C-BF were inactive against *S. Enteritidis*. However, this change from the inactive monomers to an active dimer could be due to the variability seen in the MBC assay, which was mostly confined to the dimeric peptides and C-BF. It is possible that upon further testing C-BF could display a limited activity towards *S. Enteritidis* within the MBC assay, as inter-assay results from C-BF or the dimeric peptides were often above an eight-fold difference.

The dimeric peptide 36-CBF, in contrast to 35-CBF, displayed an activity level more similar to the phage-derived peptide rather than C-BF. This was exhibited by most of the AMP's activity being confined to the MBC assay, and the inter-assay results from the subsequent replicates displaying a less than eight-fold variation. The peptide was active in the MBC assay against *S. Enteritidis*, yet both monomeric peptides displayed no activity towards the *Enteritidis* serovar in this assay. The dimeric peptide also displayed inhibitory activity against *E. coli* O78 whereas the monomer #36 did not, conversely, the dimer had no activity against the O2 serotype whereas both #36 and

C-BF did. Conjugating #36 to C-BF also inhibited any activity against Gram-positives, with neither *L. innocua* nor *B. subtilis* affected by 36-CBF. This is another positive indicator for the potential of altering an AMPs specificity via dimerization. Overall, the heterodimer 36-CBF has demonstrated effective antimicrobial action against both *Salmonella* serovars tested, and an *E. coli* serotype (O78). The dimer's bacteriostatic action was limited to *E. coli* O78, in contrast to the component monomers: C-BF displays a robust inhibitory activity against all Gram-negative isolates tested whereas #36 failed to inhibit the growth of any.

Peptide 45-CBF was another potent dimeric peptide, although generally with slightly higher values than 35-CBF. Intriguingly, this was also the only heterodimer which was inactive in the MBC assay against *S. Enteritidis* despite being active against every other isolate. In this regard, the dimer retained much of the activity C-BF displays, yet with even lower MBC values for the other three isolates tested. However, the slightly lower MIC values for *E. coli* over *Salmonella* could reflect retention of some of 45's targeting ability as C-BF displayed no preference for either genus.

Dimerization of peptides #35, #36, and #45 into homodimers generally inhibited their antimicrobial activity, while potentially retaining their haemolytic behaviour at similar levels. While peptides #35 and #36 displayed a spike in haemolysis at 68 μM , their homodimers exhibited no haemolytic behaviour, with the highest concentration tested being 46 μM . This is in comparison to a notable drop in antimicrobial activity seen in their MBC assay results. Peptide #45 lysed 37% of RBCs at 34 μM , whereas 45-45 lysed 63% at 46 μM and 24% at 23 μM . This shows a comparable level of haemolytic activity, yet 45-45 also did not exhibit the inhibitory activity shown by peptide #45. Considering peptide #35 and C-BF's relatively low levels of haemolysis, even at

higher concentrations, the heterodimer 35-CBF had a very high haemolytic activity, the highest recorded. The other two heterodimers, 36-CBF and 45-CBF, showed high levels of haemolysis at higher concentrations of peptide. However, while it took 35-CBF until 1 μM of peptide to reach <10% haemolysis, 36-CBF reached this milestone at 9 μM and 45-CBF reached it at 4.5 μM . Out of all the TIs calculated for the dimeric peptides, 45-CBF provided the best option for host safety. With its robust performance in both broth and a buffered solution, 45-CBF may represent the best target for use within a more complex matrix.

By looking at the differences in the haemolytic behaviour of C-BF and CBF-CBF, there is evidence that the linker sequence chosen could be contributing to the high haemolysis seen in the dimers. At the highest concentration, 256 mg L^{-1} , CBF-CBF is almost four-fold more haemolytic than C-BF. This difference becomes more noticeable when the molar concentration is accounted for, CBF-CBF at 28 μM is four-fold more haemolytic than C-BF at 70 μM . However, the low haemolytic activity of 35-35 and 36-36 does not support the conclusion that the linker is solely to blame. Lee *et al.* (2001) has also previously found that a homodimer can be more haemolytic than its monomer, even when using a single disulphide bond to link the two monomers. This raises questions about what causes this increased attraction to erythrocytes. Potentially, there may be an alteration to the peptide's structure and response to a membrane where the AMP bypasses the initial electrostatic interaction, which causes the selective targeting to bacteria, and hydrophobic residues are more easily integrated into the membrane.

Overall, without in-depth mechanistic studies determining the cause of this increased haemolysis is impossible. However, this may not be necessary to improve the dimer's

haemolytic activity. Recent studies have suggested that amino acid modifications which disrupt the α -helical structure of AMPs can reduce their cytotoxic activities (Chen *et al.* 2005; Hawrani *et al.* 2008; Zhang *et al.* 2016). Haemolysis as an indicator of cytotoxicity is useful due to the rapid results and relative ease of the technique, but the activity of AMPs against erythrocytes may be very different to the activity of the AMPs against host cells found within the GI tract. This is of importance as the likely route of administration for these peptides would be oral. By dimerising the peptides, there were changes in efficacy and the spectrum of action when tested against pure cultures of bacterial isolates. However, to further investigate the AMPs as therapeutic agents their use in real-world environments should be simulated. Therefore, their efficacies in more complex cultures need to be tested.

5 Function of AMPs in mixed culture models

5.1 Introduction

Ensuring AMPs can work within a mixed culture environment is important, and selectivity against pathogens would provide a significant advantage over the indiscriminate therapeutic use of antibiotics which can affect large groups of bacteria, some of which may provide a beneficial effect to the host. Further to being active within a more complex culture, an ideal AMP would also be active when the bacterial density is high. If the AMP is administered orally then not only would the peptide have to retain its activity despite the presence of proteases and other harsh, abiotic factors, it also would have to be able to make a significant difference to the level of target pathogen within the gut: numbers of bacteria within the chicken gastrointestinal tract can get very high; from the crop to the caeca numbers of bacteria can range from 10^7 – 10^{11} CFU g⁻¹, with the caecal tonsils containing the highest densities (Rychlik 2020; Shang *et al.* 2018).

Viability testing of bacteria is routinely done by colony counts after plating on agar. This technique is simple and cost-effective. However, many bacteria cannot currently be cultured on available media. This means for more complex cultures, e.g. gut microbiota, background bacteria could not be comprehensively detected. Methods to identify the large number of bacterial communities which cannot be propagated on agar rely on DNA or RNA detection methods, but these cannot differentiate between live or dead cells as they will amplify whatever genetic information is present. PMA is a dye that has been developed to differentiate between live and dead cells, as intact cell membranes are impermeable to it. Here, it was attempted to use a PMA-based qPCR assay to determine whether it was an appropriate detection tool for viability

testing of bacteria after AMP treatment. While the assay proved too variable for reliable use, viable counting of bacteria after AMP treatments showed that the peptides were selectively targeting *E. coli* even within a high-density *Salmonella* culture.

5.2 Mixed culture assays

To further validate the MIC assays, and to investigate the activities of the heterodimeric AMPs in a more complex environment, the peptides were assayed in a two-bacterium mixed culture model. Adapted from the MIC assay, AMPs were used at the following concentrations: four-fold above their MIC, four-fold below their MIC, and at their MIC. These were altered in some assays based on the results from previous iterations, so a clear break between inhibition and no inhibition could be detected. The peptides were then incubated with a mixed culture of an *E. coli* serotype (either O2 or O78) and a *Salmonella* serovar (either Enteritidis or Typhimurium). A viable count was taken of both bacteria before and after incubation, and the result used to determine if the peptides remained active at the same level as seen in the Broth MIC monocultures when more than one bacterial species is present. To allow selective counting, bacteria were plated on both MHA and Brilliant Green agar. Alongside the mixed culture assay, a Broth MIC assay was performed to ensure MIC values used to determine the peptide concentrations for use were valid for that assay. All peptides investigated were found to be bactericidal when used above their MIC, demonstrating they are able to kill in growth media as well as buffered solution (as seen in the previous MBC assay), and all peptides maintained their activity against each pathogen when in a mixed culture (Figure 5-1, Figure 5-2, Figure 5-3, Figure 5-4). To ensure there were no inhibitory effects caused by growing these strains in a mixed culture, the bacteria were incubated by themselves and in mixed culture and the viable counts

after overnight incubation compared. No statistical significances were found (Figure 5-5).

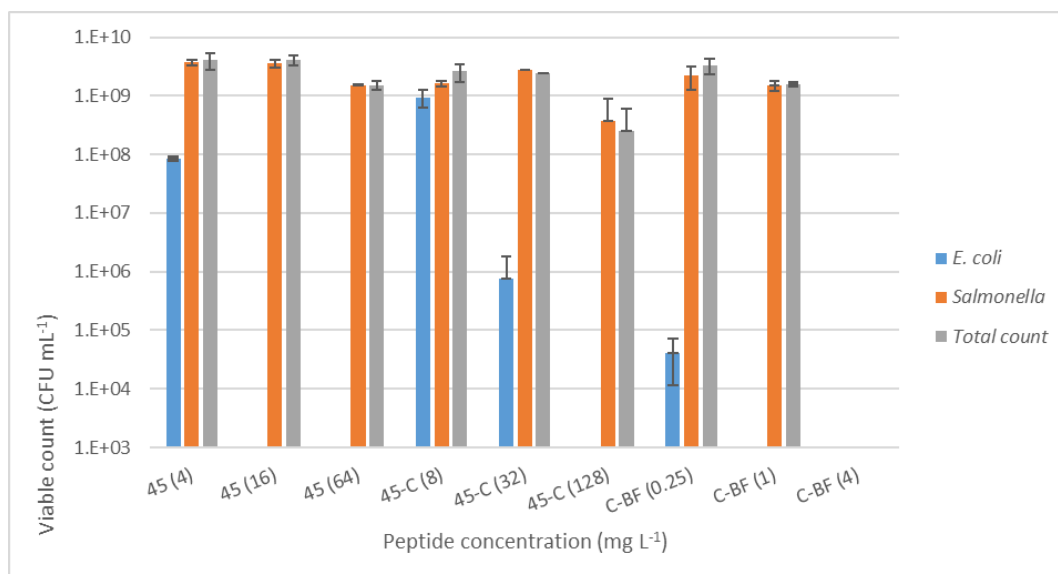


Figure 5-1. Effect of C-BF on #45's activity against *E. coli* O78 and *S. Enteritidis* in a mixed culture.

Peptides #45, 45-CBF, and C-BF were added to a mixed culture of *E. coli* O78 and *S. Enteritidis* at concentrations 4× above and below their MIC against *E. coli* O78, and at their MIC. The cultures were incubated overnight and selective viable counts were taken after incubation and the mean from both results plotted with SD. Cultures were plated onto both Brilliant Green agar for selective counting, and onto MHA for a total viable count.

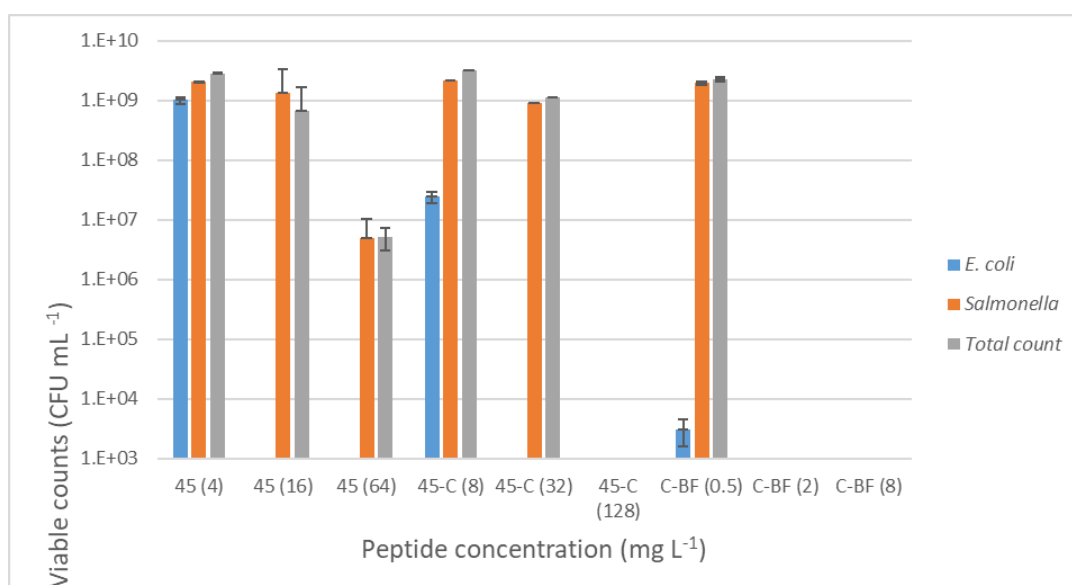


Figure 5-2. Effect of C-BF on #45's activity against *E. coli* O2 and *S. Typhimurium* in a mixed culture.

Peptides #45, 45-CBF, and C-BF were added to a mixed culture of *E. coli* O2 and *S. Typhimurium* at concentrations 4× above and below their MIC against *E. coli* O2, and at their MIC. The cultures were incubated overnight and selective viable counts were taken after incubation and the mean from both results plotted with SD. Cultures were plated onto both Brilliant Green agar for selective counting, and onto MHA for a total viable count.

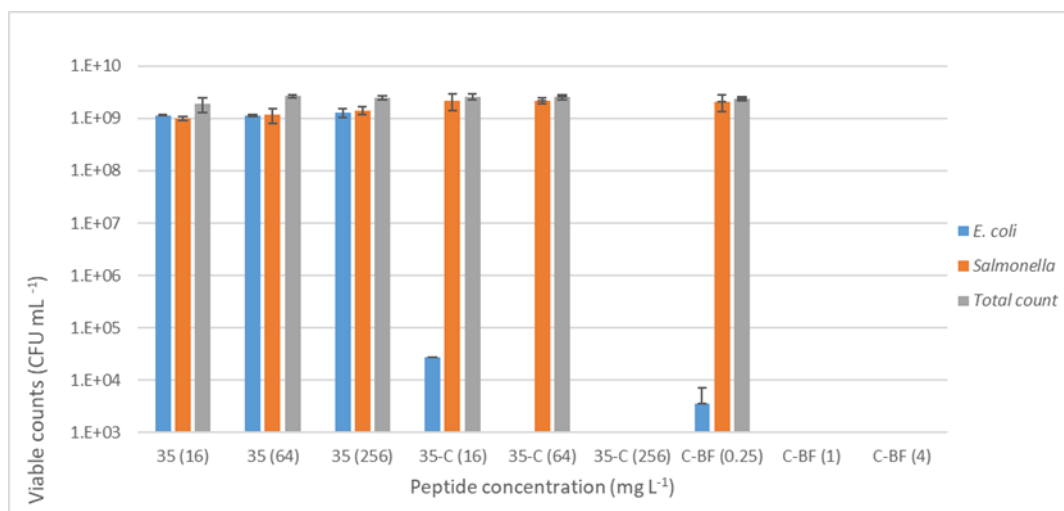


Figure 5-3. Effect of C-BF on #35's activity against *E. coli* O2 and *S. Typhimurium* in a mixed culture.

Peptides #35, 35-CBF, and C-BF were added to a mixed culture of *E. coli* O2 and *S. Typhimurium* at concentrations 4× above and below their MIC against *E. coli* O2, and at their MIC. The cultures were incubated overnight and selective viable counts were taken after incubation and the mean from both results plotted with SD. Cultures were plated onto both Brilliant Green agar for selective counting, and onto MHA for a total viable count.

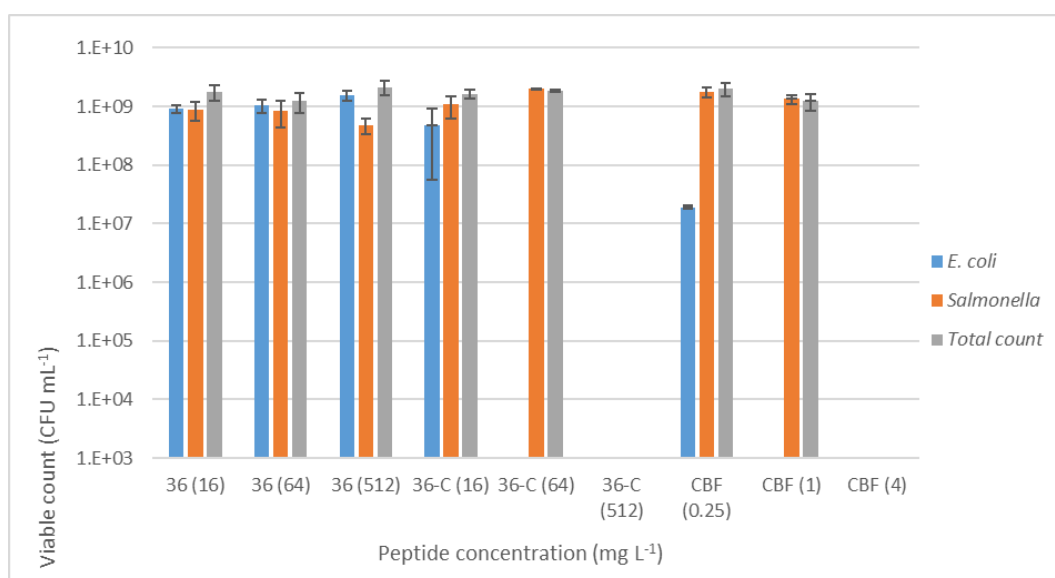


Figure 5-4. Effect of C-BF on #36's activity against *E. coli* O78 and *S. Typhimurium* in a mixed culture.

Peptides #36, 36-CBF, and C-BF were added to a mixed culture of *E. coli* O78 and *S. Typhimurium* at concentrations approximately 4× above and below their MIC against *E. coli* O78, and at their MIC. The cultures were incubated overnight and selective viable counts were taken after incubation and the mean from both results plotted with SD. Cultures were plated onto both Brilliant Green agar for selective counting, and onto MHA for a total viable count.

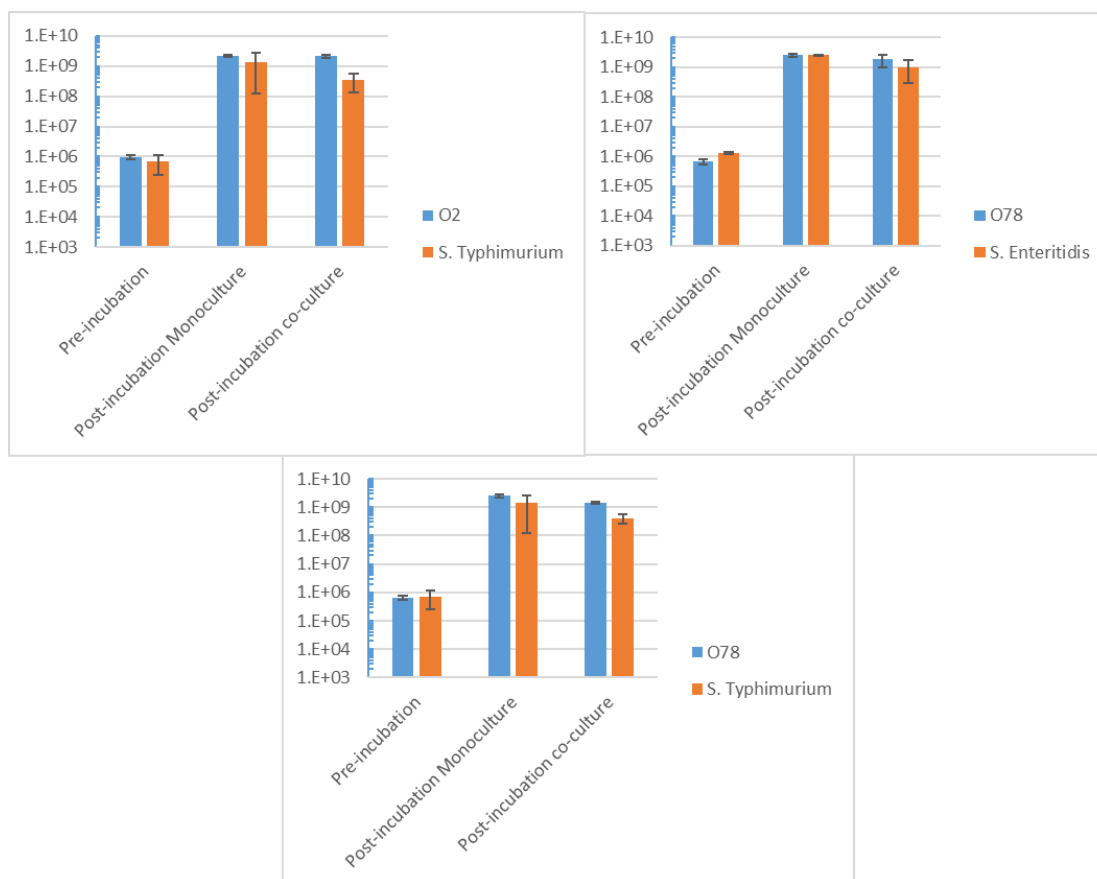


Figure 5-5. Effects of growing *E. coli* and *Salmonella* strains in co-culture on viable counts.

Each bacterial strain combination used for mixed culture assays: *E. coli* O2 and *S. Typhimurium*, *E. coli* O78 and *S. Enteritidis*, *E. coli* O78 and *S. Typhimurium*, were investigated for inhibitory effects due to the co-culturing methods. After overnight incubation in MHB, an unpaired t-test was used to determine whether the viable counts of each bacterial isolate was significantly different when grown in a mixed culture compared to in a monoculture. No statistical significances were found.

5.3 v-PCR detection and viable counts of AMP-treated *E. coli*

To investigate each dimeric AMP's effectiveness in more complex cultures, first a reliable detection method was needed. The method would need to robustly detect changes in viability of a target pathogen. The viability dye PMAxx (Biotium, US) is a commercially-produced improvement upon the viability dye PMA. PMA allows live/dead differentiation using DNA amplification techniques such as qPCR by a dual mode of action: by binding to the DNA of permeable dead bacteria PMA not only

inhibits template amplification, but also renders the DNA insoluble and so it is removed during purification (Nocker, Cheung, and Camper 2006). Plasmid DNA was extracted from *E. coli* O78 and PCR amplified using primers for various APEC-associated genes (Figure 5-6). Of those tested, *iss*, *iucC*, *cvaC* and *hylF* were amplified efficiently by PCR. The *cvaC* gene was selected as the target gene for O78 detection. Plasmids carrying this gene often carry other virulence genes which perform functions such as iron uptake or the production of temperature-sensitive haemagglutinin (Ewers *et al.* 2007; Williams and Warner 1980), both of which are often associated with APEC strains (Johnson *et al.* 2006).

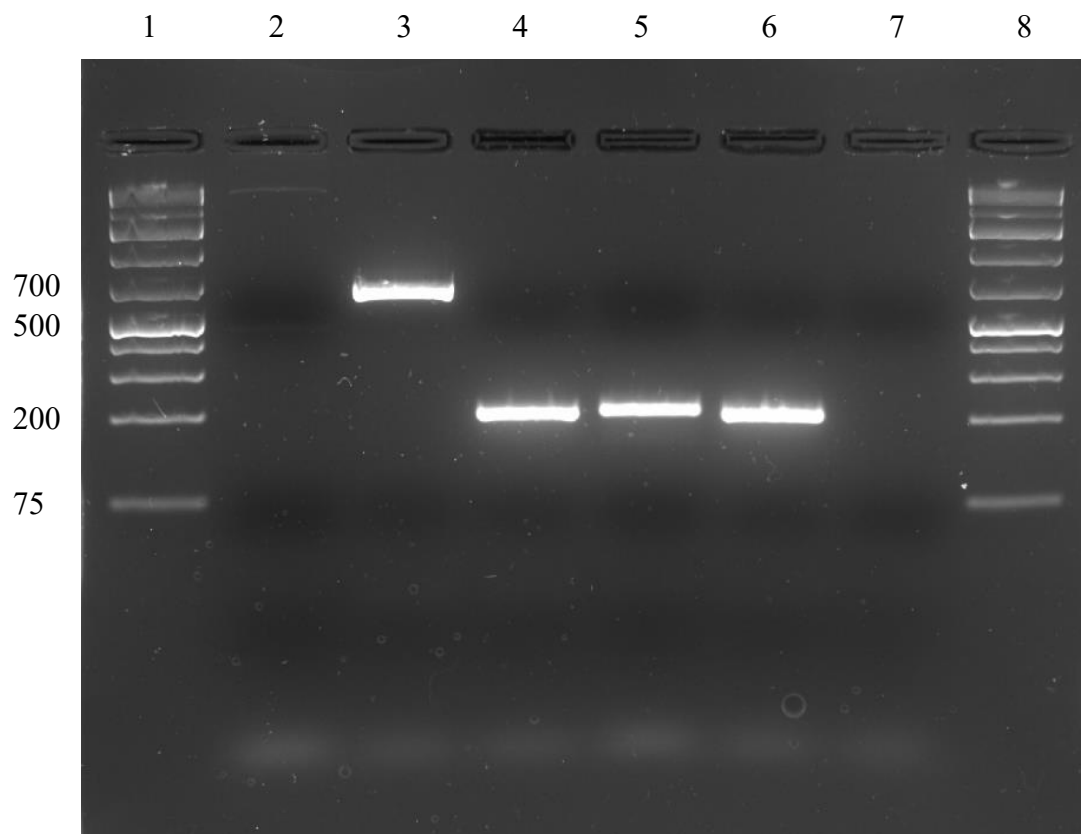


Figure 5-6. PCR amplification of various APEC-associated genes from *E. coli* O78 plasmid DNA.

Overnight cultures of *E. coli* O78 were centrifuged and plasmid DNA extracted. Then, five sets of primers were used in five separate PCR reactions to amplify various APEC-associated genes. 1, 8 – GeneRuler 1 Kb Plus DNA ladder, 2 – *tsh*, 3 – *iss*, 4 – *iucC*, 5 – *cvaC*, 6 – *hylF*, 7 – Negative control.

Once the target gene was chosen, the limit of detection for the qPCR assay was investigated by using serially diluted cultures of *E. coli* O78. Starting at 10^5 CFU and diluting by 10-fold down to 10 CFU, the plasmid DNA extracted from each was amplified via qPCR using the KiCqStart SYBR Green qPCR ReadyMix (KCQS00; Sigma-Aldrich). The resultant amplification and threshold values showed that 10^3 CFU was the minimum number of cells needed to obtain enough plasmid DNA for reliable detection (Figure 5-7).

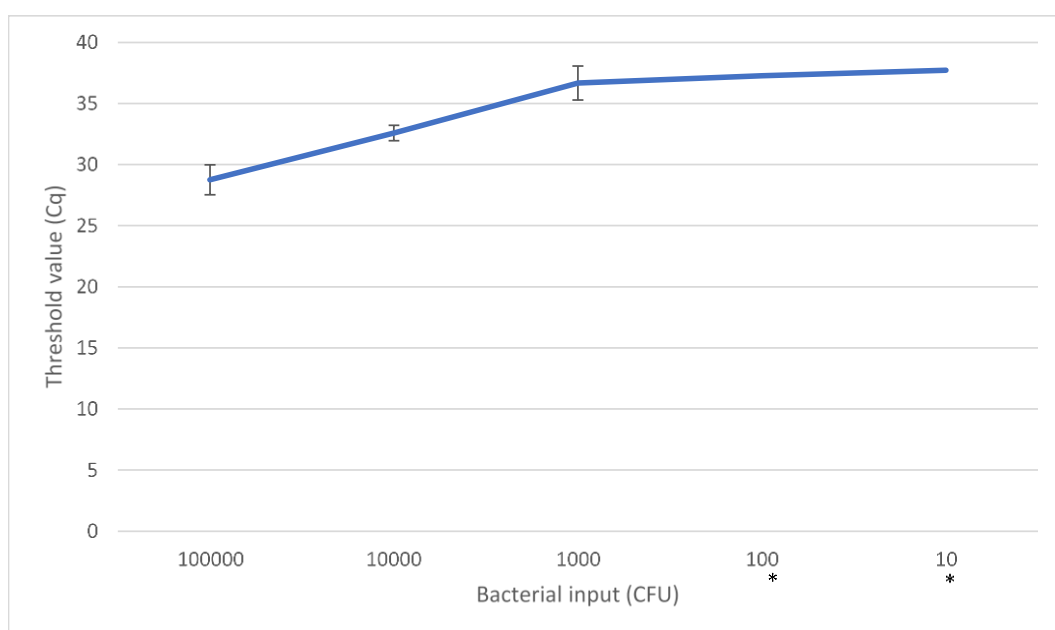


Figure 5-7. Detection threshold of *E. coli* O78 populations for reliable qPCR amplification.

A culture of *E. coli* O78 at 10^5 CFU was diluted in 10-fold steps down to 10 CFU. The cultures were then incubated for one hour, to mimic the PMA protocol, and then plasmid DNA extracted and amplified via qPCR. The mean average of the quantification cycle (Cq) for each replicate was calculated and then plotted with standard deviation.

* - only one of the replicates produced a Cq value.

With the qPCR amplification's limit of detection established, use of the PMAxx dye was then validated for use in differentiating live/dead cultures of *E. coli* O78. Cultures of *E. coli* O78 in MHB were grown to mid-log and then diluted down to between 1×10^6 CFU mL⁻¹ and 5×10^6 CFU mL⁻¹. Selected AMPs were then added at 4× MIC and

incubated for one hour. After the incubation, 10 µL from each culture was taken for viable count analysis before PMAxx treatment and the purified DNA amplified using qPCR. The Cq values showed that while the general trend followed the viable counts, there was a number of discrepancies (Figure 5-8, Figure 5-9).

For example, the Cq values for #35 indicates that this AMP has an effect on viability in MHB when the viable counts suggest otherwise. Indeed, #35 showed a significant difference between the means of PMAxx+ and PMAxx- treatments ($p=0.011$), when analysed by ANOVA (Appendix 9.6.2). Conversely, there was no significant difference between the PMAxx+/- treatments of 35-CBF and #45 ($p=0.0807$ and $p=0.9859$). A consideration of note is that for #45 this may have been due to the replacement of missing values with the maximal Cq value of 40 (ultimate cycle) for statistical purposes, as without appropriate replicates analyses could not be performed. When the two internal controls are considered, there was no significant difference between DMSO +/- PMAxx and for the DEAD control there is ($p=0.0204$), which suggests that the PMAxx treatment bound DNA appropriately according to viability status. However, when comparing only the PMAxx+ treatments to the DMSO control, there was no significant difference for #35 ($p=0.1121$) whereas every other treatment result was significantly different ($p\leq 0.032$). As the viable count data corroborated the comparisons to the DMSO control, and no significant difference was observed between the PMAxx- DMSO control and any other treatment, this was decided to be the most appropriate way to analyse v-PCR results (Appendix 9.6.2).

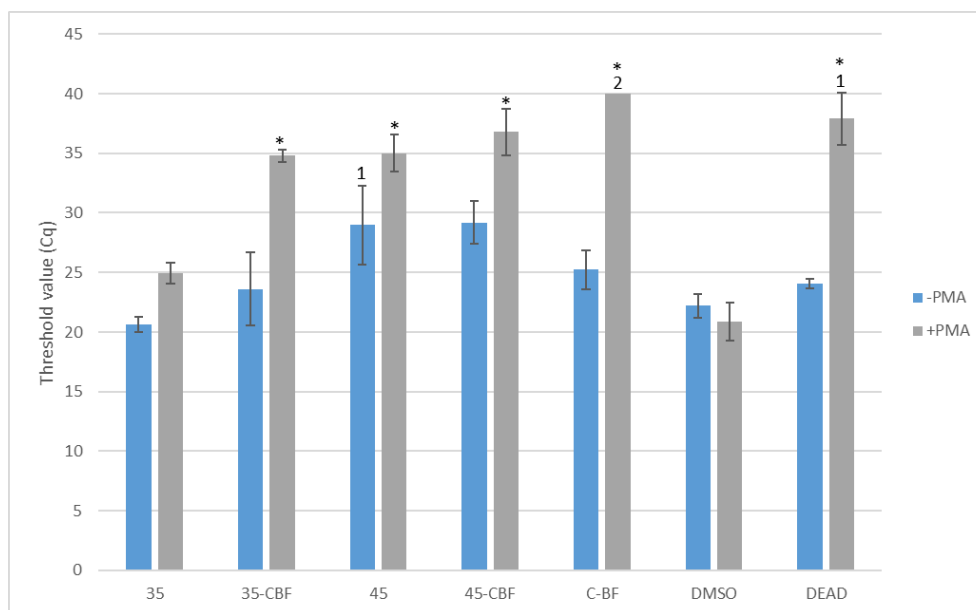


Figure 5-8. Effect of monomeric and dimeric AMP's on the viability of *E. coli* O78, as determined by v-PCR.

Cultures of *E. coli* O78 were grown to approximately 1×10^6 CFU mL⁻¹, then monomeric and dimeric AMPs were added (triplicate for each condition) at 4× MIC. The cultures were then incubated for one hour alongside a solvent (DMSO) and positive (DEAD) control before PMAxx dye and PMA enhancer were added and photoactivated. Plasmid DNA was then extracted and used as the template for v-PCR with the Cq value plotted with standard deviation. The +PMA Cq values between the DMSO-treated group and AMP-treated groups were analysed by Welch's one-way ANOVA with multiple comparisons. Only peptide 35 was not significantly different ($p=0.1121$).

1 – Only two replicates reached a Cq value.

2 – Only one replicate reached a Cq value.

* - significantly different to +PMA solvent control

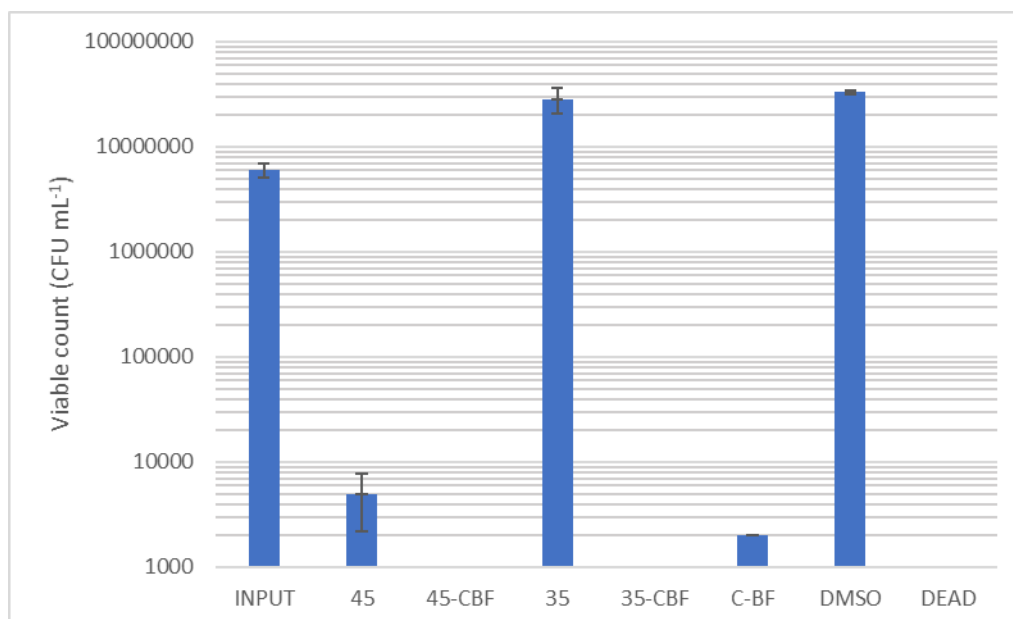


Figure 5-9. Effect of monomeric and dimeric AMP's on the viability of *E. coli* O78, as determined by viable count.

Cultures of *E. coli* O78 were grown to approximately 1×10^6 CFU mL⁻¹, then monomeric and dimeric AMPs were added in triplicate at 4× MIC and the cultures incubated for one hour alongside a solvent (DMSO) and positive (DEAD) control. Before addition of AMPs 10 µL of the *E. coli* O78 culture was taken for viable counts. After one hour incubation with each treatment, 10 µL from two of three replicates for each culture was taken for viable counting.

Interestingly, when the bacterial density of *E. coli* was increased from 10^6 CFU mL⁻¹ to $\geq 10^8$ CFU mL⁻¹ to test the AMP's efficacy in a high-density culture, the AMPs had negligible-to-no effect on the viable counts (Figure 5-10). When the bacterial density reaches concentrations of 10^8 CFU mL⁻¹, the AMPs fail to cause the same high level of killing as seen when bacterial density is at 10^6 CFU mL⁻¹. This could be due to irreversible binding to cell membranes, as to create pores in susceptible membranes AMPs often translocate into the lipid bilayer; once an AMP has inserted itself into the membrane it might not be energetically favourable for it to dissociate from the membrane. However, the statistical analyses indicated that more replicates were needed for a robust analysis: streptomycin treatment results were significantly different from the DMSO treatment across both one and 48 hour time points

($p=0.0285$, $p=0.0038$), yet not 16 ($p=0.0501$). This was likely due to a large range calculated between the three replicates of approximately 10^4 CFU mL⁻¹. Treatment with C-BF was also suggested to affect normal survival and/or propagation as there was a significant difference between C-BF and the DMSO control at the 48 hour time point. To determine if this latter difference was truly significant, and that C-BF has an effect on survivability, would need to be investigated by repeat experiments.

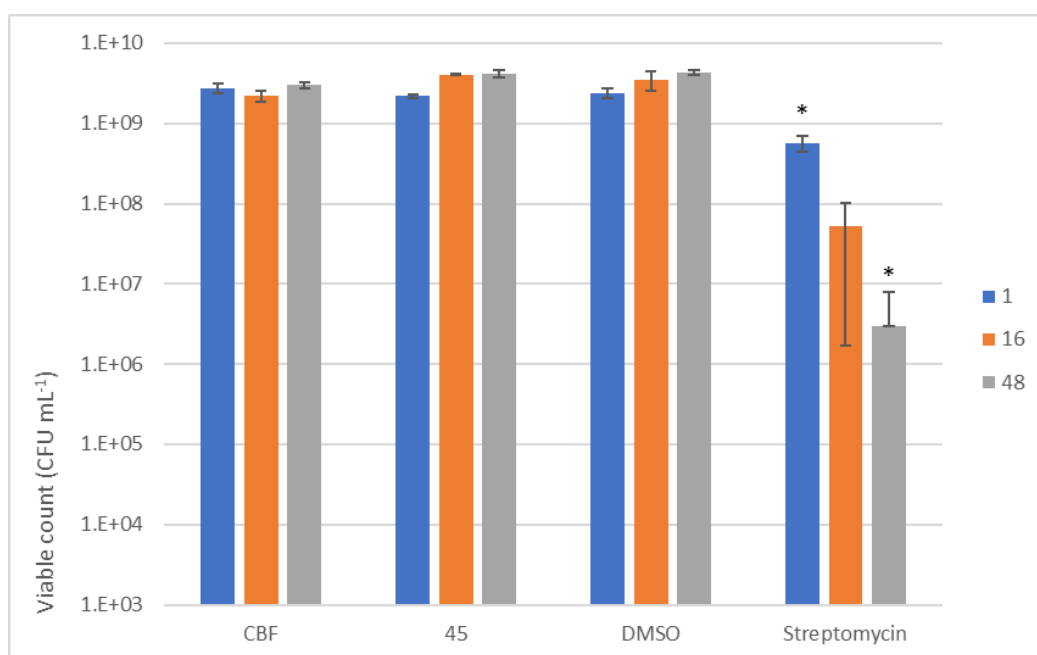


Figure 5-10. Effect of C-BF and #45 on *E. coli* O78 at culture concentration $\sim 5 \times 10^8$ CFU mL⁻¹ as determined by viable count.

Cultures of *E. coli* O78 were grown to an OD₆₀₀ of 1, which equates to $\sim 5 \times 10^8$ CFU mL⁻¹, then C-BF and #45 were added (triplicate for each condition) at 4× MIC and the cultures incubated alongside a solvent (DMSO) and positive (Streptomycin) control. Cultures were incubated for a total of 48 hours, with 10 µL from each culture taken for viable counting at 1, 16 and 48 hour post-treatment. Viable counts were obtained from a single dilution from each replicate within a triplicate. The mean average for each AMP treatment was calculated and plotted per time point with standard deviation. Welch's one-way ANOVA was used to determine significance of any differences in viable counts when compared to the DMSO control. No AMP showed any significant difference.

* - significantly different to solvent control

When this experiment was repeated to determine if the v-PCR would detect a similar trend, with the cultures centrifuged at one hour post-treatment after viability dye

mixing and photoactivation, the results displayed a discrepancy observed in a previous assay: two replicates of a PMAxx- treatment group (DMSO) failed to reach the threshold for a Cq value (Figure 5-11). As high, or non-existent, Cq values would normally indicate a high level of amplification inhibition, and therefore viability reduction, this anomalous result had implications on the analysis. No treatments were observed to be statistically different after PMAxx treatment and the missing values, which were replaced with a Cq value of 40, were likely the major contributing factor in this. Further repeats of this experiment may yield fewer discrepancies, and could require investigation.

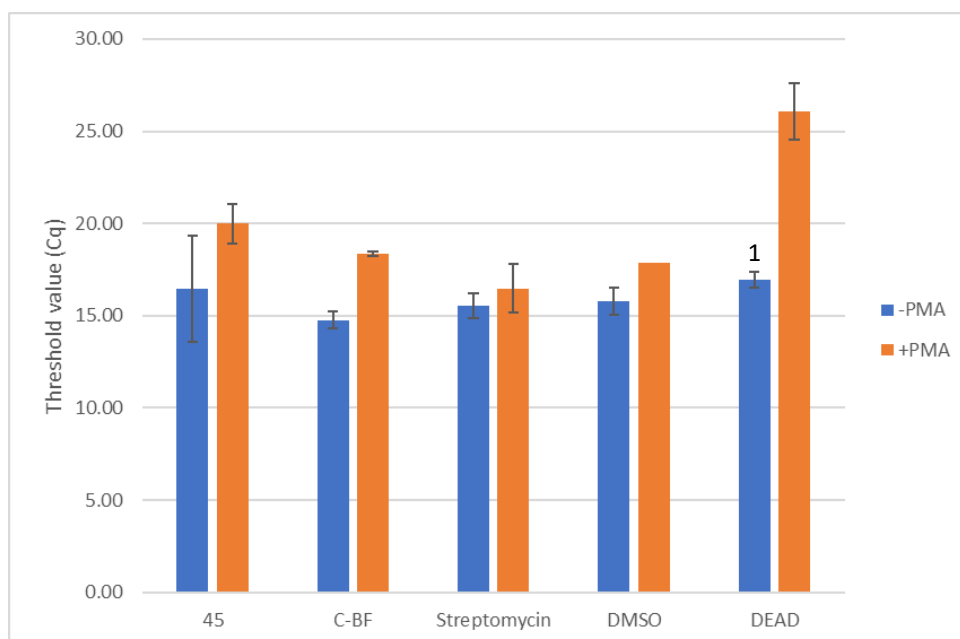


Figure 5-11. Effect of C-BF and #45 on *E. coli* O78 at culture concentration $\sim 5 \times 10^8$ CFU mL⁻¹ as determined by v-PCR.

Cultures of *E. coli* O78 were grown to an OD₆₀₀ of 1, which is equivalent to $\sim 5 \times 10^8$ CFU mL⁻¹, then C-BF and #45 were added (triplicate for each condition) at 4× MIC and the cultures incubated for one hour alongside a solvent (DMSO) and positive (DEAD) control. After incubation, the plasmid DNA was extracted and used as the template for v-PCR. The mean average of the Cq values for each AMP treatment was calculated and plotted with standard deviation. Welch's one-way ANOVA with multiple comparisons was used to determine any significant differences between the +PMA DMSO control and the +PMA peptide treatment groups. No statistically significant differences were reported.

1 – only one Cq value reported.

The general lack of activity was hypothesised to be due to the inoculum effect, where the MIC for an antimicrobial can vary depending on the density of cells present (Loffredo *et al.* 2021). While an increased number of cells can cause the MIC to increase concomitantly, a decrease in the number of cells from the standard 10^5 CFU mL⁻¹ does not tend to decrease the MIC. The authors asserted that, for AMPs, this was based on two factors: the threshold number of peptides needed to exert the necessary amount of antimicrobial activity for cell death, and the number of cell-associated peptides. To investigate, C-BF at varying concentrations was mixed with *E. coli* O78 at 10^8 CFU mL⁻¹. Peptide concentrations started at the MIC value, and were then increased to MIC $\times 10$, MIC $\times 100$, and MIC $\times 1000$. Both the viable counts and the v-PCR results suggest that the concentration of peptide needed for cell death increases linearly with cell density (Figure 5-12).

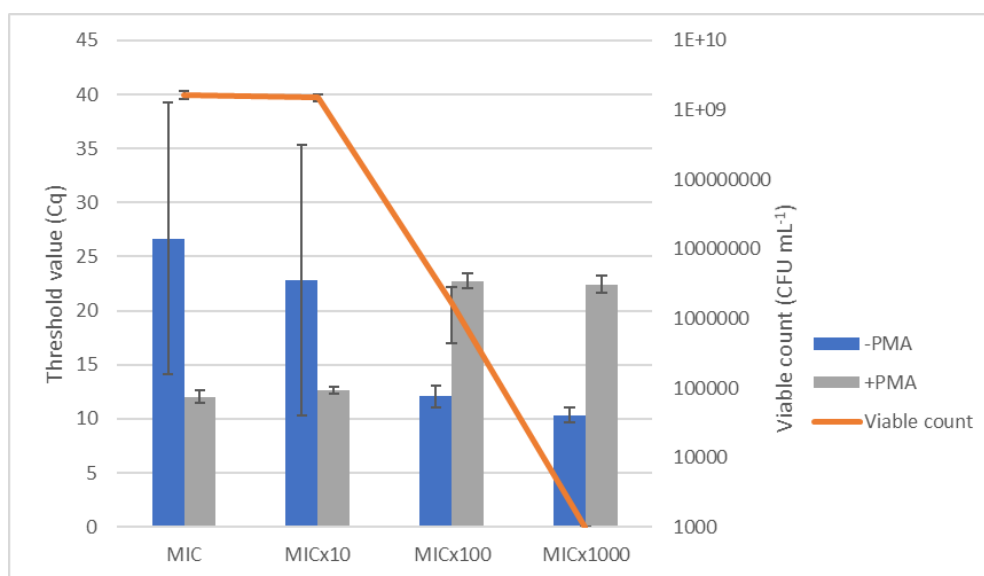


Figure 5-12 Effect of increasing C-BF concentrations on v-PCR detection and viable counts of high density *E. coli* O78 cultures

Cultures of *E. coli* O78 were grown to an OD₆₀₀ of 1, which is equivalent to $\sim 5 \times 10^8$ CFU mL⁻¹, then C-BF was added in triplicate at 1× MIC, ×10 MIC, ×100 MIC, and ×1000 MIC and the cultures incubated for one hour. After incubation, 10 µL from each culture was taken for viable counting then the plasmid DNA was extracted from the pellet and used as the template for v-PCR. The mean average of the Cq values for each AMP treatment was calculated and plotted with standard deviation on the primary axis. Viable counts were obtained from duplicate plates of three independent cultures, with the mean average calculated and plotted with standard deviation.

The loss of detectable antimicrobial activity when administered within a high bacterial density was investigated further to determine if the AMPs could show selectivity against a target bacteria within a high density background (a scenario that would mimic a natural infection model). When AMPs were previously applied in mixed cultures of equal numbers of *E. coli* and *Salmonella*, #45, #35, and C-BF and/or their dimers displayed some preferential killing of *E. coli* which related to their MIC against the separate pathogens. Here, cultures of *E. coli* O78 and *S. Enteritidis* were grown to an OD of 1. Then, a 1:100 dilution of the *E. coli* culture was made by spiking the *S. Enteritidis* culture with it. This created a high density background of *S. Enteritidis*, but with a concentration of *E. coli* O78 similar to that used in the MIC assays (10⁶ CFU mL⁻¹). A second mixed culture was also made, where the *E. coli* O78 culture was

diluted 1:100 before spiking into the *S. Enteritidis* culture. This created a final concentration of 10^4 CFU mL⁻¹ of *E. coli* within the high background of *S. Enteritidis*.

These two mixed cultures were incubated, in triplicate, with the monomeric peptides #45 and C-BF at 4× MIC. After one hour samples were taken for viable counts and the rest of the cultures centrifuged, the supernatant discarded, and the plasmid DNA extracted for v-PCR. Results suggested that the AMPs used here can still be active against a susceptible bacteria even when there is a high level of background bacteria. Data from the v-PCR suggested that only C-BF with an initial inoculum of 10^4 CFU mL⁻¹ *E. coli* O78 robustly decreased the viability of the spiked bacteria (Figure 5-13). However, when the viable counts were calculated it indicated that both C-BF and #45 were able to kill >90% of the spiked *E. coli* when the initial inoculum was 10^4 CFU mL⁻¹, and intimated that similar levels would be seen at 10^6 CFU mL⁻¹ as there was no DMSO control for this cell density (Figure 5-14). Statistical analysis of the 10^4 CFU mL⁻¹ results confirmed that only C-BF, and not #45, significantly affected the viability of *E. coli* O78 via v-PCR. Analysis also showed that both AMP treatments significantly lowered the viability of *E. coli*, without affecting *Salmonella*, when determined by viable count (#45, $p=0.004$; C-BF, $p=0.0036$).

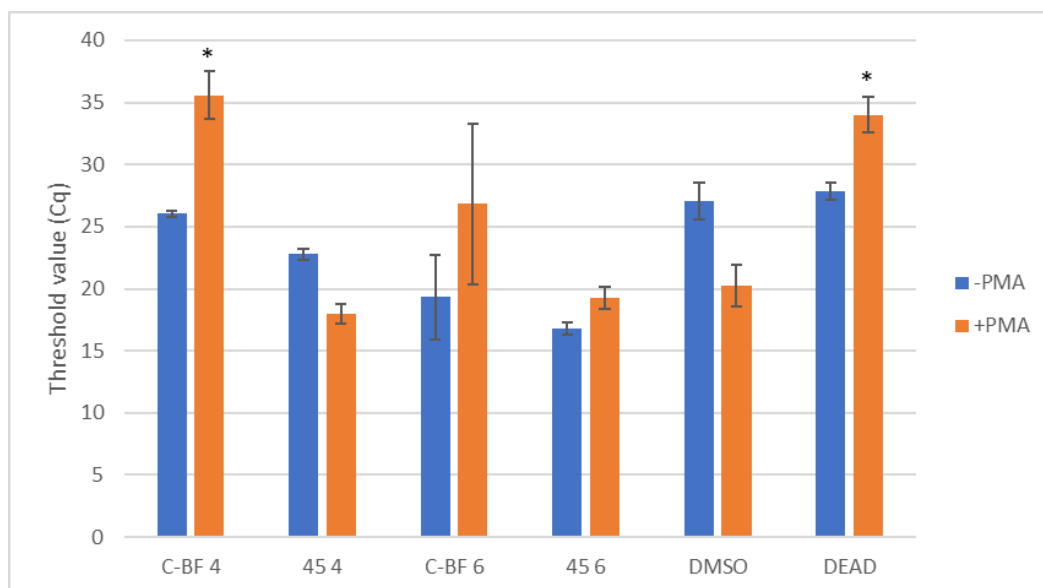


Figure 5-13. v-PCR assay of spiked *E. coli* O78 within a high density *S. Enteritidis* background

Cultures of *E. coli* O78 and *S. Enteritidis* were grown to an OD₆₀₀ of 1, then *E. coli* was spiked into *S. Enteritidis* for a final concentration of either 10⁴ *E. coli* present, or 10⁶ *E. coli* present. In triplicate analyses, C-BF and #45 were then added at 4× MIC (calculated against *E. coli* O78) and the cultures incubated for one hour with a solvent (DMSO) and positive (DEAD) control. After incubation, the plasmid DNA was used as the template in v-PCR targeting *cvaC*. The mean average of the Cq values for each AMP treatment was calculated and plotted, along with the total input bacteria before treatment, with standard deviation. Welch's one-way ANOVA with multiple comparisons was used to determine that only one AMP (C-BF), with an initial inoculum of 10⁴ CFU mL⁻¹, was significantly different to the solvent control.

* - significantly different to solvent control

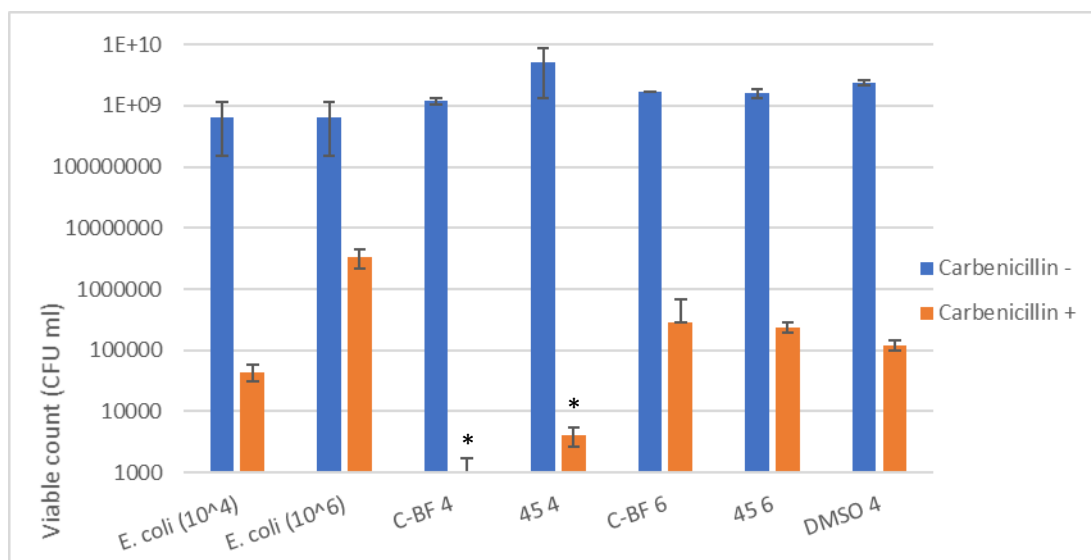


Figure 5-14. Viable counts of spiked *E. coli* O78 within a high density *S. Enteritidis* background.

Cultures of *E. coli* O78 and *S. Enteritidis* were grown to an OD₆₀₀ of 1, then *E. coli* was spiked into *S. Enteritidis* for a final concentration of either 10⁴ *E. coli* present, or 10⁶ *E. coli* present. In triplicate analyses, C-BF and #45 were then added at 4× MIC (calculated against *E. coli* O78) and the cultures incubated for one hour with a solvent (DMSO) and positive (DEAD) control. Viable counts were taken from both mixed cultures before treatment and from all treatments after the one hour incubation. Selective plating for *E. coli* occurred on MHA supplemented with 100 µg mL⁻¹ Carbenicillin. Viable counts were taken from duplicate samples. Welch's one-way ANOVA indicated significant differences between the solvent control and the two peptide-treated groups with an *E. coli* initial inoculum at 10⁴. No solvent control at 10⁶ initial inoculum prevented statistical analyses for those comparisons.

* - significantly different to solvent control

Due to the discrepancies seen in the v-PCR assays and the viable counts, optimisation of the v-PCR protocol was attempted. Cultures of *E. coli* O78 were grown to OD 1, and then diluted 1:100. The diluted culture was then split into three treatment groups: C-BF, DMSO, or DEAD (heat-treated at 95°C for five minutes). During the PMAxx treatment, the factors altered comprised volume of PMAxx dye added and the photoactivation period. By modifying the volume of PMAxx added, the number of dye molecules that successfully bind the DNA of dead cells may be increased. The photoactivation period was altered to determine if the apparatus used was efficient enough, as the recommended blue light-emitting device was not used. Results showed

that there was no difference between any of the altered treatments (Figure 5-15, Figure 5-16). By using C-BF at $4\times$ MIC against *E. coli* at 10^6 CFU mL⁻¹, to mimic assay conditions, any treatment options which could increase the difference in Cq value between the control and PMA-treated cells would be observed and utilised in further v-PCR assays. However, neither PMAxx volume nor length of photoactivation had an impact on Cq values with no significance observed between the altered treatments. Therefore, it was concluded that the v-PCR assay could not be optimised sufficiently to reduce the inter- and intra-assay variation observed in earlier assays and further experiments utilised viable counts for live/dead differentiation.

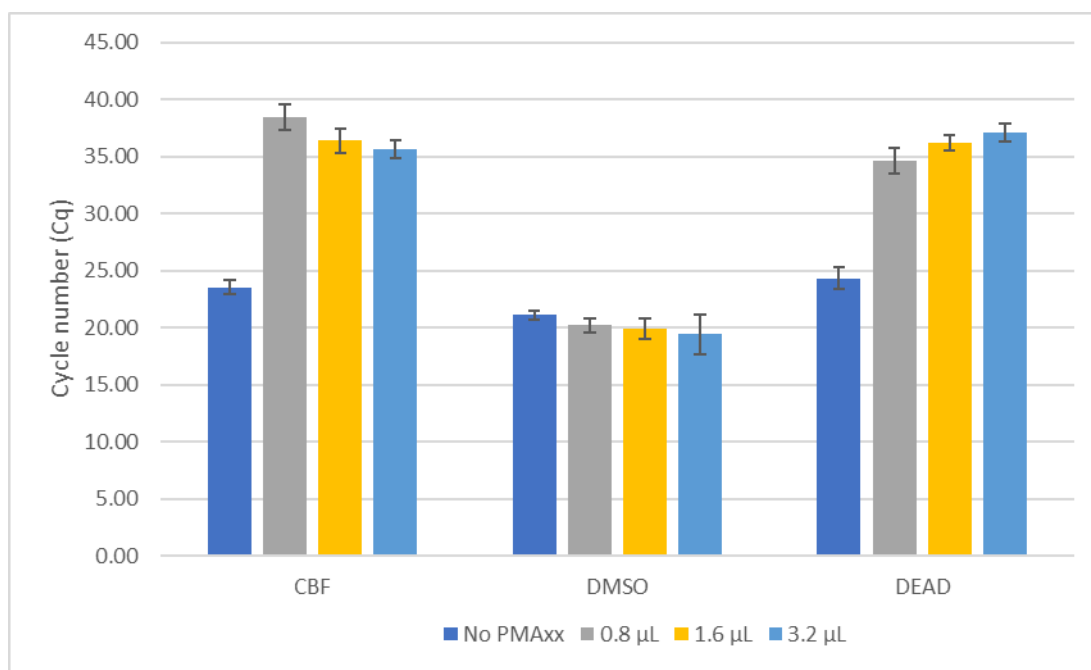


Figure 5-15. PMAxx optimisation via altered dye volumes.

Cultures of *E. coli* O78 were grown to OD 1, and then diluted 1:100. The diluted culture was split into three treatment groups: C-BF, DMSO (solvent control), and DEAD (heat-treated at 95°C for five minutes). Each group was tested in triplicate with C-BF added at $4\times$ MIC. After one hour incubation, PMAxx dye was added at either 0.8, 1.6, or 3.2 µL, photoactivated, and then the plasmid DNA was extracted for use as the template in the v-PCR assay. The mean average from each group was calculated and plotted with standard deviation. A one-way Welch's ANOVA with multiple comparisons was used to determine that there was no statistical difference between different volumes of PMAxx dye added.

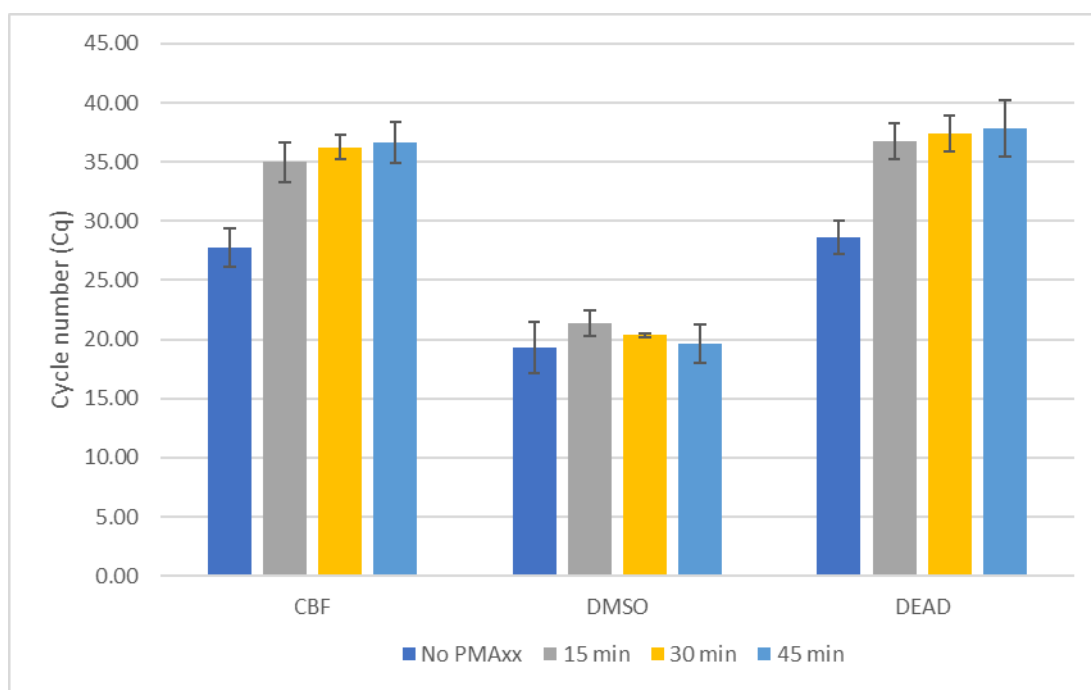


Figure 5-16. PMAxx optimisation via altered photoactivation periods.

Cultures of *E. coli* O78 were grown to OD 1, and then diluted 1:100. The diluted culture was split into three treatment groups: C-BF, DMSO (solvent control), and DEAD (heat-treated at 95°C for five minutes). Each group was tested in triplicate with C-BF added at 4× MIC. After one hour incubation, PMAxx dye was added and photoactivated for either 15, 30, or 45 minutes, and then the plasmid DNA was extracted for use as the template in the v-PCR assay. The mean average from each group was calculated and plotted with standard deviation. A one-way Welch's ANOVA with multiple comparisons was used to determine that there was no statistical difference between different volumes of PMAxx dye added.

During repeats of a spiking experiment, the activity of #45 was found to possibly be bacteriostatic rather than bactericidal in some instances, where the starting target bacterial numbers are maintained over the one hour incubation rather than being reduced (bactericidal) or allowing bacterial propagation (no effect, as for the DMSO control) (Figure 5-17). This suggested the selectivity displayed by #45 may be more susceptible to high densities than C-BF, with the necessary threshold number of peptides for cell death not being reached on every occasion. However, the statistical analyses still strongly suggest a significant difference between the DMSO control and both of the AMP treatments, regardless of starting inoculum ($p < 0.0001$).

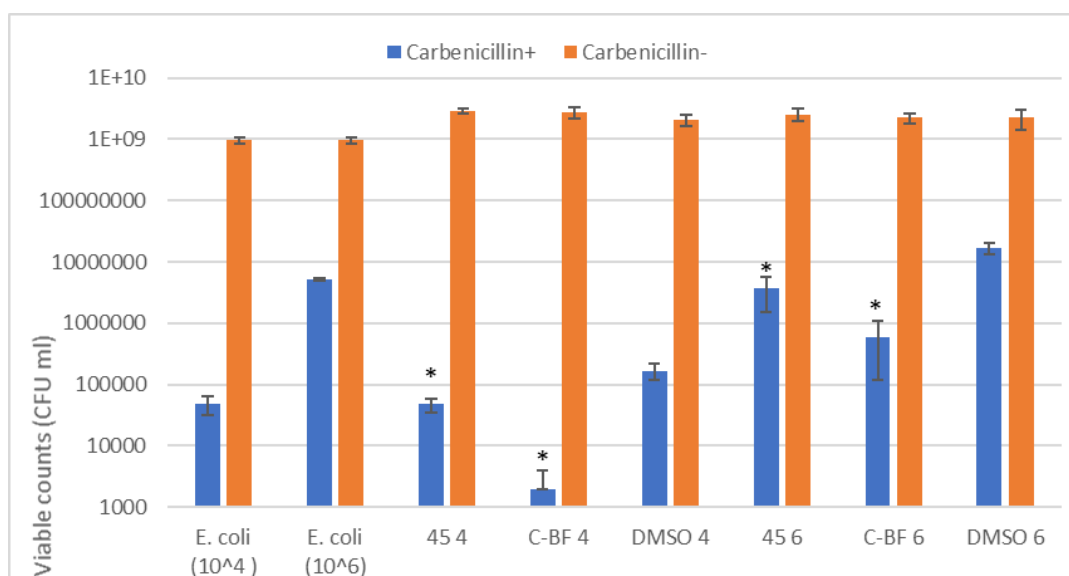


Figure 5-17. Further investigation of antimicrobial activity of #45 and C-BF in high density mixed cultures.

Cultures of *E. coli* O78 and *S. Enteritidis* were grown to an OD₆₀₀ of 1, then *E. coli* was spiked into *S. Enteritidis* for a final concentration of either 10⁴ *E. coli* present, or 10⁶ *E. coli* present. In replicates of five, C-BF and #45 were then added at 4× MIC (calculated against *E. coli* O78) and the cultures incubated for one hour with a solvent (DMSO) control. Viable counts were taken from both mixed cultures before treatment and from all treatments after the one hour incubation. Selective plating for *E. coli* occurred on MHA supplemented with 100 µg mL⁻¹ Carbenicillin. Viable counts were taken from duplicate samples for each replicate. Welch's one-way ANOVA indicated significant differences between the solvent control and the two peptide-treated groups for both initial *E. coli* inoculums.

* - significantly different to solvent control

Next, the assay was repeated with the heterodimeric AMPs 35-CBF and 45-CBF. The heterodimers were tested at 4× MIC and were found to have potent, selective activity against *E. coli* O78 (Figure 5-18). Both 35-CBF and 45-CBF reduced numbers of *E. coli* below the detectable limit for the viable counts (<1,000 CFU mL⁻¹) when the initial inoculum was 10⁴ CFU mL⁻¹. When the inoculum was increased to 10⁶ CFU mL⁻¹ the heterodimer 45-CBF successfully reduced the *E. coli* concentration by >99% when compared to the DMSO control, with 35-CBF reducing it by >99.9%. All treatments across both inoculum densities were found to reduce bacterial counts significantly (p<0.0001), with the exception of 35-CBF at 10⁴ CFU mL⁻¹ which was not included due to all counts being 0.

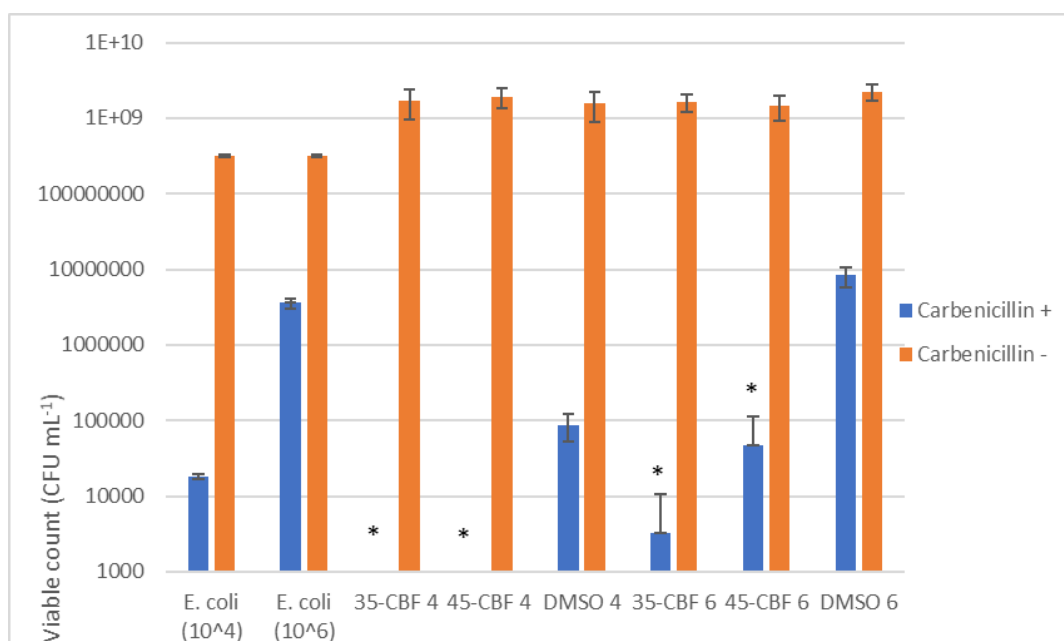


Figure 5-18. Heterodimer activity in high density mixed culture.

Cultures of *E. coli* O78 and *S. Enteritidis* were grown to an OD₆₀₀ of 1, then *E. coli* was spiked into *S. Enteritidis* for a final concentration of either 10⁴ *E. coli* present, or 10⁶ *E. coli* present. In replicates of five, 35-CBF and 45-CBF were then added at 4× MIC (calculated against *E. coli* O78) and the cultures incubated for one hour with a solvent (DMSO) control. Viable counts were taken from both mixed cultures before treatment and from all treatments after the one hour incubation. Selective plating for *E. coli* occurred on MHA supplemented with 100 µg mL⁻¹ Carbenicillin. Viable counts were taken from duplicate samples for each replicate. Welch's one-way ANOVA indicated significant differences between the solvent control and the two peptide-treated groups for both initial *E. coli* inoculums.

* - significantly different to solvent control

When the data from the bacterial spike assays were collated and the monomers C-BF and #45 compared to the heterodimers 45-CBF and 35-CBF, as the two most potent AMPs from each category in MHB, there is a clear difference between #45 and the two heterodimers in terms of inhibition of *E. coli*. A difference between C-BF and the two heterodimers is also indicated, but repeated experiments with appropriate statistical analysis would be needed to verify this. For example, where the viable counts of 45-CBF and 35-CBF-treated *E. coli* are ≥99.8% below that of the DMSO-treated group when the initial inoculum of *E. coli* was 10⁴ CFU mL⁻¹, #45 only achieved a 72% reduction (Table 5-1). The monomer C-BF achieves slightly less than

the heterodimers, at 99%, but notably more than #45. A similar trend was seen when the initial inoculum of *E. coli* was 10^6 CFU mL⁻¹, where the viable counts of *E. coli* of the heterodimer-treated groups was reduced by $\geq 99\%$ when compared to the DMSO-treated groups (Table 5-2). However, while the starting concentrations of each of the bacteria were kept as similar as possible throughout the experiments, the assays should be repeated with all AMPs used in one experiment to reduce any variability introduced by different growth rates. Interestingly, there was a slight decrease in the viable counts of *S. Enteritidis* with 10^6 CFU mL⁻¹ *E. coli* O78 spiked into the culture and treated with either 35-CBF or 45-CBF, when compared with the DMSO-treated control (Table 5-2). This reduction was only significantly different for 45-CBF ($p=0.0119$). A consideration for this putative effect is that this reduction was of a similar proportion to the positive effect on *S. Enteritidis* (when spiked with 10^4 CFU mL⁻¹ *E. coli*) growth seen in the monomer-treated groups (Table 5-1). Therefore, it may be an artefact of sampling bias and repeated experiments would be needed to determine if the effect is constant.

Table 5-1. AMP activity in high density *S. Enteritidis* when spiked *E. coli* inoculum is 10^4 CFU mL⁻¹

	<i>E. coli</i> difference (% of starting inoculum)	<i>Salmonella</i> difference (% of starting inoculum)	<i>E. coli</i> difference (% of post incubation)	<i>Salmonella</i> difference (% of post incubation)	Reference figure
C-BF	-96	191	-99	35	Figure 5-17
#45	0	132	-72	38	Figure 5-17
45-CBF	-99	500	-99.8	23	Figure 5-18
35-CBF	-100	427	-100	8	Figure 5-18

Table 5-2. AMP activity in high density *S. Enteritidis* when spiked *E. coli* inoculum is 10^6 CFU mL⁻¹

	<i>E. coli</i> difference (% of starting inoculum)	<i>Salmonella</i> difference (% of starting inoculum)	<i>E. coli</i> difference (% of post incubation)	<i>Salmonella</i> difference (% of post incubation)	Reference figure
C-BF	-89	132	-96	0	Figure 5-17
#45	-31	165	-78	14	Figure 5-17
45-CBF	-99	355	-99	-35	Figure 5-18
35-CBF	-99.9	412	-99.96	-27	Figure 5-18

To further determine whether the killing observed when *E. coli* O78 was spiked in was due to the high selectivity of the peptides, the assays were repeated with the culture concentrations reversed. Both *E. coli* O78 and *S. Enteritidis* were grown to OD 1, with the *S. Enteritidis* culture spiked 1:100 into the *E. coli* O78 culture to yield 10^6 CFU mL⁻¹ of *S. Enteritidis*. The AMPs #45, C-BF, and 45-CBF were tested against the mixed culture at 4× MIC (calculated against *S. Enteritidis*). The results show that the degree of antimicrobial action seen against *E. coli* O78 was not repeated when the spiked bacteria is *S. Enteritidis*, suggesting that the activity was specific (Figure 5-19). However, the application of #45, 45-CBF, and C-BF all resulted in significant

differences to the number of *E. coli* O78 when compared to the DMSO control ($p=0.0004$, $p=0.0016$, $p=0.0021$). This could suggest that the AMPs are still preferentially binding the *E. coli* cells, which would explain the slight loss in concentration of *E. coli*, yet no effect on the *Salmonella*.

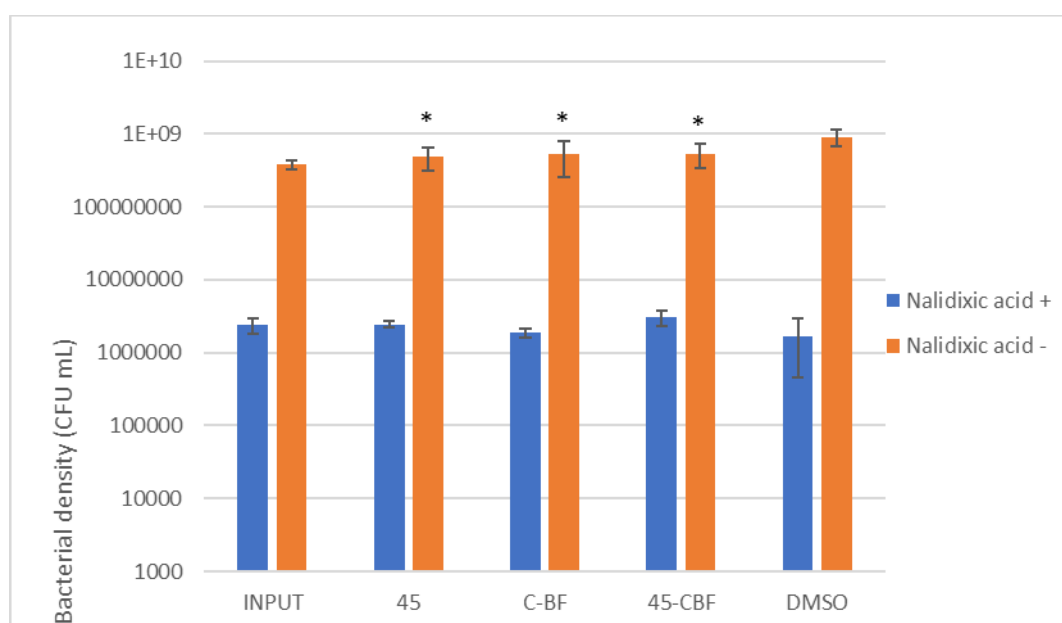


Figure 5-19. AMP effect on *S. Enteritidis* spike in high density *E. coli* O78.

Cultures of *E. coli* O78 and *S. Enteritidis* were grown to an OD₆₀₀ of 1, then *S. Enteritidis* was spiked into *E. coli* O78 for a final concentration of either 10⁴ *Salmonella* present, or 10⁶ *Salmonella* present. In replicates of five, C-BF and #45 were then added at 4× MIC (calculated against *S. Enteritidis*) and the cultures incubated for one hour with a solvent (DMSO) control. Viable counts were taken from both mixed cultures before treatment and from all treatments after the one hour incubation. Selective plating for *E. coli* occurred on MHA supplemented with 100 µg mL⁻¹ Carbenicillin with viable counts taken from duplicate samples for each replicate. A one-way ANOVA indicated significant differences between the solvent control and the peptide-treated groups for the *E. coli* counts.

* - significantly different to solvent control

These results clearly show that the AMPs studied here display a specificity towards *E. coli* and can retain their antimicrobial activity even when the target bacteria is mixed with a high (10,000-fold) excess of another bacteria. The results also show that the

heterodimeric forms of the peptides have increased antimicrobial activity in the high density mixed culture assays than their monomeric progenitors.

5.4 Discussion

Use of v-PCR was attempted as a method for quantifying the effect of AMPs on a mixed culture of bacteria where methods such as viable counting may not be suitable. Validating the experimental protocols in monocultures or simple mixed cultures would then allow further investigations in more complicated, realistic environments. Such environments could comprise *in vivo* challenge studies of bacterial infection or *ex vivo* investigations into the specificity of the AMPs against commensal gut bacteria. Colicin V is an antimicrobial compound used by *E. coli* to target sensitive, phylogenetically-related species which may compete for the same ecological niche (Cascales *et al.* 2007). It was chosen as an ideal target for a pathogenic *E. coli* indicator due to its association with APEC and the product after amplification was approximately 200 bp (Borzi *et al.* 2018; Sharif *et al.* 2018). This was an ideal length as typical q-PCR cycling targets gene fragments up to 200 bp in length.

One thing noted during the v-PCR experiments was that there was occasionally a detectable increase in Cq value in the PMAxx treated cultures compared to the control for samples where viable counts indicated the bacteria were fully viable. For instance, peptide #35 shows an increase in Cq when the viable counts display no difference (Figure 5-8). This same effect is seen in the high density experiments, where 10^8 CFU mL⁻¹ bacteria is used (Figure 5-11) Here, C-BF shows an increase in Cq value with no concomitant loss of viability. It was speculated that the v-PCR was detecting instances where the peptides are causing pores within the bacterial cells. Due to the number of

cells, there was not enough AMP present to reach a lethal concentration per cell. However, there may be enough to cause transient/non-lethal pores and dye molecules may be able to traverse these pores and bind to enough DNA to cause the discrepancies seen.

However, there were still some conclusions that could be drawn from the v-PCR assays. For example, the v-PCR was not sensitive enough to detect the changes in viability caused by AMPs, even though the number of viable bacteria after treatment were often below the detection threshold when 10^4 CFU mL⁻¹ inoculum is used. This suggests that more time might be needed within the experimental protocol, either in the incubation stage with the AMPs or after the dye was added before photoactivation as the results from the heat-treated cells were very consistent. A consideration might be that, in contrast to the heat-treated cells, the AMP-treated cells were not fully permeabilised. This is likely to be because after five minutes at 95°C, bacterial membranes would be broken apart and the dye would therefore readily access the DNA. This reasoning would then have implications on the activity of the AMPs themselves, with a detergent-like activity ruled out and perhaps a pore-forming activity more likely that does not facilitate efficient PMA entry into the cell. Furthermore, the PMA molecule also has two positive charges which may repel it from the cationic AMPs which are forming the pore (Nocker *et al.* 2006).

The use of viable counts, despite the use of five replicates, was prone to suspected statistical errors; the natural variances in diluting cultures, which can be up to 10^9 CFU mL⁻¹, down to a countable range on an agar plate may be a source of type one or type two errors in statistical analysis. It is therefore likely that multiple experiments would be needed when viable counts are utilised, and inter-assay results to be included in

future analyses, or limit their use to only approximate $\log_{10}$ changes in viability, which would still provide useful information.

The *E. coli* viable counts within the bacterial spike assays shows that the AMPs tested display a specific action, preferentially killing *E. coli*. There were either 100× or 10,000× more *Salmonella* cells for the peptides to interact with, depending on the initial inoculum of *E. coli* used. This would suggest that, without selectivity, the AMPs would associate with the first cell they encounter and with the large excess of *Salmonella* cells they would have little or no measurable effect on the total viable count of either the *E. coli* or the *Salmonella*. Despite this, there were measured effects from all peptides used at both inoculum densities. Furthermore, there was only a slight difference observed in the viable count reductions between the initial inoculum concentrations (10^4 CFU mL⁻¹ or 10^6 CFU mL⁻¹), this showed that it was likely the AMPs tested were used at near-optimum concentrations for inhibitory/bactericidal activity. However, to achieve 100% killing in both assays it is the concentration of AMP would need to be increased. Viable counts of *E. coli* within the caeca can reach $10^8 - 10^9$ CFU g⁻¹ and so for any future *in vivo* experiments with these peptides the concentration of AMP may need to be optimised for a higher bacterial density than was used here, depending on any potential intra-*E. coli* specificity (Atterbury *et al.* 2011). Nonetheless, the results displayed here clearly show that the AMPs, especially the heterodimers, could have great therapeutic potential in specifically targeting avian pathogenic *E. coli*.

6 Engineering dimeric peptides for improved efficacy and ease of production

6.1 Introduction

There are two options for producing an AMP: Solid phase peptide synthesis (SPPS) or recombinant production. SPPS is economical for small quantities used in the AMP testing stages but due to their complex requirements, e.g. hydrophobicity, production becomes prohibitively expensive when commercial quantities are needed (Behrendt, White, and Offer 2016). Length of the amino acid sequence also contributes to this issue when attempting to produce multimers of AMPs, a promising avenue of research to increase efficacy. Recombinant production offers a solution to large-scale production problems as it can be both cheap and relatively simple.

The effective isolation of AMPs from large libraries of possible sequences created by combinatorial chemistry or phage display requires stringent selection conditions ideally using high-throughput functional screening methods. Without using such high-throughput methods the researcher would need to synthesise each target peptide through standard, small-scale (~5 mg of peptide) SPPS technology and then screen for antimicrobial effects by broth microdilution or agar plating methods. To facilitate more high-throughput methods of screening often requires a significant investment in machinery and expertise. Guralp *et al.* (2013) developed a technique to combine the large libraries available in biological systems with the flexibility that synthetic methods allow using engineered *E. coli* and a plate overlay methodology. Here, it was attempted to identify methods to relatively cheaply and efficiently produce dimerized AMPs and to investigate whether a high-throughput assay designed by Guralp *et al.* (2013) could be used to find novel AMPs. Investigations into expressing recombinant

AMPs were hindered by the small nature of the peptide, with no peptide imaged by either Coomassie or Western blot. Multimerization using streptavidin and biotinylated peptide indicated that this could not be used as a method to improve AMPs, and the high-throughput overlay assay identified no novel AMPs.

6.2 Multimerization via streptavidin conjugation

To identify a quick test which could determine if a peptide has potential for improvement based on multimerization, an experiment was designed to utilise the binding capacity of biotin and streptavidin. First, a biotinylated version of peptide #45 was synthesised by Biomatik via SPPS. Peptide #45 was chosen as it was the most potent, phage-derived peptide identified. The AMP was biotinylated at its N-terminus with C-terminus amidation to avoid any change in charge. This biotinylated peptide was then used in an MIC assay against *E. coli* O78 with varying molar ratios of streptavidin added to achieve molecules of streptavidin displaying two, three, or four copies of #45. In the first assay, the streptavidin was ordered in lyophilised form with 1× PBS. To obtain a high enough molar concentration to get the correct ratios of streptavidin to biotinylated peptide, while also testing high enough concentrations of peptide for activity, the streptavidin had to be reconstituted in 1/10 of the original volume, pre-lyophilisation. Therefore, once reconstituted after the addition of water the concentration of salts present would have been equal to a 10× PBS solution. This was diluted in 10× PBS to obtain the correct concentration of streptavidin for each molar ratio. Next, the peptide was diluted in aMHB and added to the streptavidin in a 96-well plate. The plate was then incubated for 30 minutes at 37°C to ensure binding of biotinylated peptide to streptavidin. A four-fold dilution series, resulting in peptide concentrations ranging from 128 mg L⁻¹ to 4 mg L⁻¹, was then performed. The peptide

was diluted in a 50/50 mixture of 10× PBS, supplemented with 0.4% BSA and 0.02% acetic acid, and aMHB to ensure the salt concentration was consistent throughout. This created a final concentration of 5× PBS throughout the assay plate. Finally, a culture of *E. coli* O78 in aMHB was added to approximately 5×10^5 CFU mL⁻¹ in each well and incubated as per the CLSI requirements for that bacterium (Appendix 9.1). In this assay, there was no activity seen by any of the streptavidin/biotinylated peptide ratios (Figure 6-1). Intriguingly, growth seemed to be enhanced by the presence of the peptide and streptavidin with relative growth consistently over that of the growth control wells. However, as this assay was not repeated due to the expense of streptavidin, it is difficult to draw meaningful conclusions other than a lack of inhibitory activity. Another result of note from this assay was that the biotinylated #45 on its own failed to inhibit growth of the *E. coli* at 128 mg L⁻¹, despite the MIC of non-biotinylated #45 against *E. coli* O78 being 16 mg L⁻¹. This suggests one of two factors could have affected the antimicrobial activity of #45: the biotinylation, or the increased salt content due to the added 5× PBS present in the media.

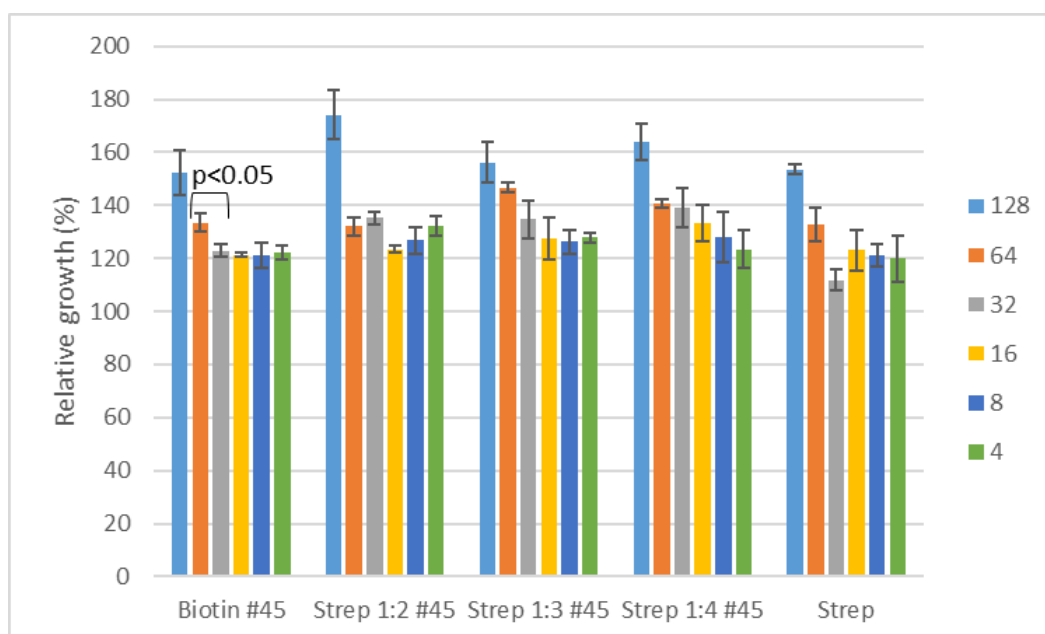


Figure 6-1. Streptavidin-based multimerization of peptide #45 and the effect on MIC.

A modified MIC assay was performed using various molar ratios of streptavidin and peptide #45. Streptavidin arrived lyophilised in $1\times$ PBS, and to obtain a high enough molar concentration to test #45 at MIC values, it was resuspended in $10\times$ less volume creating $10\times$ PBS. This was then mixed, in technical triplicates, with the appropriate volume of peptide and diluted in aMHB. This was then serially diluted in two-fold steps from 128 mg L^{-1} to 4 mg L^{-1} peptide and *E. coli* O78 added. The plate was then incubated at 37°C overnight with the OD_{600} values plotted as a percentage of the averaged growth control and the SEM calculated. The peptide was characterised as active at that concentration if the OD_{600} was less than 50% of the mean average of the growth control. The X axis labels show the results in the following order: Biotinylated #45 on its own, as a dimer with streptavidin, as a trimer, as tetramer, and finally streptavidin on its own. A two-way repeated measures ANOVA with multiple comparisons was then used to determine that the only statistically significant difference were the values of Biotin #45 at 64 mg L^{-1} and 32 mg L^{-1} .

Therefore, to determine if this reduced activity was an effect of the PBS or the biotinylation, a modified MIC assay was performed. Both the biotinylated peptide and the unmodified peptide were tested for inhibitory activity against *E. coli* O78 in two types of media. The first type of media was 50% $10\times$ PBS, supplemented with 0.4% BSA and 0.02% acetic acid, and 50% aMHB (v/v); the second media was 100% aMHB. The biotinylated peptide displayed no activity at 128 mg L^{-1} in the 50/50 aMHB (Figure 6-2), compared to an MIC of 64 mg L^{-1} μM in 100% aMHB (Figure 6-3). In comparison the unmodified #45 showed MIC values of 32 mg L^{-1} in the 50/50

media, and 16 mg L⁻¹ in 100% aMHB. This indicates that free N-termini on an AMP, or at least #45, clearly contributed to its efficacy.

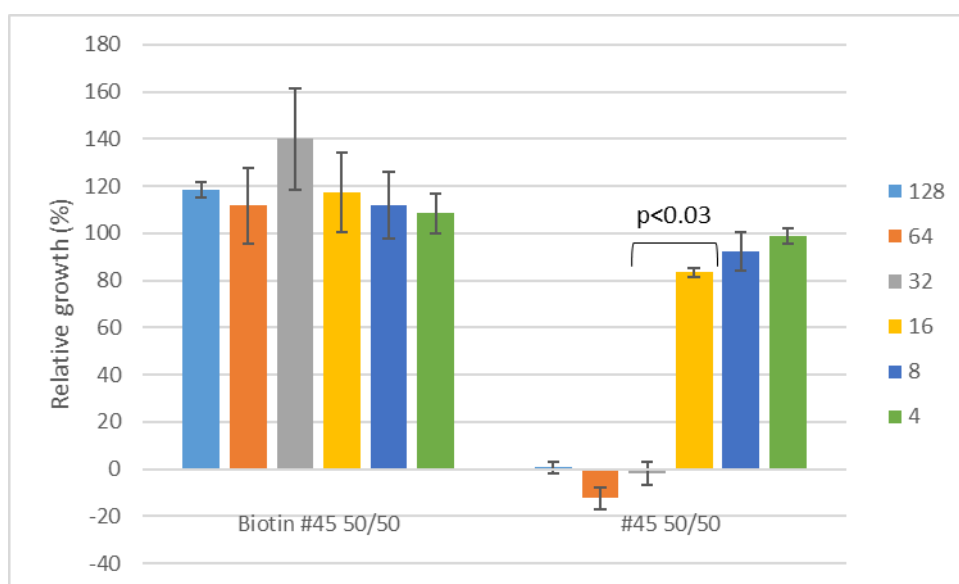


Figure 6-2. Inhibitory activity of biotinylated and non-biotinylated #45 in high salt concentrations.

A modified MIC assay was performed which used a 50/50 media comprising 10× PBS and aMHB (v/v). Biotinylated and non-biotinylated #45 were serial diluted in technical triplicates from 128 mg L⁻¹ to 4 mg L⁻¹ in this media. Then *E. coli* O78 was added and the plate was incubated overnight at 37°C and the OD₆₀₀ of each well was read before plotting as a percentage of the averaged growth control and the SEM calculated. The peptide was characterised as active at that concentration if the OD₆₀₀ was less than 50% of the mean average of the growth control. A two-way repeated measures ANOVA with multiple comparisons indicated that there were no statistically significant differences between the means of the biotinylated #45 treatments. There were significant differences within the #45 treatments, with the first significant difference between consecutive two-fold dilutions being between 32 and 16 mg L⁻¹.

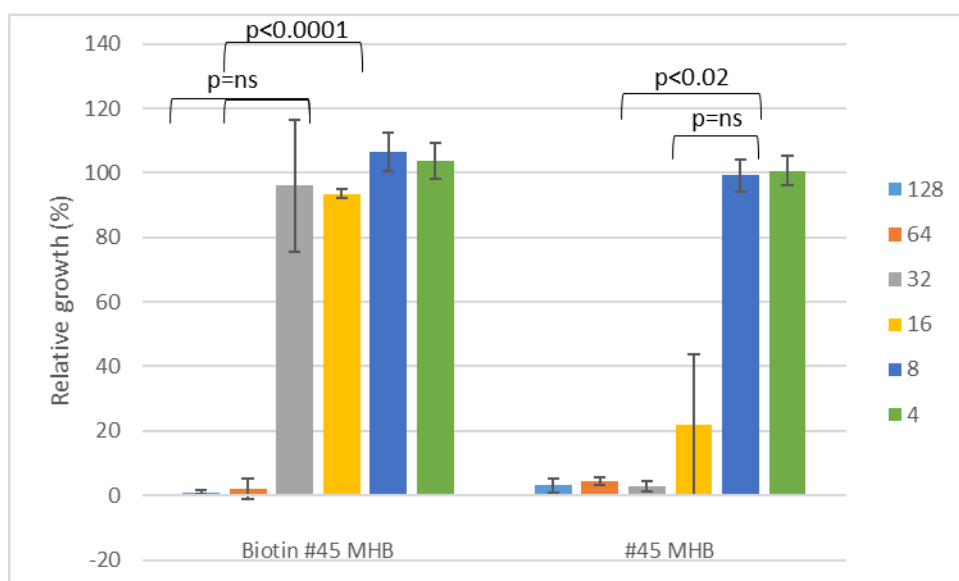


Figure 6-3. Inhibitory activity of biotinylated and non-biotinylated #45.

An MIC assay was performed using biotinylated and non-biotinylated #45. The peptides were serially diluted in technical triplicates from 128 mg L⁻¹ to 4 mg L⁻¹, and then *E. coli* O78 was added and the plate incubated overnight at 37°C. The OD₆₀₀ of each well was then read before plotting as a percentage of the averaged growth control and the SEM calculated. The peptide was characterised as active at that concentration if the OD₆₀₀ was less than 50% of the mean average of the growth control. A two-way repeated measures ANOVA with multiple comparisons indicated that there were no significant difference between the expected MIC cut-off of #45 at 16 and 8 mg L⁻¹, neither was there a significant difference between any consecutive two-fold dilutions of the biotinylated #45 treatments.

Also, it is evident that a high salt concentration reduced AMP activity in this assay.

Therefore, to avoid the decrease in activity caused by a higher salt concentration, salt-free streptavidin was used (S4762; Sigma-Aldrich). Another MIC assay was performed with varying molar ratios of salt-free streptavidin to biotinylated #45 (1:1, 1:2, 1:3, 1:4), and this showed that the addition of streptavidin increased the MIC value of biotinylated #45 to >128 mg L⁻¹ (Figure 6-4). The two versions of peptide #45 were used by themselves as controls, with the biotinylated #45 producing a similar MIC to the expected value (two-fold higher). However, the non-biotinylated #45 inhibited the *E. coli* only at 128 mg L⁻¹, which is eight-fold higher than its recorded MIC. This may indicate that the MIC assay was not optimal; however, the key finding from this assay

was the failure of streptavidin-linked #45 to produce a lower MIC value than #45 assayed on its own.

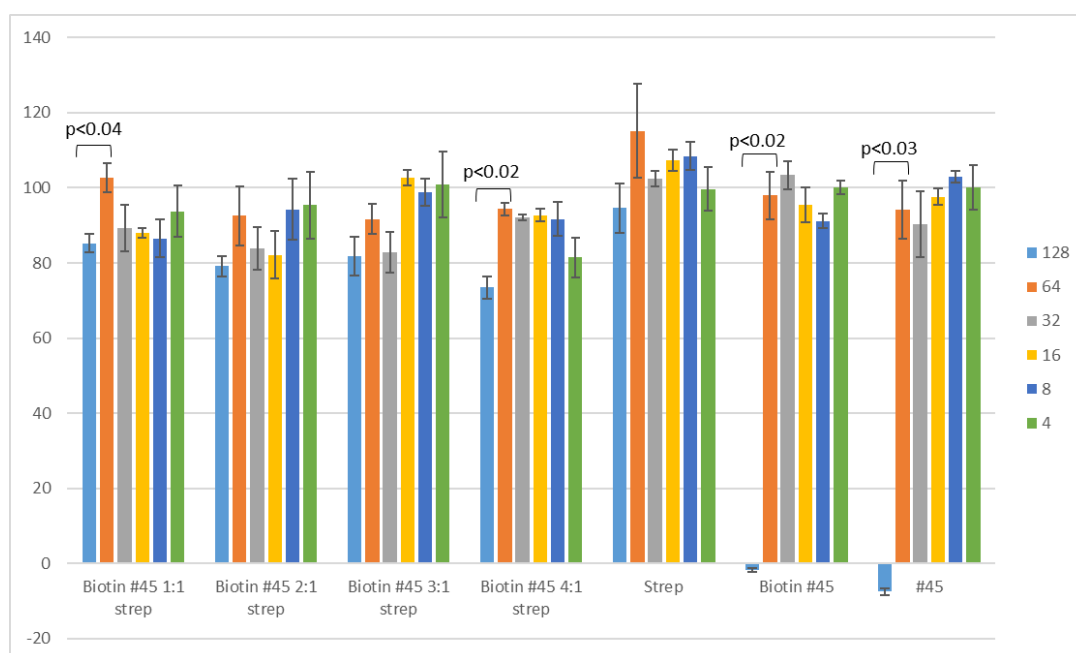


Figure 6-4. Salt-free streptavidin-based multimerization of peptide #45 and the effect on MIC.

A modified MIC assay was performed using various molar ratios of streptavidin and peptide #45. Streptavidin arrived lyophilised in salt-free media which was resuspended and mixed, in technical triplicates, with the appropriate volume of peptide. This was then serially diluted in two-fold steps from 128 mg L⁻¹ to 4 mg L⁻¹ peptide. *E. coli* O78 was then added and the plate incubated at 37°C when the OD₆₀₀ of each well was then read before plotting as a percentage of the averaged growth control and the SEM calculated. The peptide was characterised as active at that concentration if the OD₆₀₀ was less than 50% of the mean average of the growth control. The X axis provides information on the order of experimental treatments: first is biotinylated #45 as a monomer conjugated to streptavidin, next is biotinylated #45 as a dimer, then as a trimer, and then as a tetramer. The final three groups comprised the controls with streptavidin only, biotinylated #45 only, and #45 only from left to right. A two-way repeated measures ANOVA with multiple comparisons indicated that the only biologically relevant statistically significant difference between two consecutive two-fold dilutions were within the biotinylated #45 and #45 MIC control treatments.

6.3 Multimerization via MAP synthesis

Multimeric versions of peptide #45 were synthesised by Biomatik, modelled on MAPs as described by Tam (1988), by creating peptide and isopeptide bonds between the AMP's carboxyl group and the α- and ε-amine groups of lysine molecules. This creates

a branching structure allowing multimers of the AMP to be displayed in close proximity (Figure 2-1) This has been previously demonstrated, with an exponential correlation seen between number of AMPs in the MAP and the efficacy of the antimicrobial activity (Liu *et al.* 2010). Two peptides were synthesised, a dimer and a tetramer of peptide #45, which were then analysed for inhibitory activity against *E. coli* O78. Both the dimer and tetramer displayed no observable antimicrobial activity at the concentrations used (Figure 6-5).

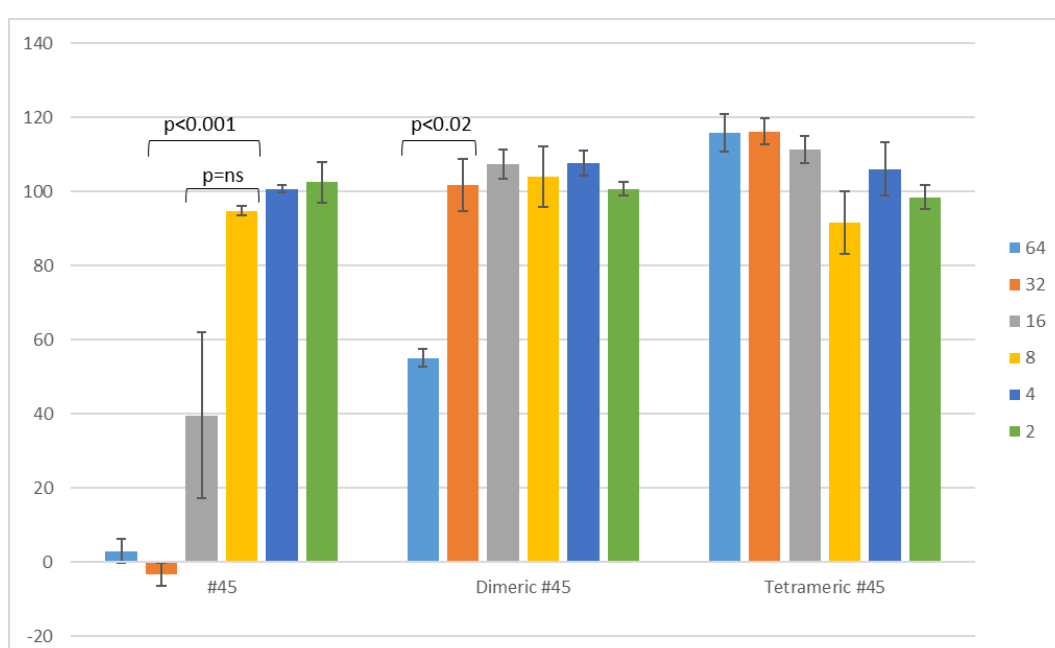


Figure 6-5. Inhibitory activity of MAP-based modifications of peptide #45.

Peptide #45 was modified based on the MAP structure: using lysine as a linker molecule #45 was synthesised as both a dimer and a tetramer. The monomer, dimer, and tetramer were then included in an MIC assay against *E. coli* O78. The peptides were added at 64 mg L⁻¹ and then serially diluted down to 2 mg L⁻¹ and the plate incubated overnight when the OD₆₀₀ of each well was read before plotting as a percentage of the averaged growth control and the SEM calculated. The peptide was characterised as active at that concentration if the OD₆₀₀ was less than 50% of the mean average of the growth control. A two-way repeated measures ANOVA with multiple comparisons indicated that while there was a significant difference between using the dimeric #45 at 64 and 32 mg L⁻¹, this did not reach the criteria of “active”.

6.4 Recombinant production and *in vivo* biotinylation

To facilitate large-scale production of AMPs, which would be needed to make larger dimeric peptides a commercially viable option, a recombinant production strategy was pursued. Utilising peptide #45, four constructs were designed which encoded a dimerised version of the AMP. The flanking peptide sequences were linked by combinations of a flexible linker and an Avi-tag, which is a 15 amino acid sequence (GLNDIFEAQKIEWHE) and acts as a site for biotinylation by the BirA enzyme. The flexible linker used was the same as outlined in Chapter 4.2, which was adapted from Chen, Zaro and Shen (2013). The four gene constructs synthesised (#45-AviTag-#45, #45-Linker-AviTag-#45, #45-AviTag-Linker-#45, #45-Linker-AviTag-Linker-#45) were expected to produce peptides that were 7, 9, 9 and 11 kDa in size. Two more constructs were also created, consisting of a phage display-derived peptide with no known AMP activity (MP_1 and MP_2; Appendix 9.4). These were used as a control for the system, in case of AMP toxicity and were expected to produce a peptide approximately 6 kDa in size.

Due to an initial delay in achieving a successful transformation of the final construct, three of the four recombinant cell lines were induced for six hours at 37°C, with samples taken at time points two, four, and six hours post-induction. Samples were analysed by Western blot for production of biotinylated peptide. There was no detection of recombinant protein, but biotinylated BSA (blotting and detection control) was detected at the correct size (Figure 6-6).

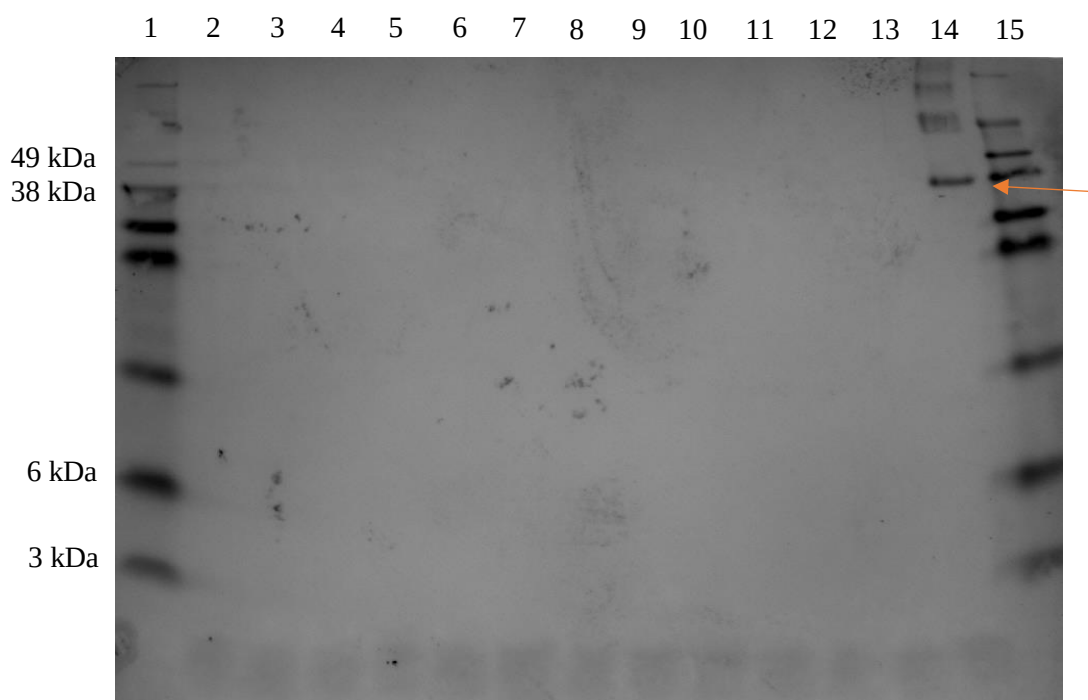


Figure 6-6. Expression of *in vivo* biotinylated dimeric #45.

Three cell lines of pBirA BL21 (pET11a_Avi, pET11a_Avi_Link, pET11a_Link_Avi_Link) were induced at 37°C by 1 mM IPTG with 50 μ M d-Biotin added. Samples were taken at time points 2, 4, and 6 hour post-induction and mixed 1:1 with SDS loading dye before heating. To visualise, 10 μ l of samples was resolved through a 16% Novex Tricine SDS gel. The protein was blotted and then visualised using an AP-conjugated anti-biotin antibody. Lanes 1, 15 – Protein standard, lane 2 – pET11a_Avi pre-induction, lanes 3-5 – pET11a_Avi 2, 4, 6 hours post-induction, lane 6 – pET11a_Avi_Link pre-induction, lanes 7-9 – pET11a_Avi_Link 2, 4, 6 hours post-induction, lane 10 – pET11a_Link_Avi_Link pre-induction, lanes 11-13 – pET11a_Link_Avi_Link 2, 4, 6 hours post-induction, lane 14 – biotinylated BSA. The arrow highlights the detected biotinylated BSA control.

Due to a failure to detect any biotinylated peptides using the western blot technique, detection methods were switched to Coomassie Blue staining. This was used to observe if the peptide was not being biotinylated or if it wasn't being produced. To determine if the protein was not produced or whether it was degraded at the induction temperature at 37°C, the same three BL21 pBirA isolates were induced at both room temperature ($18 \pm 2^\circ\text{C}$), and 30°C. Samples were stained with Coomassie Blue R-250. There was likely expression of the biotin ligase protein, but with no detectable recombinant peptide at the expected 11 kDa for the largest peptide

(pET11a_Link_Avi_Link; Figure 6-7). However, visualisation of bands below 10 kDa was not possible as the smaller markers had not separated.

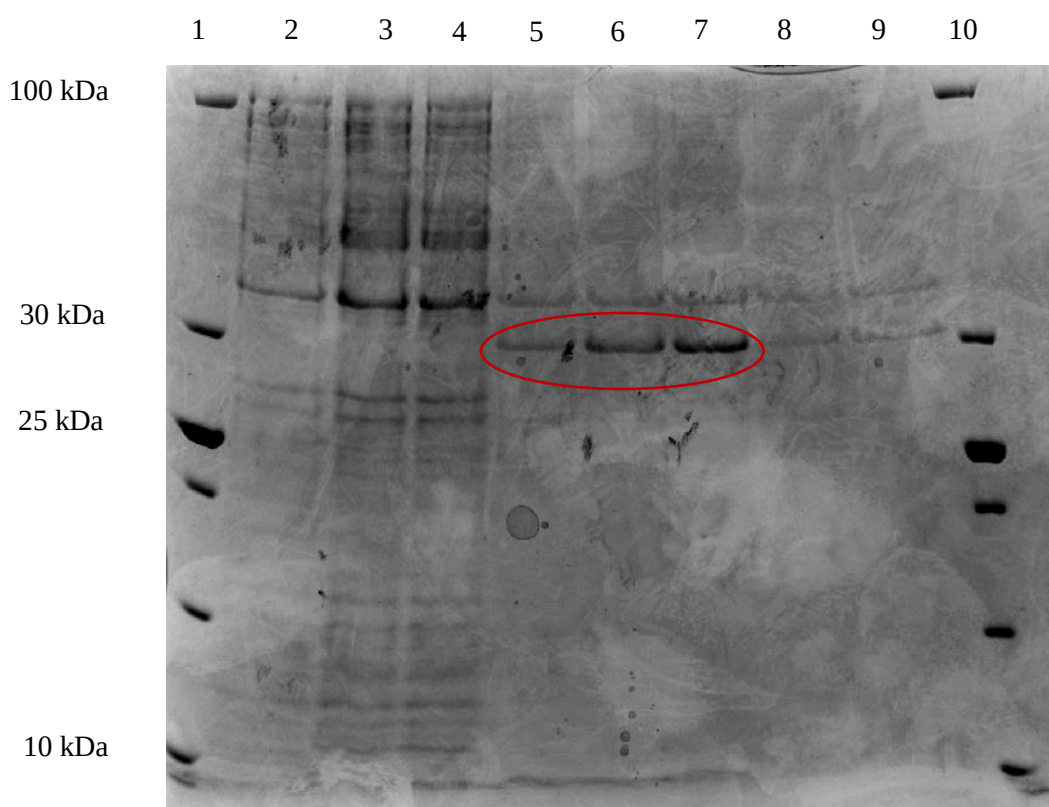


Figure 6-7. Dimeric #45 peptide overnight inductions at 30°C and 18°C.

Three cell lines of pBirA BL21 (pET11a_Avi, pET11a_Link_Avi, pET11a_Link_Avi_Link) were induced at either 18°C or 30°C by 1 mM IPTG with 50 μ M d-Biotin added. After overnight incubation, a sample was taken and mixed 1:1 with SDS loading dye before heating. To visualise, 10 μ l of sample was resolved through a 10-12% NuPAGE Tris-Bis gel. Lane 1, 10 – Protein Standard, lanes 2-4 – pET11a_Avi, pET11a_Link_Avi, pET11a_Link_Avi_Link pre-induction, lanes 5-7 – pET11a_Avi, pET11a_Link_Avi, pET11a_Link_Avi_Link post-induction at 30°C, lanes 8 and 9 – pET11a_Avi and pET11a_Link_Avi_Link post-induction at 18°C. The circle highlights the putative Biotin ligase enzyme (~35 kDa), which is clearly observed in the 30°C post-induction samples.

To investigate further, an additional induction experiment was performed with all four pBirA constructs in BL21 were used (pET11a_Avi, pET11a_Link_Avi, pET11a_Avi_Link, pET11a_Link_Avi_Link). The non-induced and induced samples were also concentrated 10-fold by centrifugation and resuspension before mixing with

SDS sample buffer. Induction was carried out at 30°C and 37°C as these were deemed to induce the cultures to the highest expression levels, with regards to the Biotin ligase protein. The largest peptide failed to show any banding around the expected size upon Coomassie staining (Figure 6-8), although there was no protein detected at this size in any sample and the 10 kDa marker was not stained well.

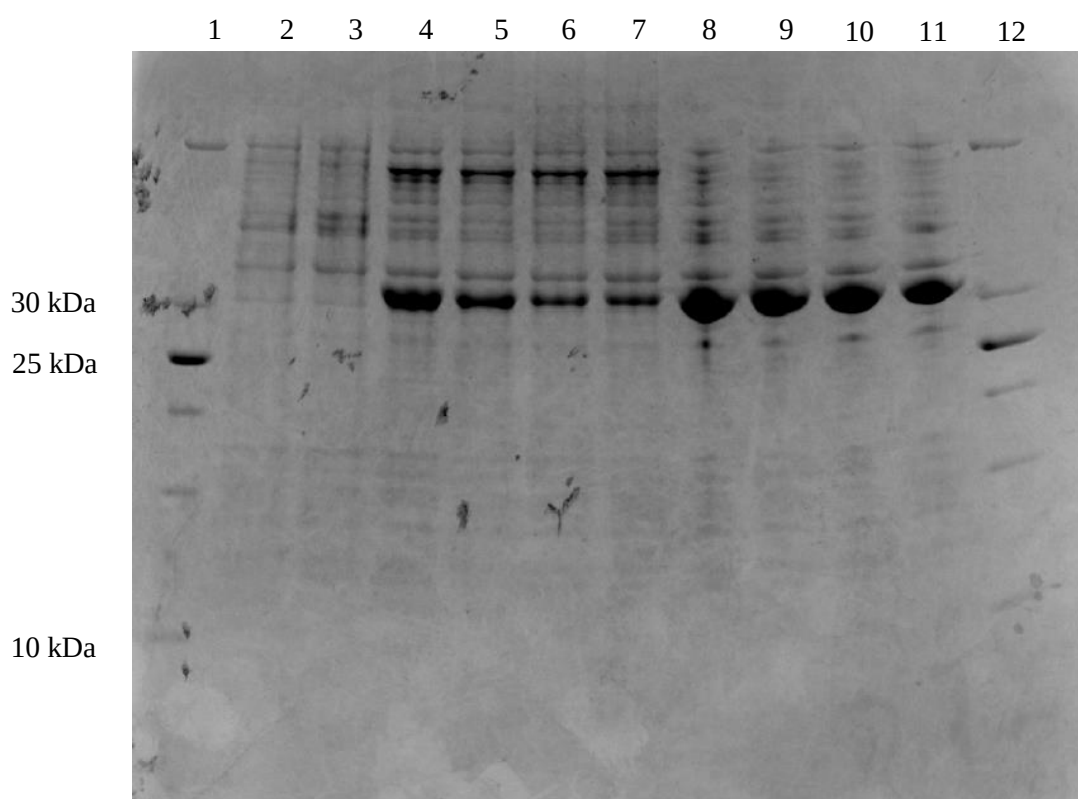


Figure 6-8. Overnight inductions of dimeric peptide #45 constructs at 37°C and 30°C with concentrated sample.

Four cultures of pBirA BL21 (pET11a_Avi, pET11a_Link_Avi, pET11a_Avi_Link, pET11a_Link_Avi_Link) were induced at either 30°C or 37°C by 1 mM IPTG with 50 µM d-Biotin added. After overnight incubation, culture media was centrifuged and the pellet resuspended in 1/10th volume and mixed 1:1 with Tricine SDS loading dye. Samples were then heated and 15 µl resolved through a 10-12% NuPAGE Tris-Bis gel. Lanes 1, 12 – Protein standard, lanes 2, 3 – pET11a_Link_Avi, pET11a_Avi pre-induction samples, lanes 4-7 – pET11a_Link_Avi_Link, pET11a_Link_Avi, pET11a_Avi_Link, pET11a_Avi post-induction samples at 37°C, lanes 8-11 – pET11a_Link_Avi_Link, pET11a_Link_Avi, pET11a_Avi_Link, pET11a_Avi post-induction samples at 30°C. All induced samples clearly show induction of Biotin ligase protein (~35 kDa).

Next, to identify if the problem was due to the cell line or co-expression with the plasmid pBirA, which encoded the Biotin ligase gene, all four constructs were transformed into a different BL21 cell line. The cell line used contained the plasmid pLys, which carries the T7 lysozyme gene. All four constructs, in both cell lines, were then induced at 30°C overnight, with 10-fold concentrated samples loaded onto a 10-12% Tris-Bis NuPAGE gel. Cell line BL21 pBirA which had not been transformed with a construct was also induced. The resultant gel images show that there was no detectable expression of any of the peptide constructs in any of the cell lines used (Figure 6-9). However, there was no detection of any protein below 10 kDa with markers below this size also not visible.

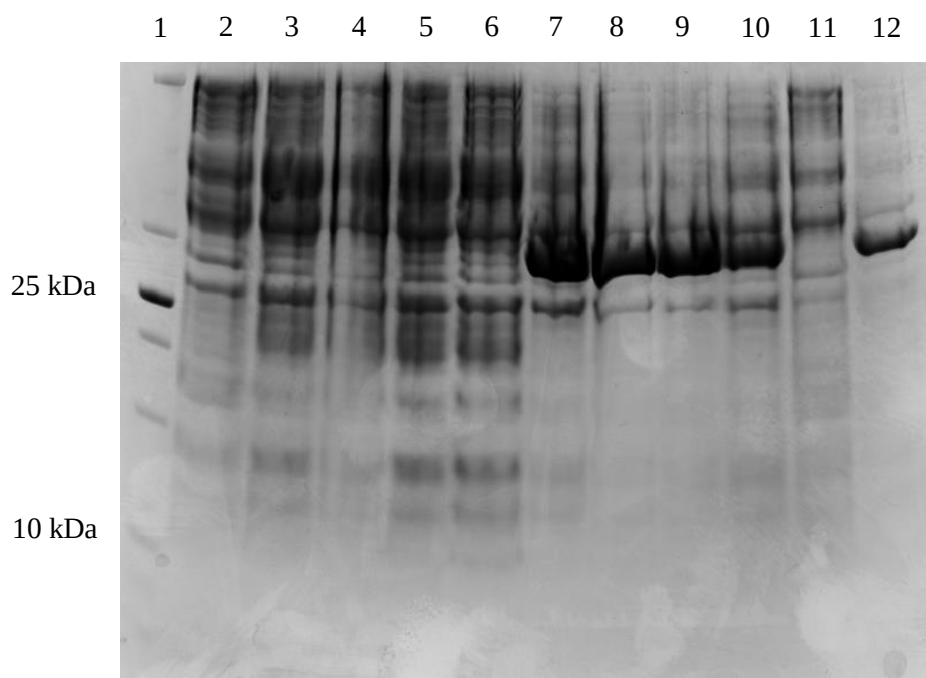


Figure 6-9. Single plasmid inductions of either Biotin ligase or peptide #45 constructs.

Cell lines BL21 and pBirA BL21 were transformed with the same four constructs as Figure 6-8 and induced by 1 mM IPTG and 50 μ M d-Biotin at 30°C overnight. One culture of pBirA BL21 was not transformed with a pET11a construct. After overnight incubation, culture media was centrifuged, the pellet resuspended in 1/10th volume and mixed 1:1 with Tricine SDS loading dye. Samples were then heated and 15 μ l resolved through a 10-12% NuPAGE Tris-Bis gel. Lane 1 – Protein standard, lane 2 – pLys (pET11a_Link_Avi_Link) pre-induction, lanes 3,4,5,6 - pLys (pET11a_Avi, pET11a_Avi_Link, pET11a_Link_Avi, pET11a_Link_Avi_Link) post-induction, lanes 7,8,9,10 – pBirA (pET11a_Avi, pET11a_Avi_Link, pET11a_Link_Avi, pET11a_Link_Avi_Link) post-induction, lane 11 – pBirA pre-induction, lane 12 – pBirA post-induction. Single plasmid induction of the pBirA constructs confirmed the successful induction of the Biotin ligase protein.

After these investigations it was hypothesised that either the overnight induction or the lysis step could have been releasing endogenous proteases which were then degrading the expressed peptides. To investigate this, three cell line/plasmid combinations were used: BL21 pLys (pET11a_Link_Avi_Link), BL21 pBirA (pET11a_Link_Avi_Link), and BL21 pBirA. The cultures were induced for five hours at 30°C and protease inhibitor was added to lysates. To try to improve staining, particularly visualisation of bands below 10 kDa, the gel was stained overnight. However, there was no visualisation of the target peptides with only the putative Biotin

ligase seen (Figure 6-10). However, 5 and 10 kDa MW controls were clearly stained indicating the absence of any expression of target peptides.

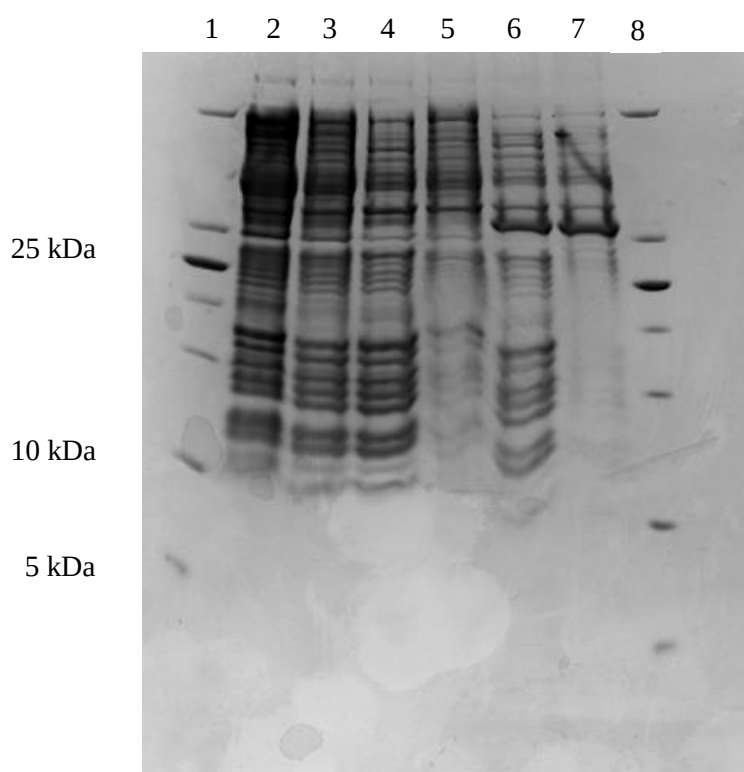


Figure 6-10. Expression of dimeric #45 constructs after cell lysis with protease inhibitor.

Cell lines BL21 pBirA and pLys were transformed with pET11a_Link_Avi_Link and induced for 5 hr at 30°C. The cultures were then pelleted by centrifugation and the pellet resuspended in BugBuster protein extraction reagent, with added protease inhibitor and benzonase nuclease. This was mixed and then clarified by centrifugation. Tricine SDS loading dye was then used to resuspend the pellet and 10 µL loaded onto a 16% Tricine NuPAGE gel. Lanes 1 and 8 – Protein marker, 2 – pLys (pET11a_Link_Avi_Link) non-induced, 3 – pBirA (pET11a_Link_Avi_Link) non-induced, 4 – pBirA non-induced, 5 – pLys (pET11a_Link_Avi_Link) induced, 6 – pBirA (pET11a_Link_Avi_Link) induced, 7 – pBirA induced.

To determine that the sequences obtained from Genscript were correct, and that no mutations had occurred during transformation and propagation, plasmid DNA for each construct was extracted from BL21 pLys cells (27104; Qiagen) and sent off for Sanger sequencing. The sequences returned showed no alteration to the expected ORF (data not shown).

6.5 Inverse PCR-mediated dimerization and screening

6.5.1 Screening of known AMPs in JE5505 bacterial strain

After attempting to produce dimeric forms of a limited number of peptides via chemical modification, without success, a more economical production and screening method was investigated. First, the database DBAASP was searched for more antimicrobial sequences that could be used (Table 6-1). Criteria for inclusion comprised potency ($<10\ \mu\text{M}$) and target bacteria (AMP must be reported as active against at least one of the bacteria used in this study).

Table 6-1. Sequence information of AMPs derived from DBAASP for insertion into the pSD3 vector.

Antimicrobial peptide	Amino acid sequence	References
Plantaracin-423 (Pln-423)	KYYGNGVTGCGKHSCSV NWGQAFSCSVSHLANF GHGKC	(Guralp <i>et al.</i> 2013)
Plantaracin-423 H28 (H28)	KYYGNGVTGCGKHSCSV NWGQAFSCSVSRLANF GEGKC	(Guralp <i>et al.</i> 2013)
Plantaracin-423 S27 (S27)	KYYGNGVTGCGKHSCSV NWGQAFSCSVDHANF GHGNC	(Guralp <i>et al.</i> 2013)
Bacteriocin E50-52 (E50-52)	TTKNYGNVCNSVNWC QCGNVWASCNLATGCA AWLCKLA	(Wang <i>et al.</i> 2013)
Cathelicidin-BF (C-BF)	KFFRKLKKS VKKRAKEF FKKPRVIGVSIPF	(Wang <i>et al.</i> 2011)
Cathelicidin 4F buCATHL4F (buC4F)	AIPWSIWWRLLFKG	(Brahma <i>et al.</i> 2015)
Caerin-2.6 (Ca2.6)	GLVSSIGKVLGGLADV VKSKGQPA	(Pukala <i>et al.</i> 2006)
Palustrin-Ca (Pal-Ca)	GFLDIKDTGKEFAVKIL NNLKCKLAGGCPP	(Helbing <i>et al.</i> 2019)
Lactoferricin-B1-15 (Lac)	FKCRRWQWRMKKLGA	(Eliassen <i>et al.</i> 2006)
NZ2114	GFGCNGPWNEDDLCH NHCKSIKGYKGGYCAK GGFVCKCY	(Chen <i>et al.</i> 2017)
MP1102	GFGCNGPWQEDDVCKH NHCKSIKGYKGGYCAK GGFVCKCY	(Zong <i>et al.</i> 2016)
Plectasin, defensin like (Po-Def)	GFGCNGPWDEDDMKC HNHCKSIKGYKGGYCA SAGFVCKCY	(Xiong <i>et al.</i> 2011)

Next, inverse PCR primers were designed to insert each sequence into the pSD3 expression vector (Appendix 9.2). Primers were designed to create monomeric and dimeric versions of the selected AMPs, with the phage-derived AMPs discovered in this study also being cloned. To create the dimeric peptides, the linker sequence previously used here was employed to separate the two monomers. The sequences were inserted downstream of a PelB leader sequence, which should result in secretion of the peptide into the periplasm. The *E. coli* strain JE5505 was chosen for the host cell due to its outer membrane mutation within the OmpT protein, which causes loss

of periplasmic contents into the extracellular space. After transformation, the *E. coli* clones were investigated for their potential to be used in an economical screening method for antimicrobial peptides, hereafter termed the Overlay assay, as described by Guralp *et al.* (2013).

Briefly, the recombinant *E. coli* are spread onto sMHA with another layer of sMHA poured over once set. A final layer of sMHA is then overlaid, inoculated with a test species and 1 mM IPTG. Initially, the already validated Plantaricin-based AMPs (Pln-423, H28, S27) were investigated in this assay, with *L. innocua* used in the top layer of sMHA. All three AMPs produced zones of inhibition consistent with those reported in Guralp *et al.* (2013), suggesting the assay is reproducible (Figure 6-11).

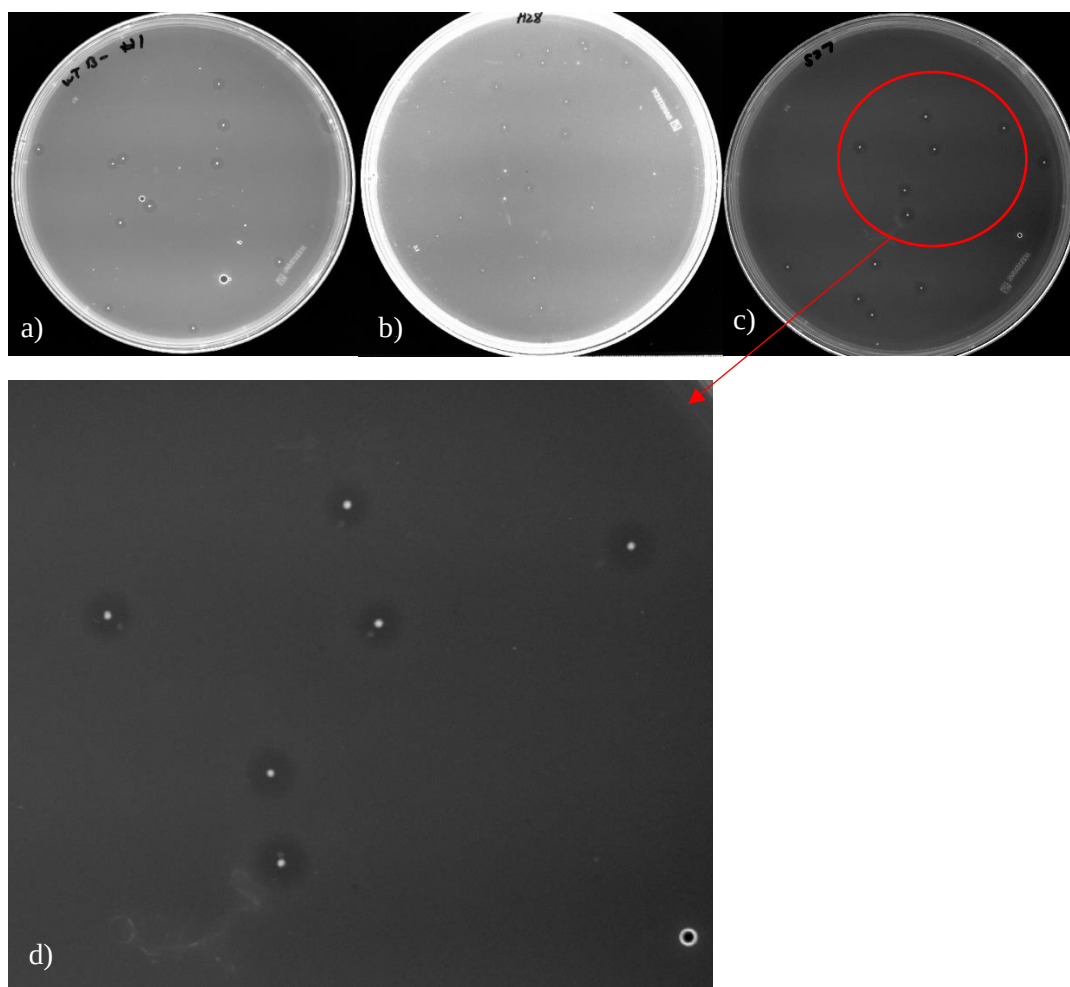


Figure 6-11. Overlay assay screening of Plantaricin-based AMPs.

Three pSD3 constructs transformed into JE5505 were chosen to validate the overlay assay, with each encoding either (a) Plantaricin-423, or the mutants (b) H28, (c, d), S27. Cultures of each JE5505 clone were spread on each plate and overlaid with sMHA. Once dry, the plates were incubated overnight at 37°C. Then, the test species (*L. innocua*) was used to inoculate molten sMHA, which was supplemented with 1 mM IPTG, and was then poured over each plate. Once set, the plate was incubated overnight at 30°C and then the plate viewed for visible zones of inhibition. Contrast settings within the images were adjusted to obtain the best picture possible for visualising the zones.

After validation of the assay, the assay was repeated with the remaining clones. Each clone was tested against at least one test species which it was reported to be active against in DBAASP. Every assay included a positive control, such as S27 or Pln-423 against *L. innocua*, and a negative control, which was the empty pSD3 vector

transformed into JE5505. Each clone was tested in technical triplicates. A total of 26 clones, in addition to the Plantaricin-derived AMPs, were tested in this assay (Appendix 9.5). No other clone, except all the plantaricin-based AMPs (Pln-423, Pln-423 dimer, H28, or S27), produced any visible zone of inhibition.

6.5.2 JE5505 *E. coli* peptide clone growth curve analysis

The growth curves of seven clones were analysed to determine if the expression of the peptides might be affecting their growth, as AMP production can be detrimental to the host cell. The JE5505 clones tested comprised no vector, Pln-423, Pln-423 dimer, #27, #35, #36, #45, and C-BF. The selected clones were chosen due to a validated activity within the Overlay assay (Pln-423 and Pln-423 dimer), or a validated activity within the MIC or MBC assays tested in this study. Each clone was tested in technical triplicates and biological duplicates with induced expression using 1 mM IPTG and suppressed expression using 1% glucose. Optical density readings were taken every 30 minutes for 20 hours and the Growthcurver package within R was utilised to produce plots of the growth rates of clones with and without induction by 1 mM IPTG (Sprouffske and Wagner, 2016; Figure 6-12).

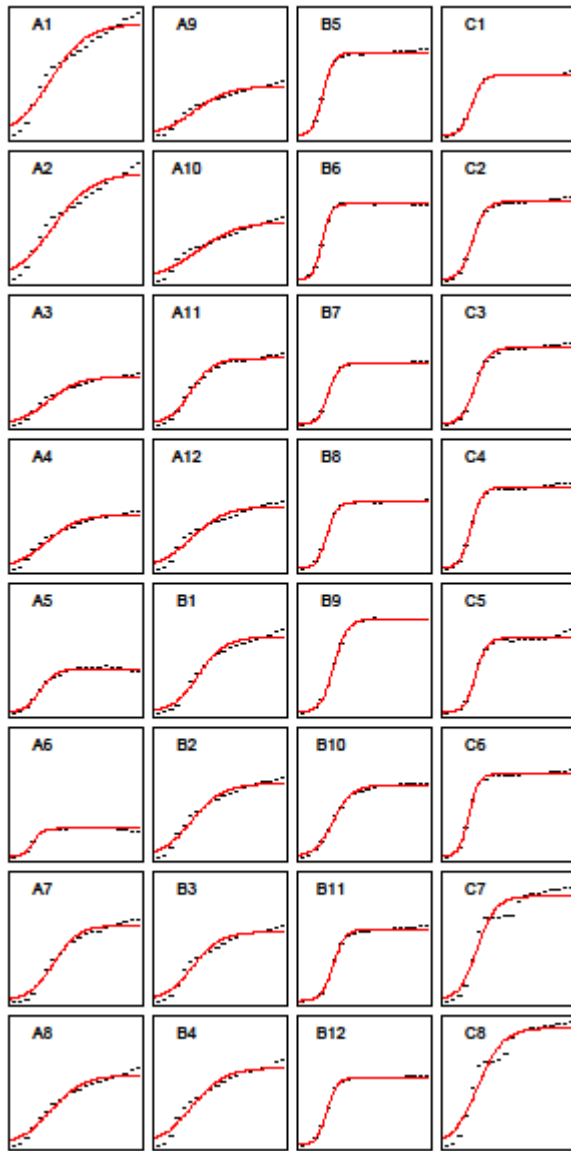


Figure 6-12. Growth curve plots of various JE5505 clones.

Peptide clones were investigated for their effect on the growth curve of their host cell, JE5505 *E. coli*. Clones were cultured overnight in biological duplicates, then centrifuged and resuspended in 2×YT supplemented with either 1% glucose or 1 mM IPTG. Each clone was then also separated into technical triplicates and incubated for 20 hours at 37°C, with OD₆₀₀ readings taken every 30 min. The mean average was then taken from each biological replicate and imported into R, with the Growthcurver package then used to create plots of each curve. The data points for each graph are plotted on the same scale, with the X axis detailing time and Y axis detailing OD₆₀₀ measurements. A1-B4 IPTG-supplemented media, B5-C8 glucose-supplemented media. A1, A2 – No vector. A3, A4 – Pln-423. A5, A6 – Pln-423 dimer. A7, A8 – #27. A9, A10 – #35. A11, A12 – #36. B1, B2 – #45. B3, B4 – #C-BF. B5, B6 – No vector. B7, B8 – Pln-423. B9, B10 – Pln-423 dimer. B11, B12 – #27. C1, C2 – #35. C3, C4 – #36. C5, C6 – #45. C7, C8 – C-BF.

To determine whether there was any statistical significance between the clones, the

OD₆₀₀ absorbance readings were inputted into GraphPad Prism. First, the area under

curve (AUC) was determined for each of the 96 replicates. Then, the results from each of the biological replicates were combined into one treatment group to reduce the degrees of freedom and improve the statistical power of further tests. A one-way Welch's ANOVA was performed on the grouped AUC results and on the optical density measurements from five time points (210, 450, 690, 930 and 1170 minutes), split into the induced and non-induced replicates. A detailed methodology can be found in Appendix 9.6.

When comparing the results from the induced and non-induced groups, it was clear that the clones with a recombinant plasmid suffered a growth penalty when compared to the no vector control. In the induced group, all of the comparisons between the AUC value of no vector and the seven recombinant clones were significantly different ($p < 0.0001$), with at least 4/5 of the optical densities at each time point also being significantly different. In the non-induced group, no AUC comparison had a p value as small. The smallest p value, comparing the no vector AUC value with recombinant clones, was associated with the monomeric Pln-423 ($p = 0.0002$). This comparison was also the only one to display significant differences in 4/5 time points, suggesting that this peptide may be toxic to the cell where even the low, 'leaky' expression may affect growth. Interestingly, there was no significant difference between the monomeric Pln-423 clone and its dimeric counterpart in any of the time points and the AUC comparison, regardless of induction status.

6.5.3 Peptide library construction and screening

The Overlay assay could not identify any AMPs that had already been validated by other methods in this study, with the exception of Pln-423 and its derivatives, and this

was not due to any obvious inhibition of *E. coli* host strain growth. The assay was also not indicated to be due to a lack of potency, as C-BF was shown to have a lower MIC value within the Agar dilution assay than Pln-423 against the bacteria also tested within the Overlay assay (Table 3-2). It was hypothesised that it is possible that AMPs which show effectiveness in the Overlay assay display relatively high AMP activity and are also able to effectively diffuse through the agar medium. There was little available information in the literature as to how to increase diffusion of a peptide through agar, but it may be possible to use this method to select such AMPs by screening high diversity libraries. To investigate this, five AMPs (#27, #35, #36, #45, and C-BF) were coupled to a high diversity naïve peptide library. The libraries were designed using inverse PCR, with primers encoding a 16-mer NNK region. The randomised region was inserted next to the linker sequence joining one of five separate lead-AMP peptide sequences. The peptides chosen were selected based on their activities across different media, and their different specificities. For example, peptide #35 specifically killed *S. Typhimurium* in the MBC assay and was inactive against the other pathogens tested. Conversely, peptides #36 and #45 were chosen for their broad range of activity in the MBC and MIC assays, respectively. *[N.B. Libraries were PCR amplified, ligated and transformed by Keith Bishop during my 3-month Professional Internship for BBSRC-funded PhD students]*. After transformation into JE5505 cells, plasmid DNA from all the libraries was extracted and prepared for NGS using the IonTorrent platform. The libraries were sequenced to determine if their construction was successful in producing a high diversity to screen for novel AMPs as well as provide useful information with regards to any amino acid biases present. Alongside the five JE5505 libraries, the naïve 16-mer pIII library used to pan for the phage-

derived peptides (Chapter 3.2) was also prepared for NGS so comparative analysis could be performed on the quality of that library.

Custom Perl scripts, kindly provided by Dr. Owen (ADAS, Nottingham), were used to interrogate the data. Each read was translated into all three frames, and all three analysed for the presence of a barcode and two motifs. The motifs were the amino acids flanking either end of the randomised region. The first motif was used to determine the start of the insert and the second for the end and to ensure the read was not prematurely truncated. At least one amino acid between the two motifs was used as the minimum insert length; reads not meeting these requirements were disregarded from the analysis. To determine the quality of the libraries produced, the data were then analysed for the proportion of sequences present in each library that were of the desired length (16 amino acids). The values for the dimeric libraries ranged from 87% (#27) to 93% (C-BF), with the pIII phage-display library having a total of 97% correctly sized reads (Figure 6-13).

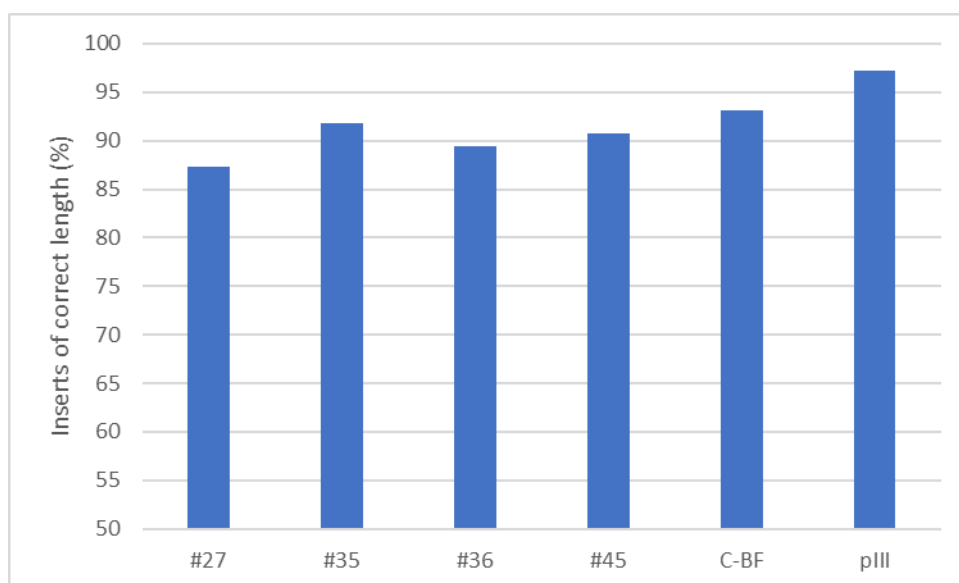


Figure 6-13. Percentage of each libraries' inserts which are of the correct length.

Each of the six libraries sent for IonTorrent analysis were analysed for inserts of at least one amino acid between two in-frame flanking sequences. Each insert was then categorised according to its length and the proportion of total sequences which were 16 amino acids long was calculated and plotted.

To calculate the diversity of each library, the titres were divided by the number of unique sequences which were of the correct length. The dimeric libraries were calculated to have a diversity of between 7.4×10^8 and 5.2×10^9 (Table 6-2).

Table 6-2. Diversity of NNK dimeric libraries

Library	Diversity
NNK_27	$1.7 \times 10^9 - 1.9 \times 10^9$
NNK_35	$7.4 \times 10^8 - 8.3 \times 10^8$
NNK_36	$3.1 \times 10^9 - 3.5 \times 10^9$
NNK_45	$4.6 \times 10^9 - 5.2 \times 10^9$
NNK_C-BF	$2 \times 10^9 - 2.3 \times 10^9$

The analysis also determined the level of unique sequences and distinct sequences in the libraries. In any given library there will be sequences that are different from each other, termed “distinct sequences”. However, distinct sequences can be repeated within the dataset; for example, a small percentage of distinct sequences may be seen

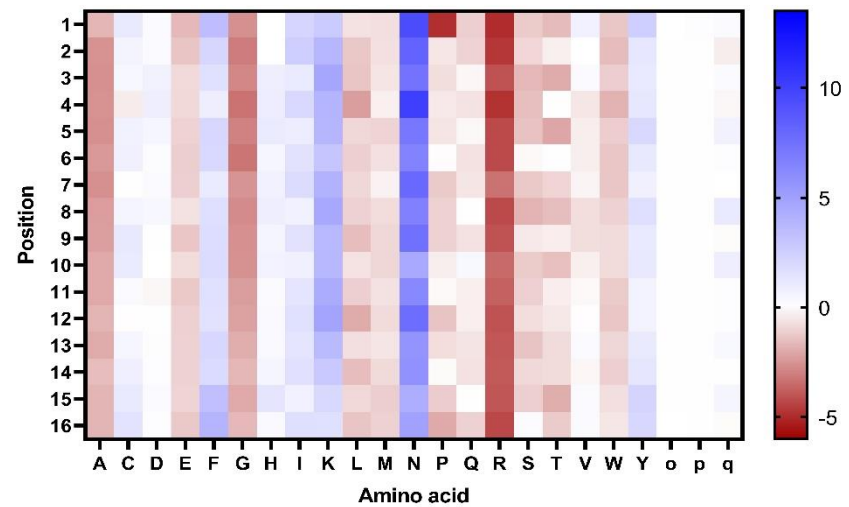
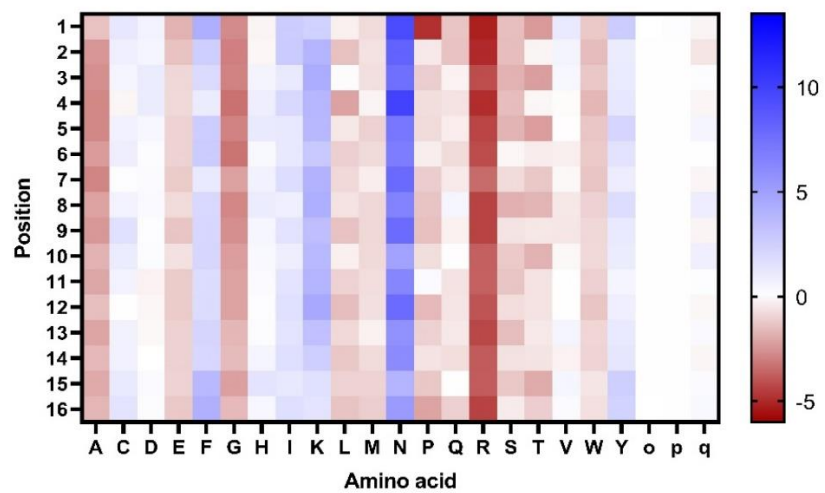
over 100 times within a single library. “Unique sequences” are also distinct, but have the additional requirement of being only seen once within the dataset. A library with a high number of distinct sequences, which were seen multiple times, may suggest that there was a bias within the primer sequences during construction or that certain sequences are favoured during bacterial propagation. Therefore, each library was assessed for distinct sequences and how many times each were repeated. As expected, the dimeric libraries produced similar results (Table 6-3). This was likely due to the same primers being used to insert the randomised region into each library, with the only difference between them being the encoded peptide sequence the randomised 16-mer was linked to. The pIII library showed much smaller amounts of repeated distinct sequences, with less than 1% of sequences being seen 3-5 times compared to over 10% in the dimeric libraries.

Table 6-3. Proportion of libraries containing repeated distinct sequences

Copies of each sequence	#27	#35	#36	#45	C-BF	pIII
>100	0.046%	0.031%	0.075%	0.038%	0.106%	0%
25-100	0.089%	0.117%	0.129%	0.185%	0.331%	0%
6-24	2.068%	2.882%	1.871%	2.985%	3.132%	0.004%
3-5	10.101%	13.083%	10.631%	12.309%	11.796%	0.848%
2	17.862%	19.242%	20.742%	19.972%	20.176%	8.575%

Next, the libraries were analysed for any biases within the primary structure of the sequences present. The theoretical expected total of each amino acid was calculated based on the NNK codon usage, and then compared against the actual total of each amino acid present in all the sequences. This showed that within the dimeric libraries there was some bias in the incorporation of particular amino acids over others, in particular asparagine (data not shown). Levels of amino acid bias within the pIII phage

display library was much lower, with only proline displaying a consistently high variation from expected percentage representation ($>2\%$; data not shown). For the dimeric libraries, amino acid bias was further investigated at the positional level to determine if there were specific positions which were highly likely to have certain amino acids either absent or present. The positional comparison revealed that the libraries could be grouped into two types, due to broadly similar amino acid representation, with #27, #35, and C-BF being similar (type 1, Figure 6-14), and #36 and #45 together (type 2, Figure 6-15).



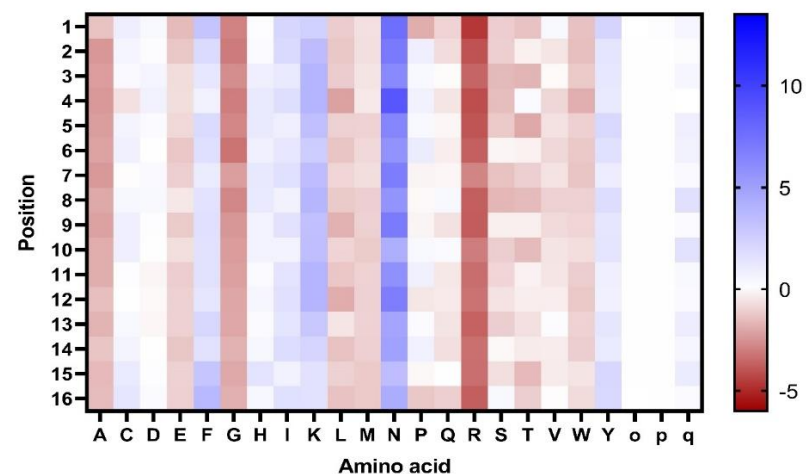


Figure 6-14. NNK library type group one.

Dimeric peptide libraries were analysed for positional biases within their primary structure using custom Perl scripts. The theoretical expected representation (%) of each amino acid was calculated based on the NNK randomisation and then it was subtracted from the actual representation (%). The resulting value was then plotted with overrepresented amino acids in blue and underrepresented residues in white. The results indicated two distinct groups, with group one displayed. Top left – #27, top right - #35, bottom middle – C-BF. Stop codons were represented by o (ochre), p (opal), and q (amber).

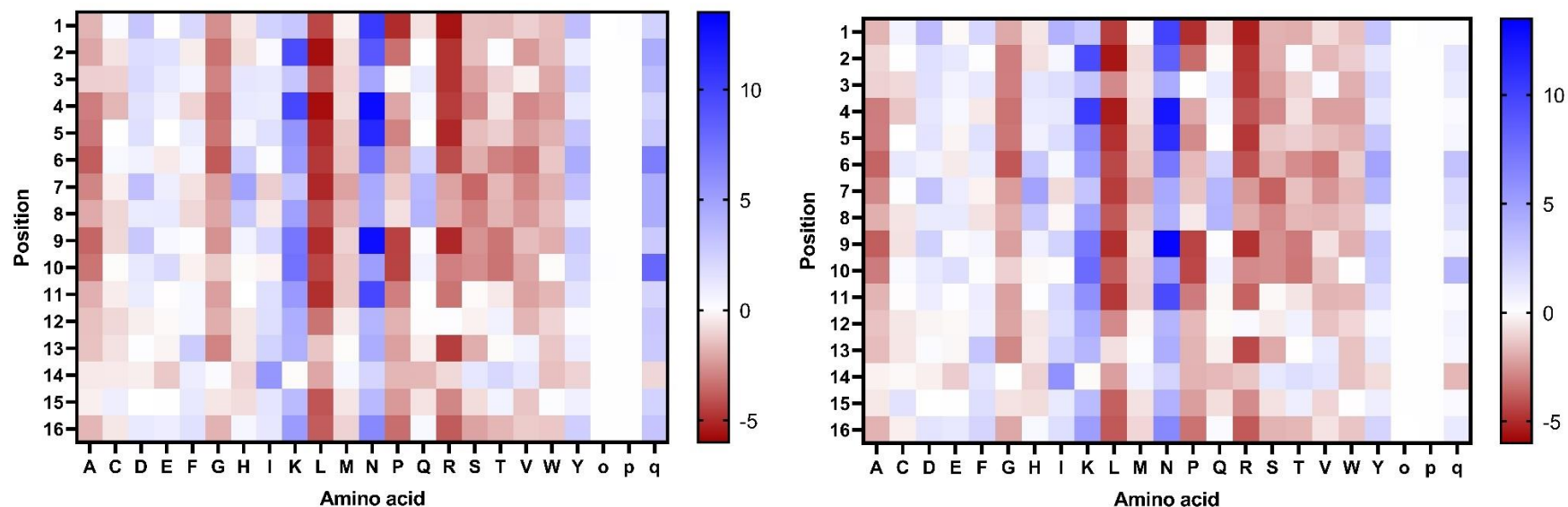


Figure 6-15. NNK library type group two.

Dimeric peptide libraries were analysed for positional biases within their primary structure using custom Perl scripts. The theoretical expected representation (%) of each amino acid was calculated based on the NNK randomisation and then it was subtracted from the actual representation (%). The resulting value was then plotted with overrepresented amino acids in blue and underrepresented residues in white. The results indicated two distinct groups, with group two displayed. Left - #36, right - #45. Stop codons were represented by o (ochre), p (opal), and q (amber).

One notable difference was the representation of leucine residues within the libraries, with group two displaying low representation across the majority of positions while group one was much closer to expected. Group one was also more consistent across all positions. For example, asparagine was generally overrepresented across all libraries, but the calculated standard deviation at each position for group one was 1.61, 1.65, and 1.41. For group two this rose to 3.5 and 3.4. If the standard deviations for each amino acid are averaged across groups and compared, group two is higher in every case (data not shown). Another amino acid found to be overrepresented was lysine, which was prevalent in both type groups. Arginine was underrepresented within both types, but was only consistently so across all residues in type one. Glycine was also found to be underrepresented in type 1, but this was less noticeable in type two. Conversely, proline was underrepresented within type two, but was less noticeable in type one. Strong positional bias was noticeable with proline, with residue one highly underrepresented within both types, although this was slightly less distinct in C-BF. Within type two, proline was also noticeably underrepresented at positions nine and ten.

After analysis, the dimeric libraries were screened via the Overlay assay. Each library was screened against *E. coli* O78 and *S. Typhimurium* in eight technical replicates. Eight replicates were chosen as each Overlay assay can accommodate approximately 12,000 colonies of peptide-producing *E. coli*, this allows for $\sim 10^5$ colonies to be screened for activity. The target 10^5 was chosen as an appropriate compromise between cost and benefit of the assay. Screening of the libraries did not successfully isolate any novel dimeric peptides which could produce a zone of inhibition within this assay.

6.6 Discussion

Based on previous work that showed AMPs can be biotinylated *in vivo* (Li and Sousa 2012), a similar recombinant production and purification method was assessed for the AMPs in this study. By using the Avi-Tag, a site for biotinylation, it was hypothesised that AMPs could be produced, biotinylated *in vivo*, and purified using the biotin-streptavidin interaction. Use of harsh, denaturing conditions to elute the biotinylated peptide from a streptavidin column would be inconsequential for a peptide without a complex structure. As α -helical AMPs tend to be unstructured in aqueous media, and only form their structures upon contact with a membrane, this was decided to be an optimal route of purification (Houston *et al.* 1998). However, it was never determined that an AMP could be produced from the six constructs created. Western blotting detected no biotinylated peptides and investigations into expression levels via Coomassie staining proved problematic as there was a failure to clearly visualise proteins below 10 kDa. This was hypothesised to be due to a low concentration of produced peptide, as due to their size more copies of the protein is needed to reach the mass threshold for detection. The low levels of protein produced was speculated to be due to host toxicity, which then interfered with high levels of production. However, if the peptide was toxic to the host expected levels of Biotin ligase expression would have been low, and the cultures not expected to grow to high density after induction. Both high levels of ligase expression and high density growth were observed. Alternatively, endogenous proteases may have degraded the peptides during the induction period. Further studies would need to optimise the staining procedure and electrophoresis conditions for each gel type, attempt other techniques such as the more sensitive silver stain, and include more positive controls at various molecular weights and concentrations to determine the sensitivity cut-off of such detection.

Attempts to create multimers of peptide #45 *in vitro* were also unsuccessful in improving the AMP's antimicrobial efficacy. Using streptavidin as a linker molecule, biotinylated #45 was conjugated to link two, three, or four copies together. The different configurations were then tested for any increase in antimicrobial efficacy, but due to the high salt content in the re-suspended streptavidin the AMPs were ineffective. Further investigations showed that biotinylation caused a possible increased sensitivity to salt concentrations, potentially by changing the hydrophobicity of the termini, which has been shown to correlate with salt sensitivity (Chu *et al.* 2020). One consideration of note is that while the biotinylated peptide was more susceptible to the effects of increased salt concentrations, it was not determined if it was specifically the biotinylation which caused it or whether free termini are an essential factor for robust activity. This might be especially true for the N-termini, as this would have been the end displayed during the initial panning experiments and therefore might play an important role in binding. The small nature of biotin, ~0.2 kDa, might explain why this effect was only minimal within MIC assays performed in aMHB. However, the reason why the effect is potentiated by an increased salt concentration is unknown. After conjugation with streptavidin in salt-free conditions, the biotinylated peptide lost its antimicrobial activity regardless of the molar ratio. This suggests that streptavidin had a strongly negative effect on the AMP's mode of action. This could be due to the size of the streptavidin molecule, which is about 60 kDa. The size could exclude other peptides from binding to the cell membrane in close proximity, meaning the AMPs would not reach a critical threshold necessary for antimicrobial action.

Next, peptide #45 was conjugated *in vitro* via lysine residues. If size was the reason that the streptavidin-conjugated AMP failed to inhibit *E. coli*, then the addition of only one amino acid (for the dimer) should not hinder the antimicrobial activity. This process was based on the validated MAP system, which has been shown to exponentially increase AMP activity (Pini *et al.* 2005). When tested with peptide #45, as a dimer and tetramer, neither compound achieved a 50% inhibition. The dimer exhibited slight antimicrobial activity, but not enough to be registered as active at 128 mg L⁻¹. One reason for the failure of #45 to retain its antimicrobial activity in either the streptavidin or the MAP-based assay may be that it is acting intracellularly where the added mass of additional copies prevents it from translocating across the membrane. However, time-kill experiments in 3.4.5 show that #45's activity is rapid suggesting that it acts on the membrane. However, even when acting on the membrane, it might still need to insert itself into the membrane to form pores, which could be adversely affected by the increased size of the multimers.

After recombinant production of dimeric forms of #45 was unsuccessful, an alternative peptide expression system was assessed. Instead of attempting to produce and purify recombinant AMPs, this expression system was used to determine its suitability as a low-cost, high-throughput screening method for novel AMPs which might then be synthesised and investigated in greater detail. The *E. coli* strain used, JE5505, had an outer membrane mutation allowing for secretion of periplasmic proteins into the extracellular space. In the first instance, the system was used to assess the effectiveness of various AMP constructs in an Overlay assay, adapted from Guralp *et al.* (2013). However, the failure of the system to detect any antimicrobial peptides aside from the Plantaricin-derived peptides already validated in the literature, suggests either a

problem with expression or the practicalities of the assay. In this system, the peptides were attached to a PelB leader sequence which should secrete the peptide to the periplasm. This should have limited the effect of endogenous proteases on the nascent peptide, however this cannot be ruled out as the reason the expressed AMPs produced no zones of inhibition within the Overlay assay. Due to the small nature of the peptides inserted into the vector, from ~2 to ~10 kDa, visualisation of the expression levels by SDS-PAGE would be problematic when previous attempts at small peptide detection by this method failed.

Another possible reason for a lack of zones of inhibition is the ability of each peptide to diffuse through agar. One effect noted upon review of Agar MIC results was a potentially larger zone produced by Pln-423 than other peptides. However, zone sizes were not recorded empirically and no other peptide was active against *L. innocua*, so a bacteria-specific effect could not be ruled out. While more evidence is needed to determine if peptide-specific changes to the degree of diffusion may be contributing to the lack of zones of inhibition, it has been noted that peptides of an amphipathic nature can significantly deviate from the expected behaviour during agar-diffusion experiments (Bonev, Hooper, and Parisot 2008). What causes this change to the free diffusion assumption in classical agar diffusion assays is unknown, but hypotheses such as binding to the medium and inactivation by the bacterial film in the upper layer were asserted by the authors.

Host cell toxicity was another possible issue with this expression system. This was investigated by use of growth curve data, by comparing growth rate differences between a clone of JE5505 with no vector inserted and those with recombinant plasmids. Both induced and non-induced clones growth rates were investigated. One

result of note was that the growth of monomeric Pln-423 was significantly different from the control even without induction. This could suggest that Pln-423 is toxic, but it was indicated to be inactive against JE5505 *E. coli* by broth MIC assay (data not shown).

Comparisons of the growth rates of induced clones such as Pln-423, which was successfully validated in the Overlay assay, and clones encoding peptides with no detectable activity and/or production were used to corroborate whether toxicity was the issue. Intriguingly, two of the phage-derived peptides expressed, which have been characterised as inactive in broth, showed the fewest significant differences between time points when compared with the Pln-423 peptides. This could then indicate that they are being expressed at a higher rate, and therefore having a greater effect on the growth rate, than the other phage-derived peptides. However, a consideration of note is that these phage-derived peptides, #35 and #36, may be toxic when produced directly inside the cell but non-toxic when added to the cell culture media.

Another consideration for the significant differences in growth rates within the induced group could be that the Pln-423 clones and #35 and #36 may be successfully completing extensive production of their associated peptides. Endogenous proteases may be degrading the other peptides during the induction period, placing less strain on the cell. The Pln-423 clones and #35 and #36 may be more resistant to proteases within the host cell and be successfully secreted out, but Pln-423's sensitivity to other proteases indicates that there might be another reason for its successful detection within the Overlay assay (Van Reenen, Dicks, and Chikindas 1998). More investigations are needed to determine the exact reasons for the failure of the clones

to successfully express and secrete peptides to cause inhibition within the Overlay assay.

Even though the number of established AMPs that were functional in the Overlay assay was very limited, it was thought that screening hundreds of thousands of peptides may identify the rare peptides that can function as AMPs in this assay. Peptides that present effective antimicrobial activity, have low toxicity to the host cell and are effectively secreted through the agar medium. To this end, random 16-mer peptide libraries were designed where this region of variability was anchored via a flexible linker to each of five known AMPs (C-BF, #27, #35, #36, #45). Bioinformatic analysis of the peptide libraries revealed that the levels of bias within the libraries were kept to a relative minimum, although the original pIII library described in 3.2 displayed lower levels of bias and fewer repeated copies of each distinct sequence. Bias within these naïve libraries such as those described can be predominantly attributed to PCR errors within the initial construction or cell toxicity due to the minimal background expression of the peptides, as the analysis was carried out before induced expression of the peptides. Other sources of bias can include poor nucleotide ratios during primer synthesis, as DNA bias has been noted in previous library constructions despite equimolar expectations within the NNK stipulation (He *et al.* 2018). Initial analysis showed that the estimated diversity of all the libraries was at least similar to naïve phage-based peptide libraries, suggesting that the protocols used for these libraries were appropriate (Kumar *et al.* 2019). Further analysis looking at the amino acid positional biases present within the libraries revealed that there were distinct differences. When heatmaps of the results were compared, two groups could be created in which the five peptide libraries could be distributed: group one consisted

of #27, #35, and C-BF, and group two comprised #36 and #45. When comparing the two groups it was noted that group two contained more positional bias, rather than censorship or overabundance of particular amino acids. Due to the same primer-design being used for construction of the libraries, the noted differences between the two groups indicates the level of primer synthesis bias that can be present in machine-mixed primer sets. The highest averaged over-represented amino acid in any library was asparagine at +7% and the lowest averaged underrepresented amino acid in any library was arginine at -4%. Both the presence of and similar level of bias due to base incorporation in primer synthesis has been noted before (Tsoumpeli *et al.* 2022). As seen in previous libraries, the positively charged arginine residue was underrepresented across most positions (He *et al.* 2018; Rodi *et al.* 2002). He *et al.* (2018) saw strong arginine bias after expressing their library, whereas the peptide libraries analysed here were not expressed pre-analysis. This indicates such bias is present from even minimal expression in *E. coli*. This may have influenced the detection of AMPs within the screening assay, as it is well known that most AMPs rely on an overall cationic charge for their activity and selectively designed AMPs often have lysine and arginine preferentially added (Greber *et al.* 2017; Jiang *et al.* 2008; Lei *et al.* 2019). However, lysine was over-represented across all libraries so this may have limited any adverse effects on the levels of charge across the peptide sequences.

Residue one within both groups was notably biased when compared with other positions, with proline highly under-represented (between -2% and -5%) and asparagine (between +8% and +10%) highly over-represented. Residue one has been documented to be susceptible to positional bias in other libraries, with both proline

and arginine under-represented, similar to what was observed here (Rodi *et al.* 2002). However, proline is thought to be under-represented at position one due to its inhibition of the signal peptidase enzyme (Barkocy-Gallaghers, Cannon, and Bassford 1994; Nilsson and Heijne 1992). While previous reports suggest such bias is not just due to PCR/primer bias, but is created during protein expression (toxicity) and phage display. Here, similar bias is observed before extensive bacterial expression and without phage assembly, indicating its occurrence during library construction. Asparagine was over-represented in all the peptide libraries created in this study, except the original pIII phage library. While this has been noted before in phage libraries, the highest over-representation of asparagine was <+3% compared to +7% described here (Tsoumpeli *et al.* 2022). Although asparagine was found to be over-represented across all positions, the most over-represented asparagine residues were noted at the N-terminus which has been found in other libraries (Krumpe *et al.* 2006; Kuzmicheva *et al.* 2009).

Therefore, while the dimeric libraries described here may have displayed slightly increased levels of bias with regards to the amino acids present, the diversity of amino acids present would likely not have any significant effect on the detection of novel AMPs. This is evidenced by the high diversity of the libraries, which display low levels of highly repeated distinct sequences and a relatively high number of unique sequences (65-70% unique sequences, Table 6-3). Diversity was lower than comparable libraries, with >80% unique sequences often seen, but the sequence space was still sufficiently large for screening (Stellwagen *et al.* 2019; Tsoumpeli *et al.* 2022). The high diversity within each library enabled use of the Overlay assay as a very high-throughput screening tool; approximately 100,000 clones were screened

from each library for antimicrobial activity. However, no peptides were identified from the dimeric libraries which were able to cause zones of inhibition within the assay. Investigations into recombinant production of AMPs suggested that screening assays involving the expression of the lead peptide may be sub-optimal. It is possible these lead AMPs prevent agar diffusion even when coupled to another peptide. Furthermore, problems with detecting small peptides causes difficulties when determining if the peptide is being expressed. Specific issues with the Overlay assay include it is not known whether the medium may cause issues by interfering with the diffusion of the peptide, which may be compounded by linking an AMP with another peptide. As an alternative approach, these libraries could be adapted by reinsertion of a phage pIII, or pVIII, coat protein gene to convert them into phage display libraries. These could then be used for screening against bacterial pathogens to isolate binders for subsequent screening for relative AMP activity. This iterative development of divalent peptides using phage display may yield improved AMPs but it is a much more labour and time-intensive process. Here, the strategies employed to engineer more effective AMPs through chemical modification and bacterial expression provided no increase in antibacterial efficacy and attempts to facilitate very high-throughput screening failed to identify novel AMPs.

7 General Discussion

In the presented study, AMPs from both literature and those identified by phage display in this study were tested using various methodologies. This highlighted that the testing matrix can greatly influence the activity of the tested peptides and investigations into a high-throughput agar-based screening assay indicated that the use of agar is only suitable for peptides with specific, as yet unknown, properties. Attempts to create a novel recombinant peptide production pipeline were hindered by the small molecular size of the expression proteins, with no visualisation of the peptides. However, use of a flexible linker to dimerise the AMPs showed that this is a valid technique for both improving the efficacy of AMPs and altering their specificity. Furthermore, investigations into the selective nature of the heterodimers that were synthesised showed a high degree of selectivity against *E. coli*, even in a high density *Salmonella* environment. This shows that methods followed in this study can potentially yield a viable therapeutic for use in poultry.

7.1 Use of phage display for identification of novel AMPs

The phage display methodology has been demonstrated before for AMP discovery and the high diversity of the peptide libraries should be able to overcome the lack of knowledge surrounding AMP design (Sainath Rao *et al.* 2013). The bioinformatics-based analysis used here to define binding to the target bacteria, based on z-scores from positive and negative antigen binding, also limits the need for extensive synthesis and testing to find appropriate lead-peptides. This method of ranking specific enrichment has been demonstrated before (Naqid *et al.* 2016; Zhang *et al.* 2011). This approach does assume that binding is related to AMP activity, but phage display-based

techniques have been used across multiple studies and have identified various peptide sequences with antimicrobial potential.

For example, Tanaka, Kokuryu and Matsunaga (2008) identified 6 peptides by screening against bacterial magnetic particles; the peptides were active against *B. subtilis* but only at 100 μM . Compared to the MBC values of peptides in this study, as the authors tested their peptides in TBS, the peptides identified here had a much higher activity, with MBC values as low as 4.2 μM . Using whole cell panning Pini *et al.* (2005) identified only one AMP with efficacy of 125 mg L^{-1} , and Flachbartova *et al.* (2016) found two peptides which inhibited *L. innocua* at 30 μM , but both of these publications did not utilise NGS as described here to identify their AMPs. In comparison to the published literature, the number of AMPs identified and their established efficacy shows that the method described in the present study is a useful tool in improving AMP discovery.

The process of producing phage display peptide libraries and the subsequent panning rounds to select binders can all introduce bias into the peptides that are available for selection. This bias can come from not only the construction of the libraries, due largely to errors or biases in primer synthesis, but also during expression in a bacterial host selecting fitness mutants, post-translational processing of peptides and phage packaging (Plessers *et al.* 2021; Tsoumpeli *et al.* 2022; Zade *et al.* 2017). Overall there has been a noted bias against positively charged residues (He *et al.* 2018). Here, following NGS analysis of comparative binding analysis to a target and control bacteria, *in silico* bioinformatic analysis can discount any peptides that are enriched during panning due to a competitive advantage in host-bacterial growth and phage packaging. However, selection bias against positive charges is harder to overcome.

Here, bias against only arginine was confirmed in the NNK libraries used and/or created within this study as lysine was overrepresented overall in each of the libraries. It is unlikely the bias against arginine described here would have an impact on the discovery of novel AMPs, as the proportion of arginine residues are within 1% to that found across the AMPs present within the AMP database ADP3 (Personal communication, Prof K. Gough and Miss B. Clarke, University of Nottingham). Interestingly, despite the overrepresentation of lysine observed in the libraries they are still ~5-6% below the proportion observed within the AMP database. With regards to hydrophobic residues, isoleucine and alanine were also underrepresented compared to the proportion within ADP3 with a 5-6% and 4% difference between that observed within the libraries, respectively. However, proportions of leucine, phenylalanine, valine, and tryptophan were all similar (within 1%). Further investigations could involve using a more optimal system for designing an AMP-biased library, rather than the NNK system utilised here which is primarily for avoiding premature stop codons.

The present study identified five novel AMPs through the process of phage display. The five AMPs showed variable levels of potency and specificity. For example, peptide #35 was only active against *S. Typhimurium* at 32 mg L⁻¹ (16.9 µM) when activity was investigated in a buffered solution of Tris-HCl and NaCl. Conversely, peptide #45 displayed robust activity against all pathogenic bacteria tested when MHB was the medium, and only failed to kill *S. Enteritidis* in the buffered solution. When considering the results from the three different susceptibility assays, including the testing of known AMPs (identified in the literature), this clearly shows that AMP activity is matrix-dependent, a trait that is corroborated within the literature (Desriac *et al.* 2020).

7.2 Matrix impacts on AMP efficacy

Due to the lack of data surrounding the assay impact on AMPs, it is unknown how many lead-peptides have been disregarded because of high MICs within a particular medium, yet could be efficacious *in vivo*. There might also be unknown factors which are needed to obtain reliable results from AMPs during susceptibility testing. According to CLSI guidelines, some antibiotics need supplementation to ensure a robust testing method (CLSI 2015). For example, 2% NaCl is required when testing oxacillin and 0.002% polysorbate-80 when testing dalbavancin. It is possible the BSA and acetic acid supplementation used here was not enough to limit inter-assay variation, as there were over 8-fold differences seen in the MBC values for some peptides. The fact that AMP activity can be subject to changes in matrix conditions such as pH or ionic strength presents difficulties in identifying peptides which will have activity *in vivo*. The varying efficacies of AMPs across different mediums and test conditions in this study highlights the importance of utilising different experimental formats to determine whether a peptide has antimicrobial activity. Without using the right medium, peptides that have relatively low MIC values *in vitro* yet are efficacious in challenge studies *in vivo* can be overlooked (Arenas *et al.* 2020). Testing peptides in diluted media, such as the ~10% MHB used for the AMP Oncocin, has been shown to be effective (López-Pérez *et al.* 2017). It is possible such a strategy, instead of the buffered solution used here, could help limit the inter-assay variation seen and should be investigated.

Various factors involved in MIC testing contribute to potential inter-study variation. Albumin has been indicated to reduce the efficacy of some AMPs, but it is useful in limiting the binding of AMPs to laboratory plastics (Manabe and Kawasaki 2017;

Sovadinová, Kuroda, and Palermo 2021). Ion concentration is also important, as different MHB/MHA from different manufacturers might influence MIC's across studies and should be investigated further (Girardello *et al.* 2012). Use of cation-adjusted MHB can help obviate discrepancies, and is recommended in most susceptibility testing guidance, but increased concentrations of cations can result in higher MIC values (Anderegg, Fritsche, and Jones 2004; Schwab *et al.* 1999). This is useful in the standardisation of AMP testing but may mean novel antimicrobial sequences are excluded, even though they may be able to be improved.

The use of PBS or other salt-containing media must be critically examined within AMP testing. The results from this study suggest that peptide #45 was less active in high salt concentrations compared to standard broth MIC conditions, but the effect on biotinylated peptide #45 was greater. This increased susceptibility may be due to the modified termini, as such modifications have been shown to have effects, both positive and negative, on antimicrobial activity (Huan *et al.* 2020; Saikia *et al.* 2017). This suggests the problem is individualised, with both the peptide and exact nature of the modification affecting the outcome. Biotin, due to its small size, seems to have a minimal effect on the antimicrobial activity when tested under standard conditions. This is evidenced by Powers *et al.* (2006) who found a two-fold increase in MIC, which was the same as seen here. The link between salt resistance and the termini of AMPs has been speculated to be due to the hydrophobicity of the termini, which allows deeper penetration into the membrane (Yu *et al.* 2013). Therefore, the hydrophilicity of biotin may be the reason that biotinylated peptides have a propensity for a susceptibility to elevated salt concentrations.

7.3 Alternative high-throughput AMP discovery tools

As an alternative novel AMP identification process, the Overlay assay was speculated to be a high-throughput, low-cost method when compared to others. An example is the method employed by Fisher *et al.* (2016) who impregnated agar with beads containing cleavable peptides which would then diffuse and create a zone of inhibition. However, the lack of positive hits from the Overlay assay screening performed here and no antimicrobial activity seen by the AMP dimers in the agar dilution assays both suggest that AMP activity is affected by agar. This is despite activity identified in each of the dimeric AMPs, with the exception of 35-35, when using MHB or in the bactericidal assay. This has been noted before in the literature, and would suggest that agar-based approaches are not appropriate for MIC screening of positively charged antimicrobials (Andersson *et al.* 2016; Kunin and Edmondson 2016; Matuschek *et al.* 2018). Even when high-throughput methods are not predicated on activity within agar they have one significant disadvantage which the screening processes in this study have also not considered: AMPs often utilise immunomodulatory properties as an indirect form of antimicrobial action.

The potential immunomodulatory properties of synthetic AMPs are ignored using standard methods of screening; there is currently no standardised protocol for determining presence and the extent of a peptide's immunomodulation. One suggested protocol uses the SPOT synthesis method described earlier (6.1) and peripheral blood mononuclear cells (PBMCs). By incubating peptides together with the PBMCs, toxicity can be assessed by using the lactate dehydrogenase assay and immunomodulation via chemokine/cytokine quantification by ELISA. This screening assay would be useful for a systemically-administered AMP, but if the route of

administration was oral, then the cells used would need to be changed. Furthermore, orienting techniques such as phage display towards identifying immunomodulatory peptides would be challenging, due to the many different ligands and cell types which AMPs have been indicated to interact with (Haney and Hancock 2013; E. Y. Lee, Lee, and Wong 2019).

However, information may be gleaned from studies such as Liu *et al.* (2008) and Bao *et al.* (2009), which studied the effect of swine AMPs on the chicken intestinal mucosal immunity. Increased IgA-secreting cells observed in both studies suggests a common stimulatory mode of action, which could be utilised when choosing a positive ligand to pan a phage library against. Increasing IgA secretion may help maintain a balance of beneficial gut microflora when there is competition from a pathogen, as IgA has a documented role in homeostasis of the gut microflora (Strugnell and Wijburg 2010). Furthermore, AMPs can be induced to secretion in the gastrointestinal tract of humans by bacterial stimuli and have been documented to be sources of chemoattractants for T-lymphocytes (Chertov *et al.* 1996; Yang *et al.* 1999). This suggests that exogenously added AMPs may have positive immunostimulatory effects, but more research is needed to understand the mechanisms and whether it may be possible to design a strategy to bias lead-peptides towards immunomodulatory activity. One consideration of note will be that AMPs can have both pro- and anti-inflammatory effects so careful design will be needed to prevent adverse effects if immune modulation becomes the target for AMP design. For example, sustained expression of AMPs have been implicated in the autoimmune skin disease psoriasis by activation of the Toll-like receptor nine (TLR9; Lande *et al.*, 2015).

7.4 Potential of AMPs as therapeutic drugs in poultry

With the continued prescription of antibiotics for use in agriculture, there will always be the chance for resistance to develop. However, it is not appropriate for all antibiotic prescription to halt. At the moment there are high priority, critically important antibiotics which have been classed as such due to their use as last resort antibiotics within human therapeutic use. If further antibiotics can be constrained to only human use or the tonnage used reduced, without affecting animal welfare, then this may help to reduce resistance development events. By utilising AMPs as the first line of treatment options in animals, this might prevent a substantial amount of antibiotics being used and therefore lower the associated resistance risk. In previous AMP administration examples they were given via feed or water where there are several efficacy hurdles to overcome: protease activity, pH and salt sensitivity, bacterial density, and toxicity. These have to be taken into account to ensure enough active AMP arrives at the site of action in the gut, as sublethal doses of AMPs can cause higher levels of survival in bacteria which are subsequently challenged with a higher dose (Rodríguez-Rojas *et al.* 2021). This is thought to be due to LPS modifications either via global regulators such as PhoPQ and MisR or more targeted approaches such as inducing capsule modifications via the *cps* genes (Duperthuy 2020).

In addition to discovering five novel AMPs, the capacity to improve AMP efficacy and refine their specificity was proven here. By linking peptides together, either as a heterodimer with C-BF, or by making homodimers, alterations were observed to both the potency and range of activity. Within the context of the results from the broth MIC assays, peptides #35 and #45 were dissimilar. Peptide #35 was inactive, only being active against *S. Typhimurium* within the MBC assay, and peptide #45 was a broad-

spectrum agent active against all bacterial isolates tested except for the Gram-positive *L. innocua*. Peptide #45 did show a selectivity towards *E. coli*, with both O2 and O78 serotypes inhibited effectively at 16 mg L⁻¹ while *S. Typhimurium* and *S. Enteritidis* were inhibited at 64 and 128 mg L⁻¹, respectively. The AMP C-BF was the most potent peptide tested in the broth MIC assay, with no selectivity seen between *E. coli* or *Salmonella*. A two-fold difference was seen between the MICs against *E. coli* O78, *S. Typhimurium* (0.5 mg L⁻¹) and *E. coli* O2, *S. Enteritidis* (1 mg L⁻¹), but this may have been caused by inter-assay variation. Both #35 and #45 were inactive as homodimers within the broth MIC assay, whereas CBF-CBF exhibited a slight specificity towards the *E. coli* serotypes with the greatest difference in MIC observed between *E. coli* O78 (4 mg L⁻¹) and *S. Enteritidis* (32 mg L⁻¹). Intriguingly, when C-BF was conjugated to the *S. Typhimurium*-specific #35, the heterodimer exhibited similar levels of specificity towards *E. coli* to CBF-CBF, albeit at higher relative concentrations. The heterodimer 45-CBF also had lower MIC values against *E. coli* serotypes than it did against the two *Salmonella* serovars tested, which suggested size may influence the activity of these peptides.

When *E. coli* and *Salmonella* isolates were incubated together at similar densities with the AMPs, selective killing of the *E. coli* cells still occurred by all peptides tested when at or above its relative MIC (first defined by broth microdilution assay in monocultures), yet below the MIC for the *Salmonella* isolate within the mixed culture. Furthermore, inhibition of *Salmonella* cells also occurred at the same MIC as defined by broth microdilution using monocultures. If the AMPs tested had true selectivity, it would be expected that the AMP would be sequestered away by the *E. coli* cells, leaving less available for the *Salmonella* cells present and thereby causing an apparent

increase in the concentration of peptide needed to kill, or inhibit the *Salmonella*. The AMP was expected to be sequestered and influence the MIC as preliminary data, which used a modified assay from Udekwu *et al.* (2009) where filtrate from a one hour incubation of C-BF (20× MIC) with *E. coli* O78 at $\sim 5 \times 10^5$ CFU mL⁻¹ was used in a standard broth MIC assay against the same isolate, suggests that the AMP likely permanently associates with the bacterial cell membrane until a saturation point is reached and any molecules left remain free in solution (data not shown). This saturation point was indicated to be higher than the concentration necessary for bacteriostatic/bactericidal behaviour.

There could be several factors influencing this result. For example, Loffredo *et al.* (2021) stipulated that the MIC is predicated on the combined effect of two factors: the number of AMP molecules needed to cause cell death and the number of AMP molecules that are cell-associated. The MIC of an AMP reaches a plateau as bacterial density is reduced, eventually with no further reduction in the MIC value regardless of bacterial density (Jones *et al.* 1994; Levison *et al.* 1993). This is postulated to be due to less cell-associated peptides and more in free solution (Morelli *et al.* 2018). The MIC determined in the monocultures may be valid even in slightly higher densities of bacteria tested. When the bacteria were added at $\leq 10^6$ CFU mL⁻¹, it may have allowed for enough peptides to become cell-associated to *Salmonella* despite the selectivity for the *E. coli*. One method to test if there was specificity would require repeating the broth MIC at gradually higher concentrations of monocultures until the concentration of peptide is no longer enough to inhibit the bacteria by 50%. Once the maximum density of bacteria is known before increases are found to the MIC of peptide, adding a mixed culture of *E. coli* and *Salmonella* to attain the same concentration and then

adding peptides at its MIC for *Salmonella* may display no inhibition of *Salmonella* due to the selectivity towards *E. coli*.

Further evidence for selectivity of the peptides is indicated by the results from the bacterial spike assays. Here, it was shown that when *E. coli* O78 was added for a final concentration of either 10^4 or 10^6 CFU mL⁻¹ to *S. Enteritidis*, at a concentration of 10^8 CFU mL⁻¹, the *E. coli* cells were selectively targeted by the AMP added. The selectivity was strongest, out of the peptides tested, with the two heterodimers 35-CBF and 45-CBF which reduced viable counts of *E. coli* O78 by over 99.9% and 98.7% from an initial inoculum of 10^6 CFU mL⁻¹, respectively. This suggests that the heterodimers have promising potential as a therapeutic drug.

Colibacillosis is thought to be the most common bacterial disease of poultry, with significant impacts on human health (Barnes, Nolan, and Vaillancourt 2008). The efficacy of the AMPs described here at killing >90% of the target APEC, suggests they could be of use as a short prophylactic course when an infection has been detected or is expected. Recently, it has been postulated that APEC strains can reside in the intestine as opportunistic pathogens until the bird is stressed, where translocation of intestinal bacteria can be facilitated by an increased permeability of the intestinal lining (Kathayat *et al.* 2021; Mandal *et al.* 2020). Stressors can include heat, exacerbated by climate change, or a primary infection e.g. from the protozoan parasite *Histomonas meleagris*. This organism can cause various symptoms such as inflamed and lesioned caeca, but can also increase relative populations of *E. coli* and the chances of extraintestinal infection (Abdelhamid *et al.* 2020, 2021). The peptides could be effective at modulating dysbiosis, by selectively lowering the proportion of *E. coli* if concentrations of AMPs can be optimised for the bacterial densities present.

Alternatively, the potential immunomodulatory properties of AMPs described earlier may help maintain the intestinal barrier preventing systemic disease (Robinson *et al.* 2015). As stable resistance mechanisms can be difficult to achieve, and require a high fitness cost, appropriate AMP usage has the potential to reduce any long-term resistance problems (Andersson *et al.* 2016).

While there is potential for AMPs to work as a therapeutic once administered, the difficulties in administering should be discussed; a commercial poultry farm can have hundreds of thousands of birds in a single shed where individual administration would be impossible. Within the context of commercial farming, the most cost-effective solution would be to supply the AMP in the drinking water. However, the effects of this environment on the stability and efficacy of the AMP would need to be investigated as the activity of these peptides is matrix-dependent. Another option would be to include the AMP within the milled feed, which is more expensive, but avoids suspending the peptide in water.

7.5 Limitations of study

There are limitations to the current study, which should be addressed. Firstly, AMP activity was investigated at 37°C which is slightly colder than the internal temperature of poultry (42°C). This temperature was used as it is the standard for broth microdilution and agar dilution methods when testing the antimicrobial susceptibility of the bacterial species used here. Secondly, only *in vitro* results are discussed; no *ex vivo* or *in vivo* models were used to test the AMPs discovered. Therefore, it is unknown how the AMPs would function other than in laboratory settings. No models were used due to impacts on the research timeline by the COVID-19 pandemic and the

biosecurity measures in place for an avian influenza outbreak. This also impacted investigations into AMP efficacy against *Campylobacter*, as a prominent poultry pathogen it was introduced but was neglected within this study after the time constraints imposed by COVID-19.

7.6 Further research

To further investigate the results detailed here, one of the most important experiments would be to determine the mode of action of the described AMPs. By investigating the mode of action, the potential for resistance to develop can be hypothesised and what pathways might be altered to develop it. This would allow the possibility of the design of multimodal AMPs, or to inform further experiments on synergy with either other known AMPs or antibiotics as there are examples of both (Aoki *et al.* 2009; Kim, Rajasekaran, and Shin 2017; Pöppel *et al.* 2015). Confocal microscopy studies using biotinylated AMPs and streptavidin-conjugated fluorophores could help to determine where the AMPs localise on susceptible bacteria. However, there have been concerns about how the conjugation of fluorophores may affect small peptide interactions with membranes (Hedegaard *et al.* 2018). This is further evidenced by the increased salt sensitivity due to the addition of biotin to peptide #45, as previously discussed. Therefore, other fluorescent techniques may be utilised instead.

For example, Farha *et al.* (2013) developed a protocol which could be used to determine any deterioration of the proton motive force (PMF), an effect of a membrane-active AMP. The PMF is governed by two factors, the electric potential and the transmembrane proton gradient. By adding a dye known as DiSC₃(5), which accumulates and self-quenches in the cytoplasmic membrane in response to the

electric potential, information can be gained on whether the AMP is membrane-active as a lysed membrane releases the dye and allows fluorescence. Further information can also be gained, based on whether the AMP specifically affects one factor over another. As a decrease in one factor can be compensated for by an increase in another, understanding which factor is affected can inform on potent synergistic interactions of AMPs (Bakker and Mangericht 1981).

Investigations into other methods for increasing AMP efficacy could also yield insights into the potential for the AMPs discovered here. For example, further investigations into the possible synergistic effects of mixing the heterodimeric AMPs with each other or with antibiotics could yield potent combinations. Synergistic interactions have been well-documented between AMPs and antibiotics, often thought to be due to the permeabilizing activity of the AMP allowing access to the antibiotic (Zhu *et al.* 2022). Having identified novel AMPs, optimisation of their amino acid sequence could also improve their potency, reduce the haemolysis levels observed and retain the specificity. Little is known about the rules governing AMP activity, but broad guidelines do exist for the amino acid structure. For example, cationicity is an important factor with peptides less than +3 having weak antimicrobial efficacy while those with +8 or above tended to be more haemolytic and/or have a reduced spectrum of activity (Zelezetsky and Tossi 2006).

The peptides discovered using phage display all consist of very low overall charge (< +3), relative to the majority of reported AMPs. Therefore, improving the overall cationic charge for these AMPs is likely to have a positive impact on the antimicrobial potency. The amphipathic and hydrophobic characteristics are also important for AMPs with an α -helix structure, yet the optimal proportion of both is ~50%: increases

over this for either trait tends to cause an concomitant increase in haemolytic activity while decreases for either trait under 50% cause a concomitant decrease in antimicrobial activity (Chen *et al.* 2005; Dathe *et al.* 1997). The phage-derived peptides discovered here contain between 38% and 56% hydrophobic residues, with the DBAASP-derived C-BF containing 47%. The linker sequence has a lower hydrophobicity proportion at 33%, which may affect how the peptides act in aqueous solution and on contact with a membrane. Haemolysis assays also indicated a high level of cytotoxicity of the AMPs identified here, therefore optimisation of the linker sequence, and/or the monomer sequence, may be a viable route for improving antimicrobial efficacy and selectivity.

One way of determining important residues within an AMP is called alanine scanning. By replacing one residue at a time with an alanine, it might be determined what residues could be replaced without adversely affecting the peptide's properties. Replacements with charged residues, such as lysine and arginine, could increase the antimicrobial activity. Arginine in particular is seen often in membrane-active peptides, which could be due to an enhanced penetration and interfacial binding (Cutrona *et al.* 2015; Hristova and Wimley 2011; Li, Vorobyov, and Allen 2013). Use of tryptophan could be employed within the hydrophobic face of the peptides, as tryptophan is well-known as a membrane-active residue and is often used as a substituted amino acid to increase potency (Deslouches *et al.* 2005; Vogel *et al.* 2002).

Further experiments should also include testing the peptides for resistance to proteases found in the gut, changes in salt concentration, and sensitivity to pH. The administered AMP would need to be able to transit within the chicken's digestive tract while retaining its activity; the proventriculus and gizzard are both acidic in pH and so the

AMP's resistance to this should be investigated (Mabelebele *et al.* 2014). Interestingly, a recent review by Malik *et al.* (2016) describes multiple studies which show that some AMPs can be enhanced by low pH, which could be connected to histidine residues as they are uncharged at physiological pH yet cationic at acidic pH. This could be applicable to the histidine-containing phage-derived AMPs discovered here (#27, #36 #59), the results of which might be of consequence if used *in vivo*. Resistance to pepsin, trypsin, and chymotrypsin should be tested as these are common proteolytic enzymes found in the digestive tract (Tabata *et al.* 2017). AMPs are often highly susceptible to proteolysis, as by their nature they are high in basic residues and tryptophan which are targeted by trypsin and chymotrypsin, respectively (Vajda and Szabo 1976).

A significant problem for applying AMPs as a potential therapeutic within poultry farming is the cost of production. Due to the low cost per bird for farmers, unless the AMPs can be produced cheaply the most economical avenue for a potential outbreak would be antibiotics and/or culling. Recombinant production offers an economic method to circumvent this issue. However, within this study there was significant difficulty in detecting any expressed peptide. Perhaps to circumvent this, further experiments would include a small tag into the recombinant peptide, such as the 6× His-tag used in a previous *in vivo* biotinylation attempt for recombinant scygonadin (Li and Sousa 2012). Other tags which could be utilised include the SUMO tag, as it has been used successfully in producing recombinant C-BF in *B. subtilis* (Luan *et al.* 2014). The use of protease cleavage sites between the tag and AMP would then leave functional AMPs, which could also then be further purified by processes such as size

exclusion. However, the extra purification steps add complexity and cost which the experimental plan attempted in the original study was designed to avoid.

To test the AMPs used in this study within a more realistic environment, investigations into their effect on cultures isolated from the chicken gastrointestinal tract would yield important information on their efficacy. Although *E. coli* can colonise throughout the gut, the caeca are an appropriate target for such a study as they contain high levels of *Salmonella* and *E. coli* during infections and are also easily used in *ex vivo* studies as they can be tied with the original contents still in place (Atterbury *et al.* 2011; Beery, Doyle, and Schoeni 1985; Hudault, Guignot, and Servin 2001). Viable counts of prominent bacterial species after incubating the contents of the caeca with an artificially spiked pathogen, such as APEC, and an AMP would help elucidate levels of killing of the target bacteria as well as any off-target effects. Furthermore, histological evidence of any intestinal morphological changes may provide evidence of immunomodulation (Daneshmand *et al.* 2019).

It would also be beneficial to test the cytotoxicity of the AMPs against more relevant host cells than RBCs, for example there are immortalised chicken embryo intestinal epithelial cells that are commercially available. However, this cell line is new and has not been as comprehensively used as other cell lines such as human colonic adenocarcinoma cells. Before the AMPs identified in this study could be taken further, more toxicity data would need to be collated. Both toxicity to the host and to beneficial microorganisms would need to be investigated as an important consideration is the dosage that would be utilised in studies such as these. For example, current antimicrobial therapy currently has adverse effects on the beneficial microflora within the gastrointestinal tract. Shown here are AMPs that have some selectivity for

pathogenic *E. coli* even when in a minority population, by optimising the dosage used it could be possible to further increase the effective killing potential of these AMPs, reducing bacterial load even more than the >99.9% described while still retaining the selectivity and limiting adverse effects on non-target species.

8 Concluding remarks

It is well known that antibiotic resistance is rising, and deaths attributed to failed treatments will also rise as a consequence. In one recent publication, deaths associated with bacterial AMR were an estimated 4,950,000 (Murray *et al.* 2022). Data in low-income countries was still found to have many gaps, which will have to be addressed if the extent of the problem is to be known. AMPs, such as the ones discovered here, offer a promising solution for bacterial control in both humans and animals. There is much still to learn about AMP activity, but research has continually improved the knowledge surrounding AMP design, activity, and screening methods. Here, the medium for AMP testing was found to be important with peptides active in a buffered solution but not within MHB. Interestingly, the majority of AMPs' activity in agar was suggested to be compromised. This has implications for any screening assays for novel AMPs. As agar-based assays may not be appropriate and to minimise lead-peptide loss based on matrix-dependent activity, use of a small number of varying concentrations of MHB (10, 25, 50, 100%) may be an ideal method to screen putative AMPs. The standardised broth microdilution assay would be easily modified to include these concentrations of growth medium.

Here, we have described five novel AMPs and characterised them in terms of their antimicrobial activity and other pertinent characteristics. It has also been shown that it is possible to alter the specificity of an AMP by linkage with another AMP, as is corroborated by various publications. The novel dimeric AMPs produced in the presented study can reduce APEC viability within a very high excess of non-target bacteria to a greater degree than their related monomers, indicating a specific activity. Indeed, as the non-target bacteria was a closely related Gram-negative bacteria, these

data indicate a high level of specificity that may allow such AMPs to target APEC yet have limited effects on the beneficial microflora within the gut. Currently, it has been shown that genes linked to multidrug resistance can be positively associated with a number of virulence genes in APEC (Johnson *et al.* 2012). This may be due to antibiotic use against resistant strains, which would reduce the competition and improve the growth conditions for the resistant strain thereby propagating the related genes (O'Brien 2002). Further research on the identified AMPs could elucidate their effect within a more realistic matrix, such as *ex vivo* caecal pouches, to further assess their potential as an effective and highly selective treatment for APEC infections.

9 Appendices

9.1 Bacterial requirements for MIC assays

Bacteria	OD ₆₀₀ required	Temperature required	Duration required	Reference
<i>Salmonella spp</i>	0.15-0.3	35+/-2°C	16-20 h	(CLSI 2018)
<i>E. coli</i>	0.15-0.3	35+/-2°C	16-20 h	(CLSI 2018)
<i>B. subtilis</i>	0.4-0.5	35+/-2°C	16-20 h	(CLSI 2018)
<i>L. innocua</i>	0.05-0.1	37+/-2°C	48 h	(Troxler <i>et al.</i> 2000)

9.2 Primer sequences used

9.2.1 Monomeric and dimeric peptides

Primer	DNA sequence (5'-3')
Forward – E50-52	[Phos]GTGTGGGCGAGCTGCAACCTGGCGACCGGCTGCGCGGCGTGGCTGTGCAAACTGGCGTAATCATGCCAGTTCTTTTGGCTAGCTAA
Reverse – E50-52	GTTGCCGCACTGGCACCAGTTCACGCTGTTGCACACGCCGTTGCCATAGTTTTTGGTGGTCGCCAGGCTGCGAAACTGCGC
Forward – E50-52 (dimer)	[Phos]GTTTGGGCATCTTGTAACTCTGGCAACCGGTTGTGCCGCATGGCTGTGTAACTTGCAAAAGAAAGCGGCAGCGTGAG
Reverse – E50-52 (dimer)	GTTACCACACTGACACCAATTAACAGAGTTGCAAACACCGTTACCGTAATTTTTTCGTCGTGGCCATCGCCGGCTGGGCGC
Forward – BuC4f	[Phos]TGGCGCCTGCTGTTTAAAGGCTAATCATGCCAGTTCTTTTGCTAGCTAA
Reverse – BuC4f	CCAAATGCTCCACGGAATCGCCGCCAGGCTGCGAAACTGCGC
Forward – BuC4f (dimer)	[Phos]TGGCGTCTGTTATTCAAAGGTAAAGAAAGCGGCAGCGTG
Reverse – BuC4f (dimer)	CCAGATAGACCATGGGATGGCGGCCATCGCCGGCTGGGCGC
Forward - CBF	[Phos]GAATTTTTTAAAAAACC GCGCGTGATTGGCGTGAGCATTCCGTTTTAATCATGCCAGTTCTTTTGGCTAGCTAA
Reverse - CBF	TTTCGCGCGTTTTTTCACGCTTTTTTTCAGTTTGCGAAAAAATTCGCCAGGCTGCGAAACTGCGC
Forward – CBF (dimer)	[Phos]GAGTTTTTCAAAAAGCCGCGTGTTATCGGTGTTAGTATCCCATTCAAAGAAAGCGGCAGCG
Reverse – CBF (dimer)	TTTGGCACGTTTTTTAACAGATTTTTTTCAGCTTACGGAAGAACTTGGCCATCGCCGGCTG

Forward – NZ2114	[Phos]AGCATTAAAGGCTATAAAGGCGGCTATTGCGCGAAAGGCG GCTTTGTGTGCAAATGCTATTAATCATGCCAGTTCTTTTGGCTAG CTAA
Reverse – NZ2114	TTTGCAATGGTTATGGCAGCGCAGATCATCTTCGTTCCACGGGGC GTTGCAGCCAAAGCCCCGCCAGGCTGCGAAACTGCGC
Forward – NZ2114 (dimer)	[Phos]TCGATCAAAGGTTACAAGGGTGGTTACTGTGCCAAAGGTG GTTTCGTTTGTAAAGTGTACAAAGAAAGCGGCAGCGTG
Reverse – NZ2114 (dimer)	TTTACAGTGATTGTGACAACGCAGATCGTCCTCATTCCACGGACC ATTACAACCGAAACCGGCCATCGCCGGCTGGGCGGCC
Forward – MP1102	[Phos]AGCATTAAAGGCTATAAAGGCGGCTATTGCGCGAAAGGCG GCTTTGTGTGCAAATGCTATTAATCATGCCAGTTCTTTTGGCTAG CTAA
Reverse – MP1102	GCGCAGTTTCGCAGCCTGGCGGGCTTTGGCTGCAACGGCCCCGTG GCAGGAAGATGATGTGAAATGCCATAACCATTGCAAA
Forward – MP1102 (dimer)	[Phos]TCTATCAAAGGTTACAAGGTGGTTACTGTGCCAAAGGAG GGTTCGTTTGTAAAGTGTACAAAGAAAGCGGCAGCGTG
Reverse MP1102 (dimer)	CTTACAGTGATTGTGACATTTAACATCGTCCTCTTGCCACGGACC ATTACAACCGAAACCGGCCATCGCCGGCTGGGCGGCC
Forward – Lac	[Phos]CGCATGAAAAAACTGGGCGCGTAATCATGCCAGTTCTTTT GGCTAGCTAA
Reverse – Lac	CCACTGCCAGCGGCGGCATTTAAACGCCAGGCTGCGAAACTGCG C
Forward – Lac (dimer)	[Phos]CGTATGAAAAAGCTGGGTGCCAAAGAAAGCGGCAGCGTG
Reverse – Lac (dimer)	CCACTGCCAACGACGACACTTGAAGGCCATCGCCGGCTGGGCGG CC
Forward – Ca2.6	[Phos]CTGGCGGATGTGGTGAAAAGCAAAGGCCAGCCGGCGTAAT CATGCCAGTTCTTTTGGCTAGCTAA
Reverse – Ca2.6	CAGGCCGCCAGCACTTTGCCAATGCTGCTCACCAGGCCCGCCA GGCTGCGAAACTGCGC
Forward – Ca2.6 (dimer)	[Phos]CTGCTGGCCGACGTTGTGAAATCTAAGGGTCAACCAGCCA AAGAAAGCGGCAGCG
Reverse – Ca2.6 (dimer)	ACCACCCAGAACTTTACCGATACTACTAACAAGACCGGCCATCG CCGGCTGGGCGGCC
Forward – Pal-Ca	[Phos]ATTCTGAACAACCTGAAATGCAAACCTGGCGGGCGGCTGCC CGCCGTAATCATGCCAGTTCTTTTGGCTAGCTAA
Reverse – Pal-Ca	TTTCACCGCAAATTCTTTGCCGGTATCTTTAATAATATCCAGAAA GCCCCGCCAGGCTGCGAAACTGCGC
Forward – Pal-Ca (dimer)	[Phos]ATCCTGAATAATCTGAAATGCAAACCTGGCCGGTGTTGCC CACCAAAAGAAAGCGGCAGCGTG
Reverse – Pal-Ca (dimer)	TTTAACCGCGAACTCTTTACCGGTGTCTTTGATGATGTCCAGGAA ACCGGCCATCGCCGGCTG
Forward – Po-Def	[Phos]AGCATTAAAGGCTATAAAGGCGGCTATTGCGCGAGCGCGG GCTTTGTGTGCAAATGCTATTAATCATGCCAGTTCTTTTGGCTAG CTAA

Reverse Po-Def	TTTGCAATGGTTATGGCATTTCATATCATCTTCATCCCACGGGCC GTTGCAGCCAAAGCCCGCCAGGCTGCGAAACTGCGC
Forward – Po-Def (dimer)	[Phos]TCTATCAAAGGTTACAAAGGTGGGTACTGTGCATCCGCCG GTTTCGTTTGTAAGTGTTACAAAGAAAGCGGCAGCGTG
Reverse – Po-Def (dimer)	TTTACAGTGATTGTGACACTTCATGTCATCTTCGTCCCACGGACC ATTACAACCGAAACCGGCCATCGCCGGCTG
Forward – Linker	[Phos]ACAGCTGGCGCAGTTTCGCAGCCTGGCGAAATATTATGGC AACGGCGTT
Reverse - Linker	TCGCTGCTCACGCTGCCGCTTCTTTGGCCATCGCCGGCTGGGCC GCCA
Forward – Plantaricin	[Phos]GGCCAGGCGTTTAGCTGCAGCGTTAGCCATCTGGCGAACT TTGGCCATGGCAAATGCTAATCATGCCAGTTCTTTTGGC
Reverse – Plantaricin	CCAGTTAACGCTGCAGCTATGTTTGCCGCAGGTAACGCCGTTGC CATAATATTTGGCCATCGCCGGCTGGGCCGC
Forward – Plantaricin (dimer)	[Phos]CAGGCGTTCTCTTGCTCTGTGTCTCACCTGGCGAACTTCGG TCACGGTAAATGCAAAGAAAGCGGCAGCGTGAGCAGCGAACAG C
Reverse – Plantaricin (dimer)	ACCCAGTTCACAGAGCAAGAGTGTTTACCGCAGGTCACACCGT TACCGTAGTATTTGGCCATCGCCGGCTGGGCCGCCAGTA
Forward – H28	GGCCAGGCGTTTAGCTGCAGCGTTAGCCGTCTGGCGAACTTTGG CGAAGGCAAATGCTAATCATGCCAGTTCTTTTGGC
Reverse – H28	CCAGTTAACGCTGCAGCTATGTTTGCCGCAGGTAACGCCGTTGC CATAATATTTGGCCATCGCCGGCTGGGCCGC
Forward – S27	GGCCAGGCGTTTAGCTGCAGCGTTGATCATCTGGCGAACTTTGG CCATGGCAACTGCTAATCATGCCAGTTCTTTTGGC
Reverse – S27	CCAGTTAACGCTGCAGCTATGTTTGCCGCAGGTAACGCCGTTGC CATAATATTTGGCCATCGCCGGCTGGGCCGC
Forward – #27	[Phos]AGCCGCATTTGGTGCCTGCTGCATTAATCATGCCAGTTCTT TTGGCTA
Reverse - #27	CGGAAAGTTCACTTTGCTGCCCCACGCCAGGCTGCGAAACTGCG CCAG
Forward - #27 (dimer)	[Phos]AGCCGTATCTGGTGTCTGTTACACAAAGAAAGCGGCAGCG TG
Reverse - #27 (dimer)	CGGGAAATTAACTTTAGAACCCCAGGCCATCGCCGGCT
Forward - #35	[Phos]GGTTGCTTCCTGGCGTACCTGCTGTAATCATGCCAGTTCTT TTGGC
Reverse - #35	CGGCGCAGACAGACGTTACGCCACGCCAGGCTGCGAA
Forward - #35 (dimer)	[Phos]GGCTGTTTTCTGGCCTATCTGTAAAAGAAAGCGGCAGCGT GA
Reverse - #35 (dimer)	CGGGGCGGACAGGCGCTCGCGCCAGGCCATCGCCGGCT
Forward - #36	[Phos]CTGGCGCATGTGAGCTGCTGGATTTAATCATGCCAGTTCTT TTGGCTA
Reverse - #36	CAGCCACGGCCAGCTGCTCACCACCGCCAGGCTGCGAAACTGCG CCAG
Forward - #36 (dimer)	[Phos]CTGGCCCACGTTTCTTGTTGGATCAAAGAAAGCGGCAGCG TG

Reverse - #36 (dimer)	CAGCCATGGCCAACTAGAAACAACGGCCATCGCCGGCTG
Forward - #45	[Phos]CAGCTGCGCGATTTTGCGGTGTGCTAATCATGCCAGTTCTT TGGCTA
Reverse - #45	GCGCAGGCCCCATTTGCCCCAGCCCGCCAGGCTGCGAAACTGCG CCAG
Forward - #45 (dimer)	[Phos]CAGCTGCGTGACTTCGCCGTTTGTAAAGAAAGCGGCAGCG
Reverse - #45 (dimer)	ACGCAGACCCCCACTTACCCCAACCGGCCATCGCCGGCT
Forward - #59	[Phos]TTTCTGCATAAACTGTTTTGCCTGTAATCATGCCAGTTCTTT TGGCTA
Reverse - #59	CGCCGGGCGCGGGCGGCACAAACGCCGCCAGGCTGCGAAACTGC GCCAG
Forward - #59 (dimer)	[Phos]TTCCTGCACAAACTGTTCTGTTTAAAAGAAAGCGGCAGCG TGA
Reverse - #59 (dimer)	CGCCGGACGCGGTGGAACGAAGGCGGCCATCGCCGGCT
Forward - #35 + Plantaricin (dimer)	[Phos] GGTGCTTCCTGGCGTACCTGCTGAAAGAAAGCGGCAGCGTGAG
Reverse - #35 + Plantaricin (dimer)	CGGCGCAGACAGACGTTACGCCAGGCCATCGCCGGCTG
Forward - #27 + Plantaricin (dimer)	[Phos]CGAGCCGCATTTGGTGCCTGCTGCATAAAGAAAGCGGCAG CGTG
Reverse - #27 + Plantaricin (dimer)	GAAAGTTCACCTTTGCTGCCCCAGGCCATCGCCGGCTGGGC

9.2.2 Preparation for NGS sequencing of NNK libraries

Round one forward NGS primer	CTAGAACATTTCACTTACATGATTACGCCAAGCTCG
Round one reverse NGS primer	GTAATCCTTGTGGTATCGGAAACTGCGCCAGCTGTT
Round two universal primer	CCTCTCTATGGGCAGTCGGTGATCTAGAACATTTCACTTAC
Round two barcode primer one	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAACGTAAT CCTTGTGGTATCG
Round two barcode primer two	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGAGAACGTAA TCTTGTGGTATCG
Round two barcode primer three	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGGATTCGTAAT CCTTGTGGTATCG
Round two barcode primer four	CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCGTAAT CCTTGTGGTATCG
Round two barcode primer five	CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACGTAA TCCTTGTGGTATCG

9.2.3 Detection of APEC genes

<i>tsh</i> forward	G TTCAGGTCTGGTTTTTG
<i>tsh</i> reverse	TCGCCCTTAACACCATT
<i>cvaC</i> forward	GGTATCCCTTCGGGTTTTTG
<i>cvaC</i> reverse	TGTTTCTGGTGGTGCTTCAG
<i>hylF</i> forward	TTAGATCCCCAGGCAAGATG
<i>hylF</i> reverse	GGTGCAACAGGATTTCTTGG
<i>iss</i> forward	CCGAACCACTTGATGTGCA
<i>iss</i> reverse	CTATGCAAAAACAACGTAG
<i>iucC</i> forward	GACGGGCTTTCAGTAGTTGC
<i>iucC</i> reverse	CTTCATCGCTGAACGTGGTA

9.3 Selection criteria for identifying AMPs from NGS data

Two panning experiments were carried out, one targeting either *E. coli* O2 or *S. Typhimurium*. At the third round of panning, a z-score of >2.0 was used to identify sequences that were specifically enriched against the target bacteria following the method described by Naqid *et al.* (2016), with some modifications. The z-scores were generated using each of five replicate screens against the target bacteria compared to each of five replicate screens of the same input phage against the control bacteria (*L. acidophilus*). Enriched sequences were then ranked by the number of experimental replicates they were observed to be enriched in. For each panning experiment, peptides that were enriched in a minimum of 15 (out of 25) of these datasets were selected as candidate binding peptides. (Personal communication with Dr. J. Owen; ADAS, Nottingham). Peptides ranked 14/25 times were also synthesised if they were predicted as AMPs by algorithms available online at <http://www.camp.bicnirrh.res.in/predict/>. In total, this comprised 60 peptide sequences with the sequences synthesised by Biomatik at crude concentrations (Personal communication with Dr. B. Maddison; ADAS, Nottingham).

9.4 Sequences inserted into pET11a vector by express cloning service

Construct and Cell line	Sequence
#45_Avi-tag_Peptide #45 pET11a_Avi	CATATGGGTTGGGGCAAGTGGGGTCTGCGTCAACTGCGTGA CTTTGCGGTGTGCGGTCTGAACGATATCTTTGAGGCGCAGAA GATTGAGTGGCACGAAGGTTGGGGCAAATGGGGTCTGCGCC AGCTGCGCGACTTCGCGGTTTGTAAAGCTGAGC
#45_Avi-tag_Flexible Linker_Peptide #45 pET11a_Avi_Link	CATATGGGTTGGGGCAAGTGGGGTCTGCGTCAACTGCGTGA CTTTGCGGTGTGCGGTCTGAACGATATCTTTGAGGCGCAAAA GATTGAATGGCACGAGAAAGAAAGCGGTAGCGTTAGCAGCG AGCAGCTGGCGCAATTCCGTAGCCTGGCGGGTTGGGGTAAA TGGGGCCTGCGTCAGCTGCGCGATTTTTCGGGTGTGTAAAGCT GAGC
#45_Flexible Linker_Avi-tag_Peptide #45 pET11a_Link_Avi	CATATGGGTTGGGGCAAGTGGGGTCTGCGTCAGCTGCGTGA CTTTGCGGTGTGCAAAGAGAGCGGCAGCGTTAGCAGCGAAC AGCTGGCGCAATTCCGTAGCCTGGCGGGTCTGAACGACATC TTTGAGGCGCAGAAGATTGAGTGGCACGAAGGTTGGGGCAA ATGGGGCCTGCGTCAACTGCGCGATTTTTCGGGTGTGTAAAGC TGAGC
#45_Flexible Linker_Avi-tag_Flexible_ Linker_Peptide #45 pET11a_Link_Avi_Link	CATATGGGTTGGGGCAAGTGGGGTCTGCGTCAGCTGCGTGA CTTTGCGGTGTGCAAAGAGAGCGGCAGCGTTAGCAGCGAAC AGCTGGCGCAATTCCGTAGCCTGGCGGGTCTGAACGATATCT TTGAGGCGCAGAAGATTGAATGGCACGAGAAAGAAAGCGG CAGCGTGAGCAGCGAACAACCTGGCGCAATTTTCGTAGCCTGG CGGGCTGGGGCAAATGGGGTCTGCGTCAACTGCGCGACTTT GCGGTTTGTAAAGCTGAGC
MP_1	CATATGTCTATGCTGCACATGCGTACGTGTGCGGCACGTAAA GTTGCGTGCGCCGACTAAATGATATATTTGAAGCTCAAAA AATCGAGTGGCACGAATCAATGTTACACATGCGCACCTGCG CAGCCCGCAAAGTGGCATGTGCTTAAGCTGAGC
MP_2	CATATGCTGAATCAACTAAAAGCGGGCCCAAGACCGAGCAT CCCACCGATTGGCGGACTAAATGATATATTTGAAGCTCAAA AAATCGAGTGGCACGAACCTGAACCAGCTGAAAGCCGGTCCA CGTCCATCTATTCCGCCAATCGGTAAAGCTGAGC

9.5 AMPs inserted into JE5505 *E. coli* using pSD3 vector

27
27-45
27-Plantaricin
35
35-Plantaricin
36
36-36
45
45-Plantaricin
59
59-59
Cathelicidin4f
Cathelicidin4f-Cathelicidin4f
E-50
E-50-E-50
NZ114
NZ114-NZ114
Ca2.6
Ca2.6-Ca2.6
Lactoferricin
Lactoferricin-Lactoferricin
Plantaricin-423
Plantaricin-Plantaricin
H28 (Plantaricin mutant)
S27 (plantaricin mutant)
Palustrin-Ca
MP1102
Po-defensin
CBF
27 –NNK
35 – NNK
36 – NNK
45 – NNK
CBF – NNK

9.6 Statistical analysis

9.6.1 Growth curves

Statistical advice was sought from a statistician for an appropriate analysis of the growth curve data (Dr. Andrea Venn, University of Nottingham). Welch's ANOVA was used as the standard deviations amongst the groups were not equal. A further

statistical test was performed, Dunnett's T3 multiple comparisons, to determine if there was a significant difference between the mean of each group. As using the AUC may not detect differences in curve shape, the optical density at five time points that were equally separated were chosen to test for differences in their means. The optical densities of each treatment at 210, 450, 690, 930 and 1170 minutes were grouped and a one-way Welch's ANOVA was performed, with Dunnett's T3 multiple comparisons test, on each replicate. The treatment groups were split into induced and non-induced replicates and analysed separately, as were the AUC results. To account for multiple comparisons, the Bonferroni correction was performed and the adjusted p value used as the indicator for significance.

9.6.2 v-PCR and viable count experiments

Welch's one-way ANOVA was chosen to compare groups when the standard deviations were not equal. This was used for every v-PCR analysis, and for comparing *E. coli* viable counts except when *E. coli* was added at 10^8 CFU mL⁻¹ (Figure 5-19). Variance within standard deviations were tested using the Brown-Forsythe and Bartlett's test. An ordinary one-way ANOVA was used to analyse *Salmonella* viable counts, as these were repeatedly shown to have similar variance. Further statistical tests were performed to enable multiple comparisons between means: for Welch's one-way ANOVA, Dunnett's T3; for ordinary one-way ANOVA, Sidak's. With regards to v-PCR experiments, the Cq results from the v-PCR experiments were split into PMAxx- and PMAxx+ groups, and each treatment was compared to its respective DMSO control. Where appropriate, viable counts of individual bacteria were analysed

separately. Each treatment group was compared to its respective DMSO control with regards to the initial inoculum level of the bacteria in that group.

9.7 Photos of MBC determination for each AMP



Figure 9-1 Bactericidal activity of peptide #45 against *S. Typhimurium* and *E. coli* serotypes O2 and O78.

For determination of the MBC value against each bacterium, a Petri dish was split into eight sections, denoted either from “0” to “-8” or “128” to “1” as the concentration of peptide spotted. The “0”, or “128”, section contains $3 \times 5 \mu\text{L}$ spots from a well containing 128 mg L^{-1} of peptide #45 and bacteria tested and with decreasing number the concentration of peptide decreases by two-fold. **a)** shows an MBC value of 16 mg L^{-1} against O2, **b)** shows an MBC value of 16 mg L^{-1} (“-3”) against O78, and **c)** shows an MBC value of 64 mg L^{-1} against *S. Typhimurium* as these are the sections which display a CFU mL^{-1} below a 3-log_{10} reduction criteria, as determined by viable count of each bacteria pre-incubation.

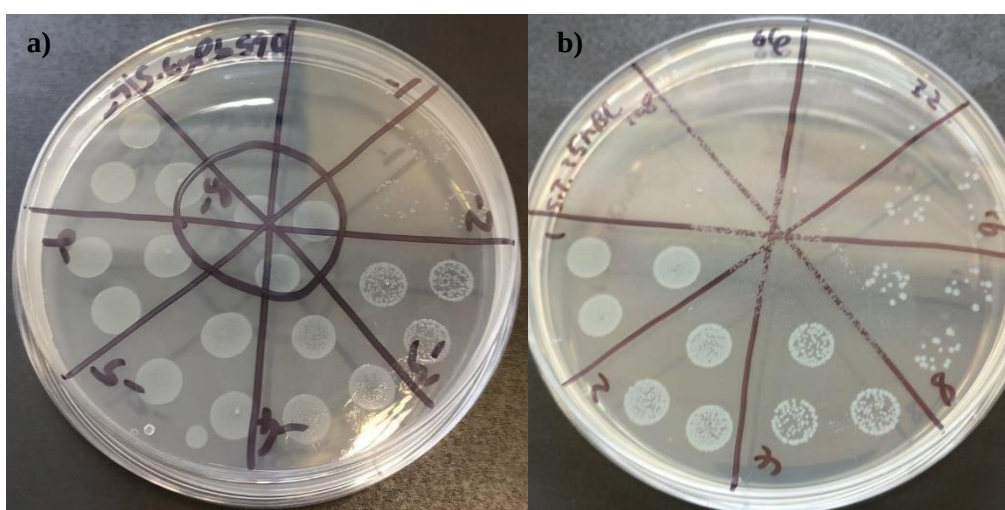


Figure 9-2. Bactericidal activity of peptides #35 and #59 against *S. Typhimurium*.

For determination of the MBC value of peptides #35 and #59 against *S. Typhimurium*, a Petri dish was split into eight sections, denoted either from “0” to “-8” or “128” to “1”. The 0, or 128, section contains $3 \times 5 \mu\text{L}$ spots from a well containing 128 mg L^{-1} of either peptide

#59 or #35, and *S. Typhimurium*. With decreasing number the concentration of peptide decreases by two-fold. **a)** shows peptide #59 has an MBC value of 64 mg L⁻¹ (“-1”) and **b)** shows peptide #35 has an MBC value of 32 mg L⁻¹ as these are the sections which display a CFU mL⁻¹ below a 3-log₁₀ reduction criteria, as determined by viable count of each bacteria pre-incubation.

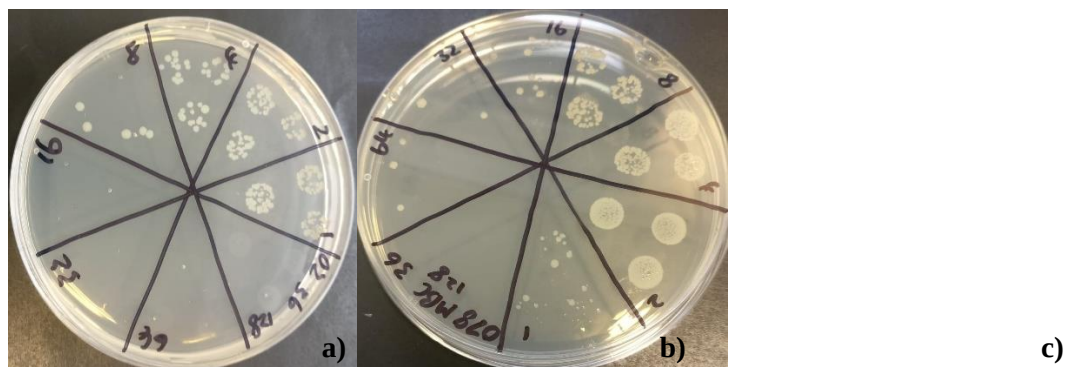


Figure 9-3. Bactericidal activity of peptide #36 against *S. Typhimurium* and *E. coli* serotypes O2 and O78.

For determination of these MBC values, Petri dishes were split into sections. Starting at 128 mg L⁻¹, in the section labelled “128”, 3 × 5 µL spots of peptide #36 and either *E. coli* serotype O78 or O2, or *S. Typhimurium*, were pipetted onto the plate. With decreasing number the concentration of peptide decreases by two-fold. **a)** shows an MBC value of 32 mg L⁻¹ for *S. Typhimurium*. **b)** shows an MBC value of 8 mg L⁻¹ against O2 and **c)** shows an MBC value of 16 mg L⁻¹ against O78, as these are the sections which display a CFU mL⁻¹ below a 3-log₁₀ reduction criteria; the 3-log₁₀ reduction limit was determined by viable count of each bacteria pre-incubation.

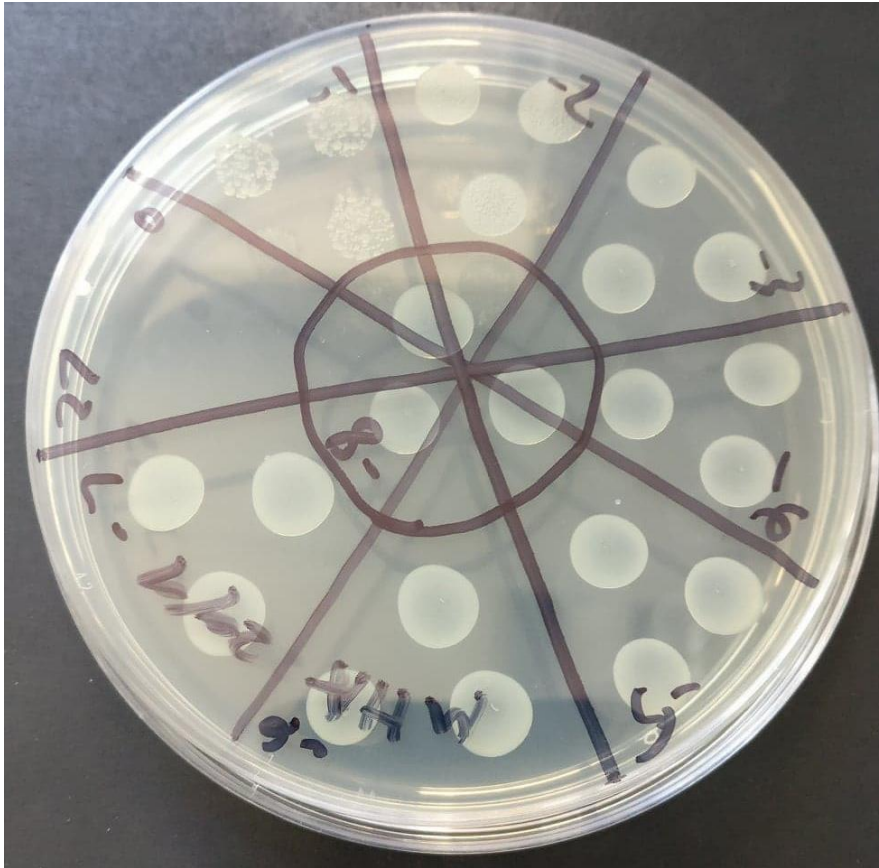


Figure 9-4. Bactericidal activity of peptide #27 against *S. Enteritidis*.

For determination of the MBC value, a Petri dish was split into eight sections, denoted from “0” to “-8”. The 0 section contains $3 \times 5 \mu\text{L}$ spots from a well containing 128 mg L^{-1} of peptide #27 and *S. Enteritidis* and with decreasing number the concentration of peptide decreases by two-fold. This shows an MBC value of 128 mg L^{-1} (“0”), as the -1 section displays too many colonies to meet a 3-log_{10} reduction criteria, as determined by viable count of *S. Enteritidis* pre-incubation.

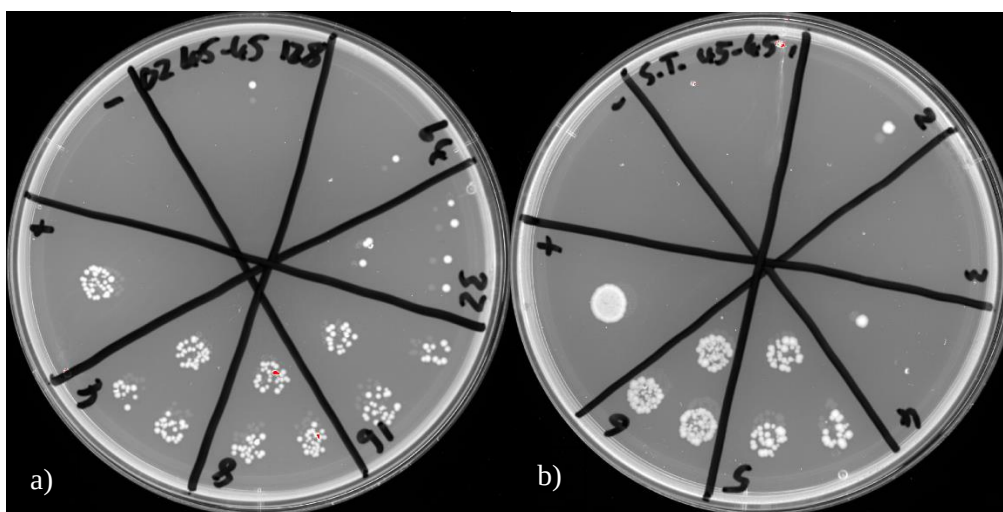


Figure 9-5. Bactericidal activity of peptide 45-45 against *E. coli* O2 and *S. Typhimurium*.

For determination of the MBC value, a petri dish was split into eight sections, with concentrations of peptide either denoted from “128” to “4” or “1” to “6”, and two controls: survival (“+”) and sterility (“-”). The 128, or 1, section contains $3 \times 5 \mu\text{L}$ spots from a well containing 128 mg L^{-1} of peptide 45-45 and either *E. coli* O2 (a) or *S. Typhimurium* (b) and the concentration of peptide decreases by two-fold in each clockwise section. This shows an MBC value of 64 mg L^{-1} and 16 mg L^{-1} for *E. coli* and *S. Typhimurium*, respectively, as the next clockwise sections display too many colonies to meet a 3-log_{10} reduction criteria, as determined by viable count of *E. coli* O2 or *S. Typhimurium* pre-incubation.

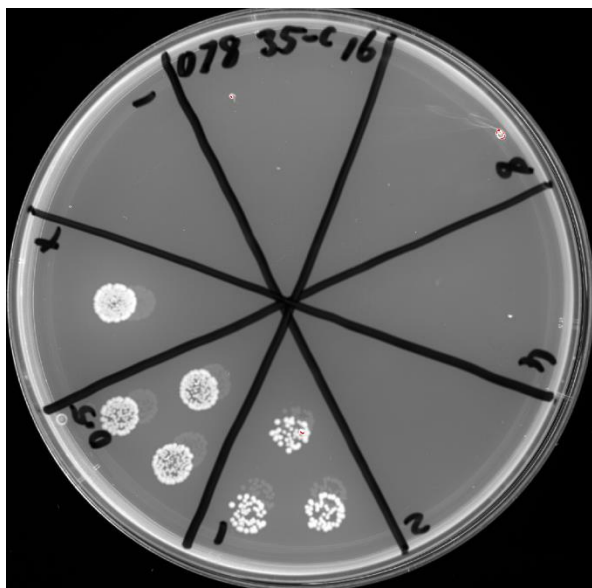


Figure 9-6. Bactericidal activity of peptide 35-CBF against *E. coli* O78.

For determination of the MBC value, a Petri dish was split into eight sections, with concentrations of peptide denoted from “16” to “0.5”, and two controls: survival (“+”) and sterility (“-”). The 16 section contains $3 \times 5 \mu\text{L}$ spots from a well containing 16 mg L^{-1} of peptide 35-CBF and *E. coli* O78 and the concentration of peptide decreases by two-fold in each clockwise section. This shows an MBC value of 2 mg L^{-1} as the next clockwise sections display too many colonies to meet a 3-log_{10} reduction criteria, as determined by viable count of *E. coli* O78 pre-incubation.

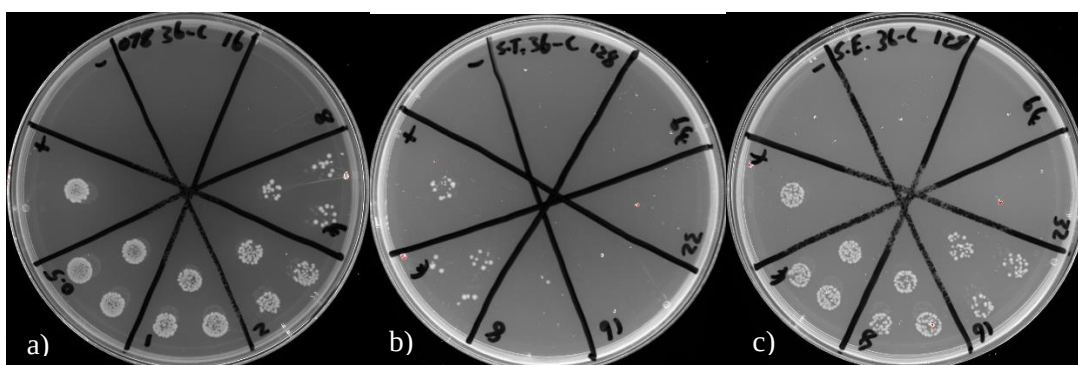


Figure 9-7. Bactericidal activity of peptide 36-CBF against *E. coli* O78, *S. Typhimurium* and *S. Enteritidis*.

For determination of the MBC value, a Petri dish was split into eight sections, with concentrations of peptide labelled in each section from “128” to “4” or “16” to “0.5”, and two controls: survival (“+”) and sterility (“-“). The 128, or 16, section contains $3 \times 5 \mu\text{L}$ spots from a well containing either 128 or 16 mg L^{-1} of peptide 36-CBF and either *E. coli* O78 (a), *S. Typhimurium* (b), or *S. Enteritidis* (c) and the concentration of peptide decreases by two-fold in each clockwise section. This shows an MBC value of 8, 8, and 32 mg L^{-1} for *E. coli* and *S. Typhimurium*, respectively, as the next clockwise sections display too many colonies to meet a 3-log_{10} reduction criteria, as determined by viable count of *E. coli* O2, *S. Typhimurium* or *S. Enteritidis* pre-incubation.

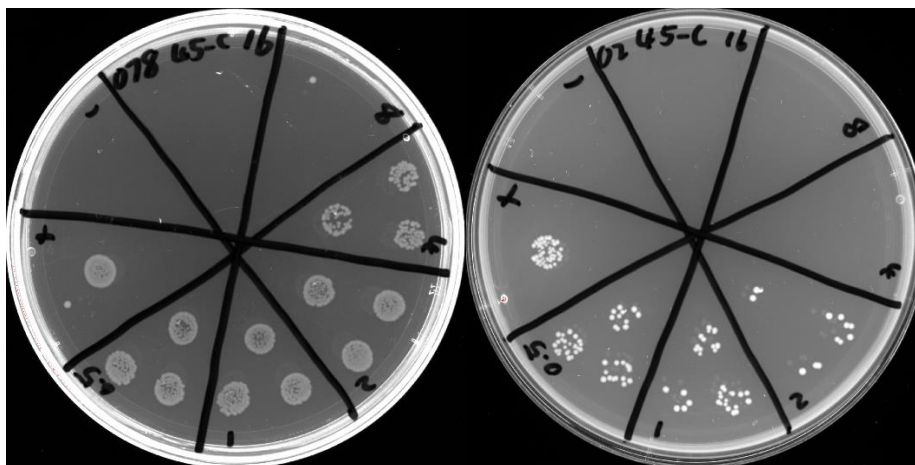


Figure 9-8. Bactericidal activity of peptide 45-CBF against *E. coli* O78 and O2.

For determination of the MBC value, a Petri dish was split into eight sections, with concentrations of peptide labelled in each section from “16” to “0.5”, and two controls: survival (“+”) and sterility (“-“). The 16 section contains $3 \times 5 \mu\text{L}$ spots from a well containing 16 mg L^{-1} of peptide 45-CBF and either *E. coli* O78 (a) or *E. coli* O2 (b) and the concentration of peptide decreases by two-fold in each clockwise section. This shows an MBC value of 8 and 4 for *E. coli* O78 and O2, respectively, as the next clockwise sections display too many colonies to meet a 3-log_{10} reduction criteria, as determined by viable count of *E. coli* O2, *S. Typhimurium* or *S. Enteritidis* pre-incubation.

9.8 BBSRC-mandated PIPS reflective statement

During my first week, I learned a lot about “Good Manufacturing Practice” (GMP).

This is very similar to Good Laboratory Practice, but much stricter with many more signatures required. I also learned the importance of enough software package training for staff, as often demonstrated in the university. I was heartened to learn just how many controls there were in the manufacture of drugs despite the examples of what

happens when controls aren't enough given during my induction. The job of a quality control microbiologist is relatively repetitive, but the career progression path is quite clear which makes the role more attractive. Once you progress past the basic role, the responsibilities and tasks are more interesting. However, you are expected to remain in the role for at least 2-3 years before progressing, which isn't appealing to someone who would have achieved a PhD seeing as you only need a degree to get the basic job. If you were able to negotiate a quicker progress, it might end up being worth it due to the likelihood of progressing further up the ladder than somebody without a doctorate.

I've been taken to a couple of meetings with higher management and the way they manage is very brusque, which can be effective (it seems). An "I don't want excuses, I want solutions" approach does work very well at focussing on the problem, if you can be confident enough to shut down people's reasoning.

I've learned about all sorts of pharmaceutical terms including deviations, change controls, pharmacopeas, validation work, and excipients. Knowledge of these will help me obtain a job in the future if I wish to work anywhere in the pharmaceutical business as they seem to be transferable across the site.

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