Discovery and Engineering of Antimicrobial Peptides to Combat Food Spoilage

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I dedicate this thesis to my children Adam and Sofija

and

to my parents Michael and Antoinette

“You were the light at the end of a very long tunnel”

Declaration

I, Michael Christopher Beckett, certify that the experimentation recorded herein represents my own work, unless otherwise stated in the text and has not been previously presented for a higher degree at this or any other University. This thesis may be lent at the discretion of the librarian.

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Michael Christopher Beckett

Summary

Food spoilage in yeast based foods is a major economic and consumer health concern worldwide. Numerous studies have been carried out to date to overcome this problem yet the most common preservation method is the addition of chemical additives to food. It had been shown in the past that the antimicrobial peptide human β-defensin-3 (HBD3) could provide effective control of the beer spoiling bacterium *Lactobacillus brevis* both *In vitro* and *In Vivo* when expressed from the lager yeast *Saccharomyces pastorianus*. It was felt that using human derived products as food preservatives would be unpalatable to the public. Consumers have long been accustomed to the use of plant additives as food preservatives.

A major aim of the research outlined in this thesis sought to discover plant based antimicrobial peptides with activity against food spoilage microorganisms. Although HBD3 would not be used as a preservative, its proven track record meant that it was a good benchmark upon which to start the discovery process. Bioinformatic analysis was used to query a database of plant antimicrobial peptides to uncover peptides which were structurally similar to the HBD3. This analysis revealed three peptides which were structurally similar to HBD3; Fabatin-1, Cp-Thionin-2 and the Mungbean defensin PDF-1. *In vitro* analysis revealed that only the first two peptides possessed antimicrobial activity under physiological relevant conditions. This analysis also revealed that effective peptide activity was sensitive to competing ions in the assay environment, thermal treatments and that the activity required intramolecular disulfide bond formation. It also revealed a clear positive correlation between antimicrobial activity and peptide net charge.

Another major aim of this research was to rationally design a set of antimicrobial peptide analogues which would have increased activity above that of the lead molecule. The initial rationale for design was based on the correlation between net charge and activity. Two peptides with increased charge were designed based on the backbone of the inactive Mungbean defensin; 3-Arg and 5-Arg analogues. It was found that increasing the cationic charge did increase antimicrobial activity against *L. brevis* but that the increased charge could not

overcome the presence of competing ions in the assay environment. It was also found that the antimicrobial activity was dependent on disulfide bond formation and was thermosensitive. The 5-Arg analogue peptide was found to have the best antimicrobial activity and at a similar level to that of HBD3.

A further set of analogue peptides, based on the 5-Arg analogue peptide, were designed which increased the structural fluidity on the molecule; All cysteine knockout analogue, internal cysteine knockout analogue and N-terminal fluid analogue. *In vitro* analysis revealed that perturbations to the structure in this manner reduced the overall antimicrobial activity of the peptide analogues. The final set of analogue peptides, also based on the 5-Arg analogue, were designed to uncover if short peptides with effective antimicrobial activity could be created. These were designed to remove either one strand or two strands from the β-sheet of the parental molecule and were designated 1-strand and 2-strand deletion analogues. *In vitro* analysis revealed that reducing the size of the analogue peptides in this manner also reduced the effectiveness of their antimicrobial activity.

The final major aim of this research outlined here was to construct a stable and modular expression platform which could be used for the *in situ* production of multiple recombinant peptide expression cassettes from *S. pastorianus* without the need for antibiotics for intracellular maintenance of the vector. The yeast artificial chromosome pYAC4 was chosen as the backbone upon which this expression platform would be designed.

Initially the vector pYAC-Kan was constructed which was compatible for use with *S. pastorianus*. Three copies of a recombinant HBD3 expression cassette, previously constructed by other members of this research group, were cloned onto this vector. ELISA analysis indicated that there was a somewhat linear relationship between the number of copies of the expression cassette and the level of recombinant peptide production but that there was an upper limit to this linearity. It also revealed that the majority of the peptide produced by this system was retained within the cell and was not secreted into the extracellular environment. Further analysis revealed however that this vector was not stably maintained within *S. pastorianus* without the use of antibiotics and that this

instability directly correlated with the number of recombinant expression cassettes integrated into the vector.

The genes for Fabatin, Cp-Thionin and the 3-Arg analogue peptides were constructed using polymerase chain assemble and cloned into the high copy number vector pRS42H. Transcription of these genes was driven by the Phosphoglycerate Kinase 1 (PGK1) promoter and terminated by an alcohol dehydrogenase 1 (ADH1) terminator. These expression vectors were transformed in *S. pastorianus.* Mass spectrometry analysis revealed that the peptides were being produced *in situ* by *S. pastorianus*. *In Vivo* antimicrobial analysis of the activity was conducted against *L. brevis* in both YEPD and brewers' wort at both 30°C and the more industrially relevant temperature of 10°C. This analysis showed that only the *in situ* produced 3-Arg analogue peptide possessed moderate antimicrobial activity and that the condition that the most effective control was seen using brewers wort at 10°C.

Overall, this work has shown that future development of recombinantly expressed antimicrobial peptides could provide a cost effective and safe alternative to current food preservation strategies for industrial scale use. This work has also expanded the repertoire of available antimicrobial peptides available for use as food preservatives.

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Chapter 1:

General Introduction

This introduction aims to give an overview of spoilage issues affecting the food industry with a specific focus on yeast based foods such as beer, wine and bread. It aims to give an overview of the current methods employed to prevent this spoilage, the drawbacks to these solutions and the current research into future technologies being investigated which may overcome these issues. It also aims to give an overview of antimicrobial peptides (AMP's) and their potential as food preservatives, including delivery mechanisms such as *in situ* production and secretion from *Saccharomyces* spp.

1.1 Introduction to Yeast Based Food and Beverage Spoilage

1.1.1 General Introduction to Food Spoilage

Food spoilage is a major concern worldwide especially with the rapidly expanding global population we are now experiencing and the fact that around one-third of all food produced for human consumption is either spoiled or wasted. According to the United Nations Food and Agricultural Organisation, around 1.3 billion tons of food is lost or wasted; this includes 40-50% of all root crops, fruits and vegetables, 35% of fish and seafood, 30% of cereals and 20% of all meat, oil seed and dairy products. In developing countries, post-harvest loss rates are high with 30–40% occurring during the post-harvest and processing stage. While in the industrialised world this figure is as high as 30% for the retail and consumer level alone (Leyva Salas *et al.*, 2017). The reasons for this massive global food loss are diverse, but microbial spoilage, which affects sensory properties, plays a major role.

This introduction will deal mainly with microbial food spoilage and prevention, with a specific focus on the microorganisms and treatment technologies affecting yeast based foods, particularly fermentation based products.

1.1.2 General Introduction to Yeast Based Food

Beer is the most commonly consumed alcoholic beverage in the world, with approximately 1.8 x 10^{11} L being produced annually worldwide, of which, around 90% is lager (Barth, 2018, Nakao *et al.*, 2009).

The ingredients of pure beer include malt, hops, water and brewery yeasts. Malt extract, made from malted barley or wheat, provides the sugars that are converted into alcohol by the action of the added yeasts. Brewers wort is composed of 50-60% maltose, 10-15% maltotriose and 10-15% glucose (Zastrow *et al.*, 2001). Hops help to improve aroma and add flavour to the beer, while also being a natural preservative (Bolton *et al*., 2019).

Brewery yeasts are classified as top or bottom fermenters, producing ale or lager respectively. Ale is predominantly fermented using *Saccharomyces cerevisiae* with fermentations usually being carried out at ~20°C. Lager is produced with the yeast *Saccharomyces pastorianus,* with fermentations carried out at the lower temperatures of 7°C-14°C (Monerawela and Bond, 2017).

Wine fermentation in comparison to this is carried out at higher temperatures, with white wine being fermented between 18°C and 20°C and red wine fermentations being carried out at up to 29°C. Typical wine musts contain glucose (8%-13%), fructose (7%-12%) and a range of pectins, acids, phenolic compounds and tannins. The primary yeast involved in wine production is *S. cerevisiae* however other, so called, wild yeasts such as *Torulaspora delbrueckii, Pichia kluyveri, Lachancea thermotolerans* and *Metschnikowia pulcherrima* act in harmony with the primary yeasts to produce complex flavours in the finished wine (Renault *et al.*, 2009, Rodriguez *et al.*, 2010, Anfang *et al.*, 2009, Gobbi *et al.*, 2013).

The contribution by non-*Saccharomyces* yeasts to wine flavour depends on the concentration of metabolites formed. This in turn is affected by how active the non-*Saccharomyces* yeasts are. The specific environmental conditions in the must, that is, high osmotic pressure; equimolar mixture of glucose and fructose; the presence of $SO_{2;}$ non-optimal growth temperature; increasing alcohol concentrations and anaerobic conditions; and decreasing nutrients all play a

role in determining what species can survive and grow (Bisson and Kunkee, 1991, Longo *et al.*, 1991)

Yeast based bakery products, in particular breads, come in a wide of forms and their production processes vary widely also but the underlying principles of their production have many similarities. Essentially they all involve mixing the raw ingredients which include flour, water, salt, other functional ingredients and most importantly yeast (De Vuyst *et al.*). The main yeast used for bread production is *Saccharomyces cerevisiae* (Carbonetto *et al.*, 2018). After mixing the gluten structure of the bread is allowed to develop and carbon dioxide from the yeast metabolism allows the bread to rise after which the bread is baked until the internal temperature is between 82°C and 99°C depending on the specific type of bread (Cauvain, 2015).

Beer and beer fermentations are hard to spoil and have good microbial stability post-brew. The reason is that beer is an unfavourable medium for many microorganisms due to the presence of ethanol (0.5–10% w/w), hop bitter compounds (approximately 17–55 ppm of *iso*-α-acids), the high content of carbon dioxide (approximately 0.5% w/w), the low pH (3.8–4.7), the extremely reduced content of oxygen (<0.1 ppm) and the presence of only traces of nutritive substances such as glucose, maltose and maltotriose. These latter carbon sources have been substrates for brewing yeast during fermentation. As a result, pathogens such as *Salmonella* Typhimurium and *Staphylococcus aureus* do not grow or survive in beer (Sakamoto and Konings, 2003a).Fermented wines typically have the same harsh chemical characteristics that beers do with regard to microbial growth but spoilage is still common, particularly by wild yeasts (Diaz *et al.,* 2013).

Bakery products on the other hand do not present the same harsh environment post baking that fermentations have and so are more prone to spoilage resulting in a shorter shelf life (Cauvain, 2015).

Given the global economic significance of the yeast based food industries, finding novel solutions to spoilage of these products is an important field of study today.

1.1.3 General Introduction to Microbial Spoilage of Yeast based Foods and Beverages

This section aims to give a brief overview of the most important microorganisms involved in the spoilage of yeast based foods particularly beer, wine and bread.

1.1.3.1 Microbial Spoilage of Beer

By far the most important spoilage organisms of beer are the lactic acid bacteria. . Lactic acid bacteria are thought to be responsible for 60% - 70% of all cases of brewery spoilage (Sakamoto and Konings, 2003a, Menz *et al.*, 2012). The reason for the prevalence of this particular group of bacteria is due to their ability to thrive in the harsh conditions present in most brewery products such as high ethanol and sugar concentrations, the presence of hop compounds with antimicrobial activity, low pH (3.8 – 4.7), reduced oxygen and nutrient content and high carbon dioxide levels (Sakamoto and Konings, 2003a, Manzano *et al.*, 2012, Rouse and van Sinderen, 2008, Menz *et al.*, 2012).

Among the many lactic acid bacteria, a few species of *Lactobacillus* and *Pediococcus,* both of which are Gram positive, are recognised as the leading cause of brewery related spoilage (Rouse and van Sinderen, 2008). Many species of *Pediococcus* have been found in breweries including *P. acidilactici, P. pentosaceus, P. dextrinicus* and *P. damnosus* with the latter being considered the most prevalent cause of spoilage from this genus (Jespersen and Jakobsen, 1996, Sakamoto and Konings, 2003a).

Among the *Lactobacillus* genus, a number of species are commonly found including *L. lindneri, L. buchneri, L. parabuchneri, L. casei, L. coryneformis, L. malefermentans*, and *L. curvatus*. By far the most important and difficult to eliminate brewery spoiling lactic acid bacterium is *L. brevis* (Leathers *et al.*, 2014). There are a number of reasons for this. Firstly, the bacterium can survive the harsh conditions in the brewery environment. More importantly however is the persistent nature of the species in the brewery setting; this is due to the bacteria's ability to form biofilms on brewery equipment (Riedl *et al.*, 2019).

Spoilage by lactic acid bacteria results in changes in colour, increased turbidity and the production of off flavours; the latter being caused by the production of diacetyl and lactic acid (Rouse and van Sinderen, 2008, Menz *et al.*, 2012). The source of this contamination can mainly be traced to the raw materials used in the brewery and to the brewery environment itself (Sakamoto and Konings, 2003a).

In additional to *Lactobacillus* and *Pediococcus* species, also, a species from the genus *Micrococcus* has been reported to be occasionally responsible for beer spoilage. *M. kristinae* can grow in beer with low ethanol and hop compounds at pH values above 4.5 (Back, 1981).

Another important genus of Gram negative bacteria involved in beer spoilage is *Pectinatus* of which the most species are *P. cerevisiiphilus* and *P. frisingensis*. The closely related genera *Zymophilus* has also been isolated from breweries (Schleifer *et al.*, 1990). Both of these genera produce toxins during fermentations below alcohol concentrations below 5% (w/v) and pH values in the range 4.3-4.6 (Jespersen and Jakobsen, 1996). Aerobic Gram negative bacteria such as *Acetobacter aceti*, which have the ability to convert alcohol into acetic acid, have also been isolated from breweries. Due to the strict aerobic nature of these bacteria, they are not considered a major problem for the brewing industry (Vriesekoop *et al.*, 2013).

Another important source of microbial spoilage in beer but one which is sometimes overlooked is *Saccharomyces*. Although the relationship between *Saccharomyces* and the food production industry is generally seen in a positive light, there are circumstances where they can spoil the product they produce (Diaz *et al.*, 2013). There are two main ways this can occur. Due to the significant metabolic diversity between *Saccharomyces* species, with certain strains producing elevated levels of sulphides and acetic acid, contaminating species can produce off flavours and aromas (Dufour *et al.*, 2003). This is a particular problem in the wine and cider industries where the starting material for fermentation is contaminated with so called “wild yeasts” (Dufour *et al.*, 2003, Morrissey *et al.*, 2004).

The second way in which the genus can spoil food is through “re-growth” after the initial fermentation process. This can result in unacceptable turbidity, sediment, gassiness and off flavours (Thomas, 1993, Loureiro and Malfeito-Ferreira, 2003).

Aside from food products, with which the genus plays a role in the production of, *Saccharomyces* can also cause spoilage of foods which it should not be involved in the production of. For example, fruit juices and soft drinks are prone to fermentative spoilage by *S. cerevisiae, S. bayanus* and *S. pastorianus* which are ubiquitous in the harvesting and processing environments of these products (Las Heras-Vazquez *et al.*, 2003, Arias *et al.*, 2002). This is due to the yeasts ability to grow at low pH, high sugar concentration, low water activity and the ability to resist heat treatments of these products (Put and De Jong, 1980, Stratford *et al.*, 2000).

Beer spoilage by non-*Saccharomyces* wild yeasts can cause super attenuation of fermentations due to the secretion of glycoamylases which enable them to ferment dextrins which the culture yeasts cannot. Some of the most common non-*Saccharomyces* spoilage yeasts include *Brettanomyces, Candida, Kluyveromyces* and *Pichia* (Steensels *et al.*, 2015, van der Aa Kuhle and Jespersen, 1998).

1.1.3.2 Microbial Spoilage of Wine

Yeast and bacteria found in grape must and wine originate from the vineyard, grapes, and winery processing equipment. One of the major differences between wine and beer fermentations, aside from the starting material, is the fact that wine must is not boiled before the fermentation process begins (Liu *et al.,* 1997). This means that the yeast and bacteria which are intrinsic to the must remain there throughout the fermentation process unless they are controlled. This ‘natural microflora’ includes several dozen species of yeast, with *S. cerevisiae* being predominant (Diaz *et al.*, 2013).

It has also been reported that sweet and sparkling wines can be spoiled by *Zygosaccharomyces* and such as *Z. bailii, Z. lentus, Z. rouxii* and *Torulaspora delbrueckii* (Loureiro and Malfeito-Ferreira, 2003).

Lactic acid and acetic acid bacteria are the only families of bacteria found in grape must and wine. These include four genera of lactic acid bacteria, *Lactobacillus, Leuconostoc, Oenococcus and Pediococcus* and two genera of acetic acid bacteria, *Acetobacter* and *Gluconobacter* (Fleet, 1993).

Acetic acid, acetaldehyde and ethyl acetate are the main spoilage compounds produced by wine-associated acetic acid species. Acetic acid and acetaldehyde are formed from the oxidative metabolism of ethanol by species such as *Acetobacter aceti* (Adachi *et al.*, 1978). Wine which undergoes prolonged barrel maturation, and is not topped up or monitored adequately, is prone to acetic acid bacterial spoilage, particularly red wine in which *Acetobacter pasteurianus* can proliferate (Bartowsky and Henschke, 2004).

The metabolism of ornithine and lysine into nitrogen-heterocylic compounds by members of the heterofermentative lactic acid bacteria, particularly *Oenococcus oeni* and *Lactobacillus* spp., can lead to mousey wines with a persistent aftertaste (Costello *et al.*, 2008, Costello and Henschke, 2002). It should be noted that the wine associated yeasts such as *Dekkera* and *Brettanomyces* also produce these compounds but are not associated with wine spoilage (Grbin and Henschke, 2008).

Sorbic acid, which is used as a chemical preservative in sweetened wines at bottling to prevent yeast fermentation after packaging, can be metabolised by *O. oeni* to form 2-ethoxyhexa-3,5-diene, which has an odour reminiscent of crushed geranium leaves (Rodriguez *et al.*, 2017).

1.1.3.3 Microbial Spoilage of Bread

Bread is the most important staple food in the western world and is consumed in vast quantities by the rich and the poor alike. As it is considered a perishable commodity, bread spoilage is a globally important problem both from a health

and economic stand point (Axel *et al.*, 2017). Bread spoilage is mainly caused molds which either remain within the product after baking or are introduced during downstream processing such as slicing and packaging, the latter being more likely. That being said, bread can also be spoiled by a range of bacteria, particularly spore forming bacteria whose spores can survive the high temperature baking process. The only problematic yeast with regard to bread spoilage is *Pichia butonii*, also known as “chalk mold” (Burgain *et al.*, 2015).

The most common bread spoilage molds are *Penicillium* spp., although *Aspergillus* spp. may be of greater significance in tropical countries (Legan, 1993). A wide range of other spoilage molds including *Cladosporium, Mucorales and Neurospora* have also been observed (Razafinarivo *et al.*, 2016, Denardi-Souza *et al.*, 2018, Habibi Najafi *et al.*, 2018). The ability for these molds to produce toxins means that they not only cause economic loss but their presence also has health implications (Axel *et al.*, 2017).

Spoilage by bacteria is mainly limited to *Bacillus* spp., where they have been implicated in causing a common spoilage condition of bread known as “Rope”. Spores of these bacteria are commonly present in flour and enter the bakery food chain *via* this route. These spores can survive in the center of the bread and continue to cause problems in hot countries (Pepe *et al.*, 2003). The main species responsible for rope are *B. subtilis*, *B. mesentericus* and *B. licheniformis* with the latter being known to cause food borne illness (Logan, 2012).

1.1.4 General Introduction to Prevention of Microbial Spoilage in Yeast based Foods and Beverages

Given the huge economic and health implications of the food spoilage discussed so far, and given the ever increasing global population, it is of critical importance to control these spoilage organisms and reduce food waste lest we prove the Malthusian catastrophe true. This section aims to give an overview of the current methods to prevent and control microbial spoilage of beer, wine and bread products. These steps can be divided into physical treatments, addition of

chemical additives and addition of natural compounds which possess antimicrobial activity.

1.1.4.1 Prevention of Microbial Spoilage in Beer

As discussed previously, beer fermentations possess many intrinsic properties such as low pH, high alcohol and carbon dioxide levels, low oxygen content and high sugar content in brewers' wort which naturally provides an environment in which most spoilage organisms cannot grow. As we have seen however, there are still a number of organisms which can thrive in these conditions and steps must be taken to prevent their proliferation.

Before discussing the current technologies employed to reduce microbial spoilage of beer it is prudent that the routes by which the spoilage organisms be discussed. The raw materials used for brewing, such as malt, hops and unmalted cereal adjuncts, carry their own microbiota (O'Sullivan *et al.*, 2012). The water used for brewing is another potential source of contamination. After the fermentation process has been completed, the remaining yeast is harvested and re-pitched into subsequent fermentations (Bamforth, 2017). This process is generally repeated up to 15 times after which the yeast is repurposed, usually as animal feed (Ocheme Julius *et al.*, 2017). This process can result in a build up over time of spoilage microorganisms (Boulton and Quain, 2001). Another important source of contamination is the brewery infrastructure itself including the fermentation vessels and related pipe work. Given that, as was already discussed, many of the beer spoilage microorganisms have the ability to form biofilms, so it is imperative that this source of contamination is rigorously controlled (Maifreni *et al.*, 2015). Packaging raw materials (bottles, casks and kegs) can all be heavily contaminated with microorganisms when returned from trade, because they contain small amounts of beer in which microbial growth may have occurred over an extended period of time (Bokulich and Bamforth, 2013).

These primary contamination routes all involve the processes and materials before fermentation; secondary contamination post-fermentation is also a major

concern. All points of contact with cleaned or filled, but unsealed bottles are possible sources of secondary contamination (Sakamoto and Konings, 2003b). Airborne microorganisms can contaminate beer in the filling department during the transport of open bottles from the bottle washer to the filler and from the filler to the point when the bottle is sealed (Storgårds, 2000).

One of the most obvious, but sometimes overlooked, processes to reduce microbial spoilage is hygiene. The beer production and packaging facilities should be hygienically designed to eliminate contamination problems that may arise in equipment such as tanks, pipelines, joints and accessories. A suitable choice of equipment and materials, and elimination or minimization of dead spaces and rough surfaces, correct construction, process layout and automation is crucial to minimise contamination risks (Storgårds, 2000, Mattila-Sandholm and Wirtanen, 1992). One of the most important aspects of beer production management is keeping all equipment that comes into contact with beer or its precursors in prime condition by regular cleaning with approved detergents, not just within the brewery, but also at the point of dispensing in the case of draught beer (Garofalo *et al.*, 2015).

Aside from good hygiene, the most important physical treatment to prevent beer spoilage is thermal treatment; this can take the form of pasteurisation, sterilisation or chilling; the latter in either storage of beer or the low temperature lagering process (Bamforth, 2017). Before fermentation is carried out, the raw ingredients are boiled which inactivates most spoilage organisms. Bacillus spores are present in malt and cereal adjuncts and will survive wort boiling but are unable to germinate during the ensuing stages of brewing due to their sensitivity to hop compounds and the low pH of the fermenting wort and beer (Portno, 1968). *Bacillus coagulans* has been reported to produce copious amounts of lactic acid in sweet wort held at 55–70°C for more than two hours, and has been linked to the formation of nitrosamine at concentrations far exceeding the recommended limit of 20 µg/L (Smith *et al.*, 1992).

The Standard unit of measurement for pasteurisation is the pasteurisation unit (PU); with one PU being equal to 60°C for 1 min. Originally it was believed that bottled beer could be processed at 65–68 °C for 20 min or 72–75 °C for 1–

4 min, equivalent to 10–20 PU (Portno, 1968). However concerns were raised that this was not adequate to inactivate spoilage organisms such as *Lactobacillus brevis*, *Pediococcus cerevisiae*, and a wild yeast *Saccharomyces diastaticus*. This was particularly a problem for ethanol free and low hop beers which presented a much less harsh environment for the spoilage organisms. Currently the beer industry uses 120-300 PU for the eradication of spoilage organisms; this however represents a large increase in energy usage (Milani *et al.*, 2015).

Filtration of the final product is another technique by which microbial spoiler can be eliminated. In most cases the beer is filtered through ceramic filters with pore sizes ranging from 0.2μm – 1.4μm. There exists a direct relationship between the flow rate and pore size. Ideally the largest pore size would be the best however 1.4 μm is not sufficient to remove many of the spoilage bacteria. This reduced flow rate filtration process adds extra time and economic costs to the brewery (Cimini and Moresi, 2016).

There is on-going research into non-traditional physical preservation methods against beer spoilage such as high hydrostatic pressure treatments and pulsed electrical fields but again these technologies add energy and economic costs to the brewery (Santos *et al.*, 2017, Galvagno *et al.*, 2007).

A range of chemical preservatives are often added to fermentations to help prevent contamination such as formaldehyde, sulphur dioxide, ethyl parahydroxybenzoate, sorbic acid, propyparaben and methyl para-hydroxybenzoate (Monakhova *et al.*, 2012, Berglund, 1978). Formaldehyde is often added to fermentations to improve the beer clarity and improve its shelf life but it is always added at concentrations below that considered dangerous by the World Health Organisation (WHO) (Monakhova *et al.*, 2012). Although sulphur dioxide is naturally occurring in beer as a by-product of fermentation, it is also additionally added to prevent microbial spoilage (Freedman, 1980). Ethyl parahydroxybenzoate, sorbic acid, propyparaben and methyl para-hydroxybenzoate are all antimicrobial agents but as ethyl parahydroxybenzoate has anaesthetic properties it has been banned for use as an additive in Australia. The use of chemical preservatives such as these is not viewed well in

the public sphere today, and so, brewers try to use alternative means to reduce microbial spoilage of beers such as naturally occurring additives.

The first natural additive to be used to improve the shelf-life of beer was hops. Resin from the flowers of the hop vine (*Humulus lupulus*) is used in the brewing industry to impart a desirable bitter flavor to beer (Bamforth, 2017). About 3 to 12% of the resin is composed of α-bitter acids including humulone (humulon), cohumulone, and adhumulone; β-bitter acids, including lupulone (lupulon), colupulone, xanthohumol, and adlupulone, are also present (Omar, 1992). Both types of bitter acids possess antimicrobial activity against bacteria and fungi although the β-acids have received greater attention and have been used in more applications in recent years (Wang *et al.*, 2016b).

Lactic acid bacteria that spoil beer, including *Lactobacillus* and *Pediococcus* spp., are resistant to the antimicrobial effects of humulone, colupulone, and trans-isohumulone, whereas strains that do not spoil beer are sensitive (Fernandez and Simpson, 1993, Simpson, 1993). There is little research on the mechanism of the antimicrobial activity of hop acids, although multiple studies have revealed that antimicrobial activity is enhanced at reduced temperature (Shen *et al.*, 2009). Interaction with the cytoplasmic membrane is suggested in light of reduced sensitivity of Gram-positive bacteria and fungi when supplemented with lipids (Larson *et al.*, 1996). Additionally, hop bitter acids inhibit Gram-positive beer spoilage microbes by dissipating transmembrane pH gradient (Simpson, 1993).

Another naturally occurring preservative used in beer fermentations is chitosan. Chitosan is derived from chitin, a by-product of shellfish processing. Chitosan comprises a series of polymers with different ratios of glucosamine and N-acetylglucosamine. Chitosan inhibits growth of foodborne fungi and bacteria (Rhoades and Roller, 2000, Kong *et al.*, 2010, Devlieghere *et al.*, 2004). It has been demonstrated that the addition of chitosan to beer fermentations inhibited the growth of beer spoilage bacteria while not inhibiting the yeast (Gil *et al.*, 2004).

The bacteriocin nisin has also been shown to inhibit a wide range of beer spoilage microorganisms but this antimicrobial peptide will be discussed in detail in a later section of this introduction (Bruno *et al.*, 1992).

Another novel approach being actively researched currently is a co-fermentation system using non-spoilage associated lactic acid bacteria. Lactic acid bacteria have been exploited for centuries in brewing and wine fermentations (Colehour *et al.*, 2014). Belgian lambic beers do not use yeast strains as the sole fermenting agents. The lambic fermentation is spontaneous and lactic acid species that are naturally present play an important role in the process (Spitaels *et al.*, 2015). The fermentation takes place initially in open vessels and subsequently in wooden casks over a period of several months up to two years and is typified by successive blooms of several distinct microbial populations including lactic acid bacteria (Boulton and Quain, 2001).

Lactic acid bacteria such as *Lactobacillus delbrueckii, Lactobacillus amylovorus, Lactobacillus casei* and *Lactobacillus plantarum* have all been used in this way. The lactic acid produced by these organisms under fermentation conditions can directly inhibit a range of spoilage bacteria (Lund and Eklund, 2000). In addition to this, many of the lactic acid bacteria produce bacteriocins. Bacteriocins produced by these bacteria usually inhibit species that are phylogenetically related to the producing strain (Marciset *et al.*, 1997). This is particularly relevant for the brewing industry, as lactic acid bacteria cause a large percentage of spoilage incidents in brewing products as has been discussed already.

The most novel area of research, and indeed the basis for this thesis, into the control of beer spoilage microorganisms, is the generation of genetically engineered fermentation yeasts which produce antimicrobial peptides (AMP's). It has been demonstrated that a *Saccharomyces pastorianus* strain engineered to produce human β-defensin-3 can inhibit the growth of the spoilage lactic acid bacteria *Lactobacillus brevis* (James *et al.*, 2014). This technology will be discussed in great detail in a proceeding section of this introduction.

1.1.4.2 Prevention of Microbial Spoilage in Wine

How best to avoid wine spoilage is not always clear-cut. Even appropriate hygiene practices and the chemically harsh nature of wine cannot be relied on as a deterrent to unwanted bacteria and winemaking regulations may further limit the options for intervention available to the winemaker (Hussain, 2016). As an initial barrier, the high ethanol concentrations (up to 16% v/v), high wine acidity (pH as low as 2·9) can inhibit development of bacterial populations, however, in wines with lower ethanol concentrations and low acidity (above pH 3·7), it can be challenging to arrest bacterial growth. Storage of wine at temperatures below 15°C might assist with minimizing the ability of bacteria to proliferate in wine, but will also delay wine maturation (Joyeux *et al.*, 1984). Unlike the treatment of wort in beer brewing, grape must is not pasteurized prior to yeast inoculation. Heating wine prior to bottling has been explored, including flash pasteurization, however, concerns on the impact of this on wine sensory characteristics have meant that this technology is not widely used (Ribéreau-Gayon *et al.*, 2006).

A range of physical treatments are now being investigated to reduce the microbial spoilage of wine including ultrahigh pressure treatments, high power ultrasound, UV irradiation and pulsed electric fields (van Wyk and Silva, 2017, Ojha *et al.*, 2017, Tahmaz and Soylemezoglu, 2017, El Darra *et al.*, 2016). As with beer fermentations however, these technologies add an increase in energy consumption for the fermentation process.

There are a range of chemical inhibitors used in the wine industry to reduce microbial spoilage. The most common additive is sulphur dioxide which acts both as an antimicrobial agent and an antioxidant (Bartowsky, 2009). However, several bacterial species are resistant to high concentrations of sulfur dioxide and there is pressure to reduce the levels of sulphur dioxide in wine due to consumer health concerns (Pozo-Bayon *et al.*, 2012). Another common chemical additive is dimethyl dicarbonate which acts by inhibiting cellular enzymes (Ancin-Azpilicueta *et al.*, 2016). It has been shown however that, at the permitted levels of this compound in wine, many of lactic and acetic acid bacteria which spoil wine are resistant to the treatment (Costa *et al.*, 2008).

Other disadvantages of the use of dimethyl dicarbonate in wine are its low solubility in water, and, potential toxicity after ingestion or inhalation during treatment of wine.

A range of natural additives have been employed in the wine industry such as lysozyme and bacteriocins (Bartowsky, 2009). Lysozyme has been used in many food industries for around 50 years. It is used as a preservative in wine at a maximum level of 500 mg/L (Ancin-Azpilicueta *et al.*, 2016). It is a small protein with muramidase activity which acts on bacterial cell walls which makes it ineffective at controlling the growth of wild yeasts such as *Dekkera* and *Brettanomyces* spp. Structural differences between the cell walls of Gram negative and Gram positive bacteria also means it is limited in its activity against acetic acid bacteria. It is mainly used to inhibit lactic acid bacteria such *Lactobacillus* spp (Bartowsky, 2009). One major drawback to the use of lysozyme is that it has the ability to bind to tannins and polyphenols in red wines which can result in a decrease in colour and the formation of haze (McKenzie and White, 1991, Bartowsky *et al.*, 2003, Bartowsky and Henschke, 2004).

Bacteriocins such as nisin, pediocin and plantaricin, which are produced by some lactic acid bacteria, can also be added to wine to reduce bacterial spoilage by other members of the lactic acid bacteria (Ndlovu *et al.*, 2015). These antimicrobial peptides (AMP's) act on the cell walls of bacteria, leading to cell lysis. Some species of *Pediococcus* and *Lactobacillus* are resistant to nisin but it has been shown that a combination of nisin and sulphur dioxide can act in a synergistic fashion to control the growth spoilage bacteria; this combination also has the potential to reduce the levels of sulphur dioxide in wine (Bruno *et al.*, 1992, Rojo-Bezares *et al.*, 2007, Fernandez-Perez *et al.*, 2018). It has also been shown *In vitro* that bovine lactoferrin derived peptides can control wine lactic acid bacteria (Enrique *et al.*, 2009).

1.1.4.3 Prevention of Microbial Spoilage in Bread

Bread and bakery products do not have as many intrinsic properties which reduce microbial spoilage as do fermentation products. As with the other yeast based foods, reduction of microbial spoilage in the bakery industry begins with good hygiene. Even with good hygiene practices, steps must still be taken to reduce microbial growth and survival in bread.

The first, and most obvious, physical treatment is the application of heat. The actual process of baking raises the internal temperature of the bread to between 82°C and 99°C. This eliminates most vegetative spoilage cells but tough spores can survive this process and further treatment must be taken to reduce or eliminate them (De Vuyst *et al.*, 2017). Commercial bakeries typically use a combination of UV light, infrared radiation, microwave heating or ultrahigh pressure treatments to achieve this (Demir and Elgun, 2014).

Ultraviolet (UV) light is a powerful anti-bacterial treatment, with the most effective wavelength being 260 nm. It is used to control the occurrence of mould spores on bread, and among applications there is direct UV irradiation of the surfaces of wrapped bakery products which allows an extension of shelf-life (Demir and Elgun, 2014). It is nevertheless worth mentioning a generally poor penetrative capacity, and the difficulty to treat a multi-surfaced product, as mould spores likely present in the air cell walls within the bread surface are protected from the irradiation (Demir and Elgun, 2014).

Microwave (MW) heating allows heating rapidly and evenly loaves of bread without major temperature gradients between the surface and the interior. Generally, a 30–60 s treatment allows making wrapped bread mould-free (Demir and Elgun, 2014). However, the application of this treatment is limited by the fact it can cause condensation problems which can hence adversely affect the appearance of the product (Cauvain and Young, 2007).

Infrared (IR) treatment can also be used to destroy mould spores, with the advantage of not adversely affecting the quality and appearance of the product or the integrity of the packaging material. Moreover, IR treatment minimizes problems due to condensation or air expansion. Among disadvantages, it is

worth mentioning it is quite costly for multi-sided products which are required either to rotate between heaters or to be treated in two separate ovens (Demir and Elgun, 2014).

A range of chemical additives are also used to extend the shelf life of bread but, as with the fermentation based foods, there are strict regulations on their use in bread (1333/2008, 2018). Weak organic acids such as propionic and sorbic acid have been used to reduce the pH and reduce the growth of spoilage organisms (Samapundo *et al.*, 2016). These compounds are required at high concentrations for antifungal activity; at these concentrations the sensory properties are altered which is undesirable. Moreover, prolonged usage of these preservatives against spoilage fungi may lead to the development of fungal resistance to them (Levinskaite, 2012, Stratford *et al.*, 2013). The addition of ethanol is another method employed by the industry with concentrations ranging from 0.2% to 12% being reported to increase shelf life (Axel *et al.*, 2017).

In the past, natural and flavoured bread with a long shelf-life was obtained instinctively, using a traditional long fermentation process: sourdough. Based on that, the bakery industry has recently started to reconsider this traditional fermentation method to possibly replace chemical preservatives and thus guarantee a clean label. Sourdough has thus become an established form of food bio-preservation and the role played by lactic acid bacteria as bio-agents and inhibitors to bread spoilage has been thoroughly researched (Corsetti *et al.*, 2000, Moroni *et al.*, 2009).

Another novel approach to combat bread spoilage is the use of active packaging. These are generally categorised as absorbing or releasing systems. The former remove undesirable compounds such as oxygen from the environment while the latter release compounds such as antioxidants, preservatives or antimicrobials (Ahvenainen, 2003).

Recent research has also shown the addition of plant AMP’s such as Cp-thionin have the ability to increase the shelf life of bread (Thery and Arendt, 2018). This technology has great potential to reduce the required level of additives to bread. It has been shown that genetically engineered strains of *Saccharomyces* can

produce AMP's such as these which can greatly extend the shelf life of bread (Thery *et al.*, 2016). This technology will be discussed in detail in a later section.

As we have seen, the chemical and physical processes employed to combat food spoilage add great cost to the industries. In addition to this, tighter regulation surrounding the additives which are licensed for use in food has meant that we need to find alternative solutions to these problems. Natural additives offer an alternative approach which is both safe and economical particularly the *in situ* production systems for AMP's which would require no further interventions once the yeast is in the food. As we have also seen, the spent yeast from the beer and wine industries are often used as animal feeds. Yeasts engineered to produce AMP's used as this feedstock may offer a potential solution to antibiotic use in the livestock industry.

1.2 The Hunt for AMP's

1.2.1 General Introduction to AMP's

AMP's (AMP) are small oligopeptides ranging in size from 0.5 – 11 kDa. They have a broad spectrum activity of antimicrobial activity against a range of target organisms from viruses to parasites (Sierra *et al.*, 2017). Historically these peptides have also been known as cationic host defence peptides, anionic AMP's, cationic amphipathic peptides, cationic AMP's, host defence peptides and α-helical AMP's (Brown and Hancock, 2006, Harris *et al.*, 2009, Groenink *et al.*, 1999, Riedl *et al.*, 2011, Huang *et al.*, 2010).

The first AMP was discovered in 1939 from a soil *Bacillus* strain, an extract of which was shown to protect mice from pneumococci infection (Dubos, 1939). The following year the peptide responsible for this activity was isolated and named gramicidin; this peptide is still in use today as a topical antimicrobial treatment (Hotchkiss and Dubos, 1940, Van Epps, 2006). The first reported animal-originated AMP was the defensin phagocytin, which was isolated from rabbit leukocytes in 1956 (Hirsch, 1956). In the following years, bombinin from epithelia and lactoferrin from cow milk were both described (Groves *et al.*, 1965,

Kiss and Michl, 1962). During the same time, it was also proven that human leukocytes contain AMPs in their lysosomes (Zeya and Spitznagel, 1963).

Since these early days more than 5000 AMP's have been discovered or designed and synthesised (Huang *et al.*, 2004). Natural AMP's are found in prokaryotes, protozoa, fungi, insects and animals (Bahar and Ren, 2013). In animals, AMP's are mainly found in the body sites which are exposed to the environment and they act as the first line of defence of the innate immune system against viruses, bacteria and fungi (Zasloff, 2002, Schauber and Gallo, 2008). Thus AMP's play an important role in stopping infectious agents before they become a problem. In the bacterial kingdom, production of AMP's offers the producer a competitive advantage and greater access to resources. The same might be said of the plant rhizosphere where plant roots may have to compete for nutrients with a range of bacteria and fungi; although production of AMP's by plants also offers the same innate immunological protection as producers from other kingdoms (Berendsen *et al.*, 2012).

AMP's can be ribosomally or non-ribosomally synthesised (Maget-Dana and Peypoux, 1994, Papagianni, 2003). The ribosomally synthesised peptides are more common in nature and are found to be produced by a wide range of organisms including mammals, insects, reptiles, birds and plants (Sierra *et al.*, 2017). Non-ribosomally synthesised AMP's include some of the most well-known antibiotics such as vancomycin and polymyxin B which are synthesised *via* peptide synthases and form cyclic peptides containing unusual amino acids. Vancomycin was first discovered in 1953 in a soil sample from the interior jungles of borneo and is produced by the soil borne bacteria *Amycolatopsis orientalis* . This is one of the last resort antibiotics used to treat Methicillin Resistant *Staphylococcus aureus* (MRSA). Polymyxin B, which is primarily used to treat Gram negative infections, is actually a mixture of a number of related compounds and is produced by *Bacillus polymyxa* (Cederlund *et al.*, 2011, Maget-Dana and Peypoux, 1994).

Most AMPs are produced by specific cells at all times, while the production of some AMPs is inducible. For example, using silk moth as a model system, Hultmark and colleagues demonstrated that P9A and P9B can be induced in

hemolymph by vaccination with *Enterobacter cloacae* (Hultmark *et al.*, 1980). In another study, epithelial cells from different tissues of mice showed increased rate of mRNA transcription for defensin production after infection with *Pseudomonas aeruginosa* PAO1 (Bals *et al.*, 1999).

Most AMPs reported to date can be characterized as one of the following four types based on their secondary structures: β-sheet, α-helix, extended, and loop (Fig. 1.1). Among these structural groups, α-helix and β-sheet structures are more common (Powers and Hancock, 2003). While most AMPs belong to one of the above four classes, some AMPs do not belong to any of these groups (McManus *et al.*, 2000). Some AMPs contain two different structural components (Uteng *et al.*, 2003). Also, many peptides form their active structure only when they interact with the membranes of target cells. For example, indolicin shows globular and amphipathic conformation in aqueous solutions while it is wedge-shaped in lipid bilayer mimicking environments (Rozek *et al.*, 2000).

Unlikely antibiotics, which target specific cellular activities (e.g., synthesis of DNA, protein, or cell wall), many AMPs target the lipopolysaccharide layer of cell membrane, which is ubiquitous in microorganisms (Sierra *et al.*, 2017). Having a high level of cholesterol and low anionic charge puts eukaryotic cells out of the target range of many AMPs. Attacking the cell membrane in this manner also reduces the likelihood of the microorganism developing high level resistance, a characteristic of global importance today (Jenssen *et al.*, 2006).

Another important feature of AMPs is their rapid killing effect. Some AMPs can kill in seconds after the initial contact with cell membrane (Loeffler *et al.*, 2001). AMPs are also known to enhance the activities of antibiotics through synergistic effects. For example, the combination of penicillin with pediocin and ampicillin with nisin Z exhibited killing of *Pseudomonas fluorescens* with 13- and 155-fold lower minimum inhibitory concentration (MIC), respectively, compared to using antibiotics alone (Wu *et al.*, 2017).

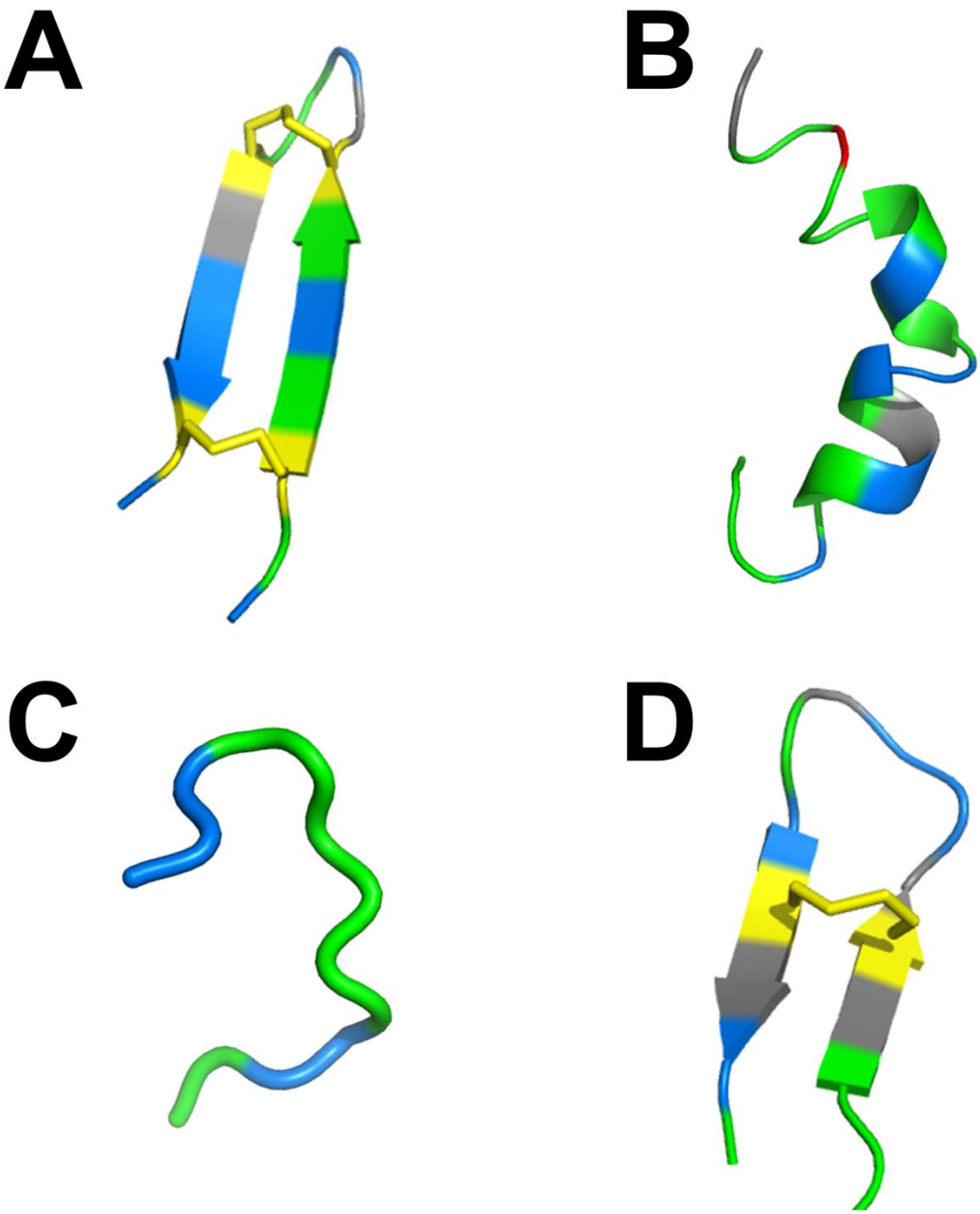


Fig 1.1 Structural Classes of Antimicrobial Peptides; (A) β-Sheet – Tachyplesin 1 (B) α-Helical – Manganin 2 (C) Extended – Indolicidin (D) Loop – Thanatin. Cationic residues are coloured blue, hydrophobic residues are coloured green, cysteine residues are displayed as sticks and are coloured yellow

Despite the advantages of AMP's as a novel therapeutic, there are still a number of problems with their development including potential toxicity to humans, sensitivity to harsh environments, high production costs and incorrect folding of large peptides (Matsuzaki, 2009, Svenson *et al.*, 2008, Bommarius *et al.*, 2010, Duquesne *et al.*, 2009). One possible solution to the production cost issue would be to produce the AMP's within yeast so it could secrete the antimicrobial into the food which it is used to produce such as beer, wine or bread; indeed it has been shown that this approach can be used to increase the shelf life of these foods (James *et al.*, 2014). Design of small analogues of AMP's could alle*via*te the peptide folding problems, as well as, the production cost issues.

1.2.2 AMP's of Microbial Origin

AMP's are produced by the majority of bacteria, archea and fungi. These peptides possess antimicrobial activity against a wide range of microorganisms including species closely related to the producer. It is believed that many of these microbial derived AMP's evolved as a means to secure an ecological niche by the producing microbe (Maróti *et al.*, 2011).

One of the most important classes of microbial derived AMP's are the bacteriocins. Bacteriocins are ribosomally synthesised AMP's produced by both Gram positive and Gram negative bacteria. Many of these AMP's are produced by lactic acid bacteria which are already approved for use in food (Ndlovu *et al.*, 2015). They have broad antimicrobial activity against a range of bacteria and fungi with many possessing sporostatic or sporicidal activity (Egan *et al.*, 2016).

Meconium, the earliest stool of newborn mammals, contains many different bacterial species such as *Lactobacilli, Bifidobacteria* and *Enterococci* which protect the newborn from pathogenic microbes through the production of bacteriocins (Al Atya *et al.*, 2015). Two good examples of these are the enterocins DD28 and DD93 which are produced by *E. faecalis* and possess antimicrobial activity against MRSA; when used in conjunction with erythromycin or kanamycin these AMP's showed synergistic activity against the

clinically relevant MRSA-1 (Al Atya *et al.*, 2016b). It has been shown that a combination of the enterocin DD14, colistin and nisin eradicated planktonic and biofilms of *E. coli* from swine with colistin-resistant strains of the bacteria (Al Atya *et al.*, 2016a).

One of the most important bacteriocins with regard to food preservation is nisin. Nisin is an antimicrobial peptide produced by certain Gram negative bacteria including *Lactococcus, Streptococcus* and *Lactobacillus* species (Lubelski *et al.*, 2008, de Arauz *et al.*, 2009). It was first identified in 1928 in fermented milk and marketed in the UK in 1953 as an antimicrobial (Rogers and Whittier, 1928, Delves-Broughton *et al.*, 1996). The originally described variant of nisin, known as nisin A, is composed of 34 amino acids and is produced by *Lactococcus lactis* (Gross and Morell, 1971) (Fig. 1.2 A).

Nisin belongs to a group of cationic antimicrobial peptides collectively called Type A (I) lantibiotics (Smith and Hillman, 2008). Characteristic of this family of antimicrobials is the fact that they are post-translationally modified and contain unusual amino acids such as dehydrated serine and threonine, and thioether amino acids (Karakas Sen *et al.*, 1999, Wiedemann *et al.*, 2001). Nisin has a broad spectrum of activity against a wide range of foodborne and pathogenic bacteria but it is most effective against Gram positive bacteria (Delves-Broughton *et al.*, 1996, Severina *et al.*, 1998, Cleveland *et al.*, 2001, Le Blay *et al.*, 2007, Shin *et al.*, 2015). Nisin has been applied as a food biopreservative in many products such as beer, wine, cheese, canned foods and pasteurised milk (Galvagno *et al.*, 2007, Henning *et al.*, 1986, Ogden *et al.*, 1988, Cotter *et al.*, 2005).

Many of the bacteriocins exhibit a dual natured mode of action against vegetative cells. Sequestering of lipid II, which is required for bacterial cell wall synthesis, by nisin results in halting of the cell wall synthesis process in a manner similar to that of vancomycin (Breukink and de Kruijff, 2006). Bacteriocins can also act directly on the bacterial cell membrane through a pore forming mechanism which results in depolarisation of the cell membrane and consequently cell death. This dual mode of action means that development of high level resistance is very unlikely (Egan *et al.*, 2016).

Another important group of bacterially derived AMP's are the colicins and microcins. Gram negative bacteria produce a number of AMP's which are generally larger than Gram positive bacteriocins including colicins and microcins (Sierra *et al.*, 2017). Colicins are produced by some strains of *E. coli* that carry a colicinogenic plasmid and are lethal for related strains of *E. coli*. (Jacob *et al.*, 1953). They have been shown to have the ability to control the growth of *E. coli* O157:H7 in both plant and animal based foods (Schulz *et al.*, 2015) (Fig. 1.2 B).

The microcins are a family of low-molecular-weight antibiotics produced by *Enterobacteriaceae* and, as with the colicins, are active against related microbial strains (Jack and Jung, 2000). Compared to the colicins, which are <20kDa, the microcins are much smaller with molecular weights below 10kDa (Papagianni, 2003). Although not currently used as a food preservative, research has shown that they have the ability to protect skimmed milk and egg yolks against contamination by *Salmonella* and *E. coli* O157:H7 (Pomares *et al.*, 2009).

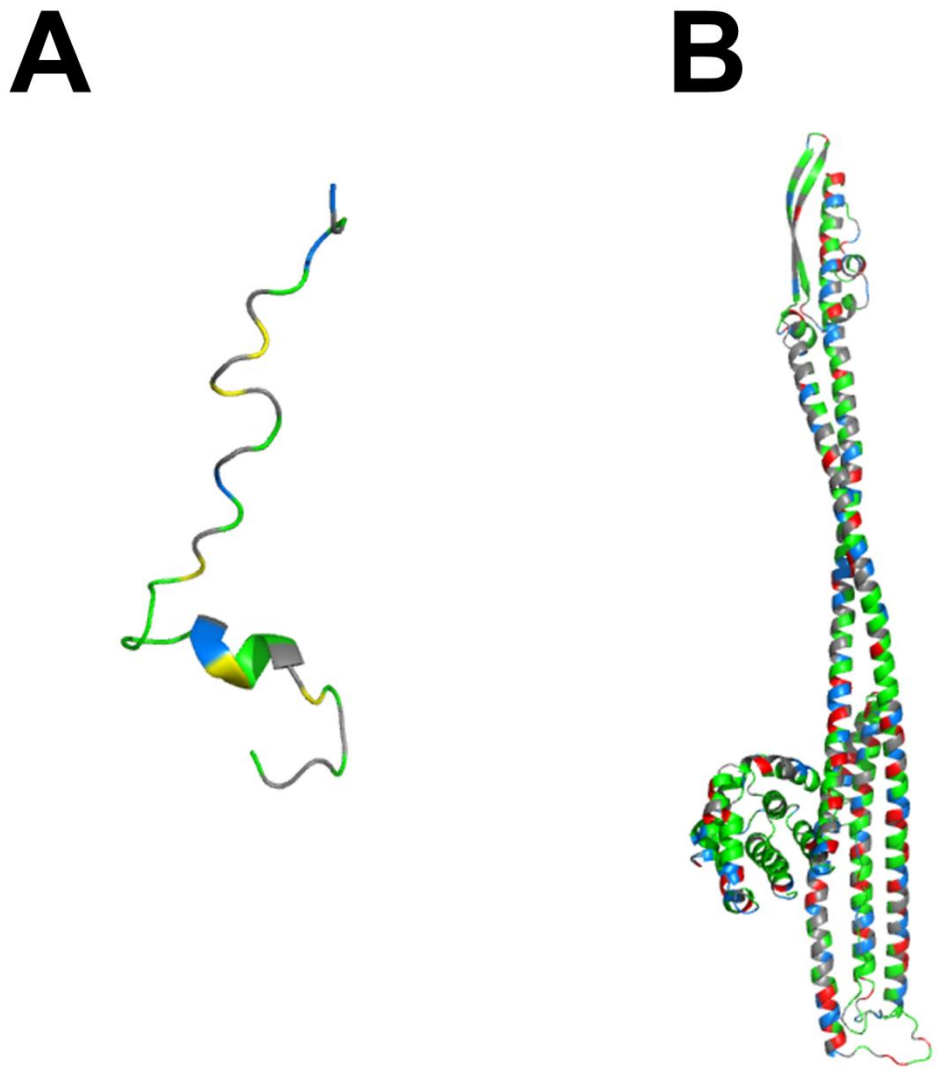


Fig 1.2 Bacterial Derived Antimicrobial Peptides (A) Nisin A from *Lactococcus lactis* **(B)** Colicin 1A from *E. coli*.

All of the AMP's discussed so far are derived from bacteria but fungi also produce a wide range of bioactive peptides; indeed many of the most antibiotics in use today were first discovered in fungi (Sierra *et al.*, 2017). The evolution of AMP's by fungi is most likely for similar reasons to that of bacteria, securing nutrients and resources in an evolutionary niche.

Fungal derived defensins are emerging as attractive antimicrobial agents due to their therapeutic efficacy, low toxicity and high serum stability (Wu *et al.*, 2014b). The first fungal derived defensin to be discovered was plectasin from the ascomycete *Pseudoplectania nigrella* (Mygind *et al.*, 2005). It has high structural similarity to many plant and insect defensins and has the classical defensin-fold composed of an α-helix and a two stranded β-sheet; this fold is stabilised by cysteine bonds (Fig. 2.3). Plectasin is ribosomally synthesised and is only active upon cleavage of a signal and pro-peptide (Wu *et al.*, 2014a). This defensin acts in a similar fashion to some of the bacteriocins in that it can inhibit cell wall synthesis by binding to lipid II. It has been shown to have similar efficacy to vancomycin in killing a range of bacteria including *Streptococcus pneumonia* (Mygind *et al.*, 2005).

In addition to the fungal derived antibacterial AMP's, there are also a number of fungal AMP's which possess antifungal activity. One such peptide is produced by *Candida intermedia* LAMAP1790 (Pena and Ganga, 2018). This peptide was found to inhibit the growth of the common wine spoilage yeast *B. bruxellensis*. It was also found not to interfere with the growth of *S. cerevisiae*. This peptide shows great potential as a preservative for use in the wine industry (Pena and Ganga, 2018).

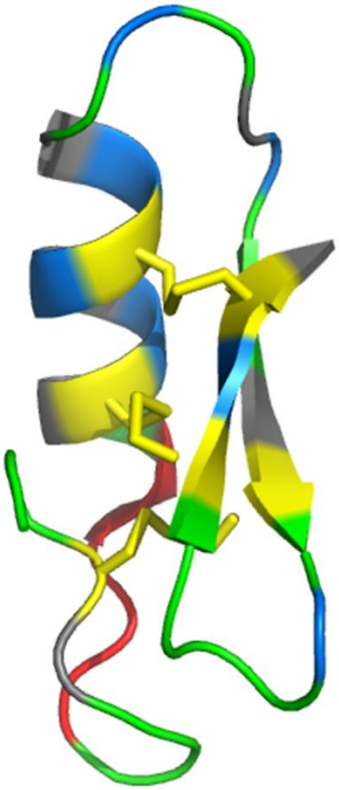


Fig 1.3 Fungal Derived Antimicrobial Peptide Plectasin. Isolated from *Pseudomonas nigrella* . Cationic residues are coloured blue, anionic residues are coloured red, hydrophobic residues are coloured green, cysteine residues are displayed as sticks and are coloured yellow

Another potential source of AMP's with potential for use in yeast based foods are produced by *Saccharomyces* itself. A good example of such a peptide is saccharomycin which is produced by *Saccharomyces cerevisiae* and has been shown to inhibit the growth of a number of non-*Saccharomyces* wine spoilage yeasts (Branco *et al.*, 2017). AMP's derived from *Saccharomyces* used to preserve yeast based foods have great potential to be exploited as they are most likely safe due to the fact that they are naturally occurring in these foods. Generation of over-producing strains would provide a safe and cost effective way to reduce alternative additives and preservation technologies.

1.2.3 AMP's of Insect Origin

It has been well established that insects are very resistant to bacterial infections due to the fact that they produce a wide range of proteins and peptides which act as the first line of defence in innate immune systems (Sierra *et al.*, 2017). Insect AMP's are divided into three main groups based on their sequence and structure; Cecropins, defensins and peptides which have unusually high numbers of proline and/or glycine residues (Makarova *et al.*, 2018). There are a vast array of insect AMP classes including cecropins, drosocin, attacins, diptericins, defensins, ponericins, drosomycin and metchnikowin. The best studied of these are the defensins and cecropins (Yi *et al.*, 2014).

Insect defensins, like other defensins, are small, ribosomally synthesised, cationic peptides with the same structural characteristics discussed previously. Defensins are produced by all insect orders with the exception of the lepidopterans (Koehbach, 2017). Unlike many defensins, from other non-insect sources, many insect defensins are exclusively inducible and not constitutively expressed (Hoffmann and Hetru, 1992). A classic example of an insect defensin is royalisin which was isolated from the royal jelly of *Apis mellifera*. This is a short (51 residue) cationic and amphipathic AMP whose structure is stabilised by three disulfide bonds. It has a broad spectrum of activity against both Gram positive and Gram negative bacteria but is particularly effective against the bee pathogen *Paenibacillus larvae*, the causative agent of foul brood (Katarína *et al.*, 2001).

Cecropins were first isolated from the hemolymph of the giant silk moth *Hyalophora cecropia* (Steiner *et al.*, 1981). These peptides are mainly structured by a large number of antibacterial and toxic peptides isolated from various lepidopteran and dipteran species, which constitute a major part of the cell-free immunity of insects. Cecropins are small peptides (around 35 amino acid residues) with activity against both Gram-positive and Gram-negative bacteria. However, Insect defensins are active mainly against Gram-positive bacteria, including *Micrococcus luteus, Aerococcus viridians, Bacillus megaterium, Bacillus subtilis, Bacillus thuringiensis*, and *Staphylococcus aureus* (Yi *et al.*, 2014). The insect cecropins (A, B and D) consist of 35–37 residues without cysteines which form stabilised α-helical structures (Hultmark *et al.*, 1982, Fu *et al.*, 2004). Cecropins can lyse bacterial cellular membranes and can also inhibit proline uptake (Zdybicka-Barabas *et al.*, 2018). Cecropin A has recently been found to have excellent antifungal activity against *Beauveria bassiana* in silkworm larvae (Lu *et al.*, 2016).

1.2.4 AMP's of Animal Origin

As with the insect AMP's, animal AMP's generally act as a first line of defence in the innate immune response against a range of pathogenic microorganisms. The main animal AMP's are the defensins, cathelicidins and histatins (Sierra *et al.*, 2017). The defensins will be discussed in detail in a later section of this introduction and will not be discussed further here.

Cathelicidins have been characterized in many phyla of animals, including mammals, birds, reptiles, amphibians, and some fish (Hemshekhar *et al.*, 2016). Cathelicidins are produced as pre-pro-peptides from which the N-terminal pro-domain which includes the cathelin domain is cleaved off to form the mature, biologically active peptide (Agier *et al.*, 2015). The active C-terminal domain is very diverse between members of the cathelicidin family which ranges from 12 – 79 amino acid residues and additionally, differential cleavage of the C-terminal can yield several different product sizes from the same pre-pre-peptide (Papagianni, 2003). An example of this is the product from the human gene hCAP-18 which can be differentially cleaved to produce three different mature

peptides; the most studied of these mature peptides is LL-37 (Fig. 1.4 A) (Sorensen *et al.*, 2003, Murakami *et al.*, 2004).

However, even without much homology at the sequence level, the mature cathelicidin peptides share certain physicochemical properties. Firstly, they are highly cationic with a charge that can vary between +4 and +13 in physiological conditions (Coorens *et al.*, 2017). Secondly, many cathelicidins have an unstructured conformation in an aqueous environment but can adopt an α-helical structure in the presence of a membrane (Wieczorek *et al.*, 2010, Bommineni *et al.*, 2007, van Dijk *et al.*, 2009). Other mature cathelicidin peptides may adopt β-sheet structures, such as the cyclic protegrins with intrachain disulfide bonds (Fig. 1.4 B). Some linear cathelicidin peptides are enriched in certain amino acids, for example, tryptophan for bovine indolicidin, or proline and arginine for porcine PR-39 (Zanetti, 2005).

Cathelicidins are antimicrobial for many pathogens, including Gram-positive and Gram negative bacteria, fungi, parasites, and enveloped viruses *In vitro* (Bommineni *et al.*, 2007, Goitsuka *et al.*, 2007, Tossi *et al.*, 1995, Veldhuizen *et al.*, 2017, Wessely-Szponder *et al.*, 2010, Xiao *et al.*, 2006). Cationic cathelicidins can bind and disrupt negatively charged membranes, leading to cell death. These peptides can also cross membranes and target intracellular processes like RNA and DNA synthesis, impair functions of enzymes and chaperones, and can stimulate protein degradation (Kosciuczuk *et al.*, 2012, Mansour *et al.*, 2014).

Although not directly added to food, the presence of naturally occurring cathelicidins in food products such as milk may help to prevent spoilage of such products (Hettinga *et al.*, 2011).

Histatins are histidine rich, cationic AMP's found mainly in saliva (De Smet and Contreras, 2005). They possess very potent antimicrobial properties against *C. albincas, A. fumigatus,* and *S. mutans* (Edgerton *et al.*, 1998, Tsai and Bobek, 1997). Although they are mainly antifungal, they show modest antimicrobial activity against *S. mutans, S. mitis, Porphyromnas gingivalis* and *Actinobacillus* spp. (MacKay *et al.*, 1984). There are known to be 12 histatin peptides produced by humans. These are differentially cleaved from

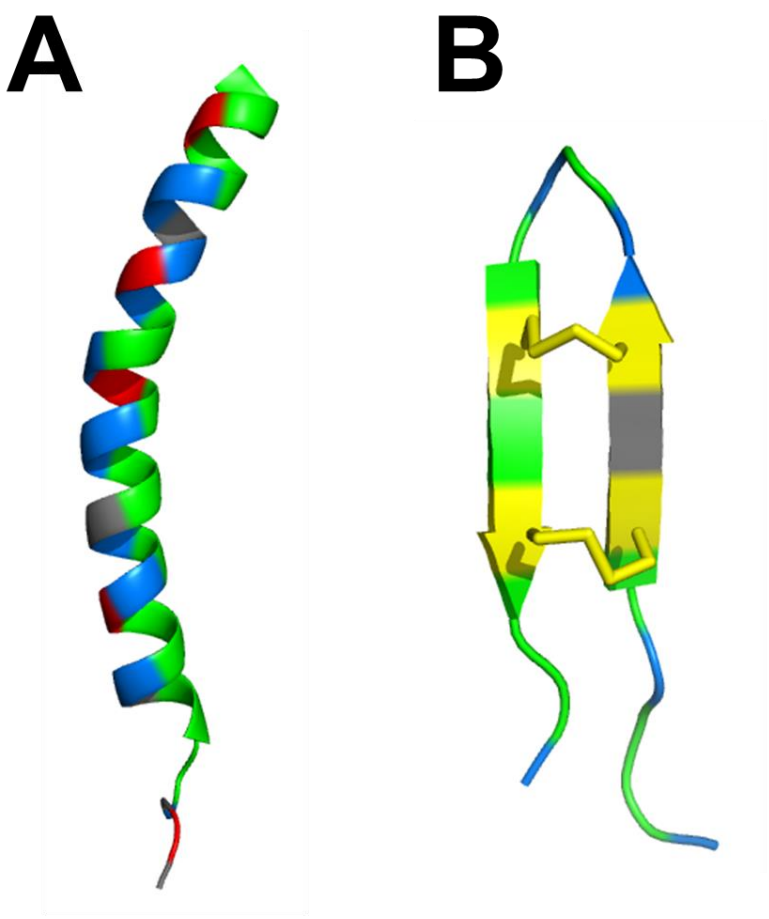


Fig 1.4 Tertiary Structure of Animal Derived Cathelicidins. (A) α-Helical Human LL-37 **(B)** .β-Sheet cyclic protegrin PG-5 from *Sus scrofa* (Wild Boar). Cationic residues are coloured blue, anionic residues are coloured red, hydrophobic residues are coloured green, cysteine residues are displayed as sticks and are coloured yellow

the products of three genes to form the mature bioactive peptides (Sabatini *et al.*, 1993, vanderSpek *et al.*, 1989, Castagnola *et al.*, 2004).

These peptides form random coil structures in aqueous environments but upon contact with hydrophobic surfaces such as membranes, they adopt an α-helical conformation (Melino *et al.*, 1999, Brewer *et al.*, 1998). Although not currently used as food preservatives, research has been conducted which shows the potential of histatins, in conjunction with sublethal ultrahigh pressure treatments, to reduce bacterial survival of *Lactobacillus plantarum* and *E. coli* species (ter Steeg *et al.*, 1999).

An interesting note however on animal derived AMP's is that many of the venoms and poisons produced by animals (and some insects) are directly evolved from AMP's (Perumal Samy *et al.*, 2017). Research into the potential of these molecules for use as novel therapeutics is on-going but due to the potential health implications, and possible public perception, these molecules will not be discussed further. Indeed, the research reported in this thesis actively sought to screen out AMP's such as these for further study.

1.2.5 AMP's of Plant Origin

Plants are constantly exposed to a vast range of pests and pathogens in nature. To counteract these, plants have developed complex defence mechanisms including the evolution of AMP's (Lacerda *et al.*, 2014). The evolution of AMP's in plants however may have occurred for a second reason. Plants cannot move in the conventional sense to acquire nutrients. AMP's may give the plant roots a competitive advantage in securing nutrients in their environmental niche in a similar fashion to microbial producers of AMP's (Berendsen *et al.*, 2012). It has also been shown that plant AMP's play important roles in cell-cell signalling and have been implicated in plant growth, symbiotic interactions and reproductive development (Murphy *et al.*, 2012). According to the PhytAMP database (the central repository for plant derived AMP's), there are 271 listed plant AMP's (Hammami *et al.*, 2009).The major classes of AMP's produced by plants are thionins, snakins, defensins, cyclotides, heveins and knottins (Nawrot *et al.*,

2014). Plant defensins will be discussed in detail in a later section and will not be described here.

Thionins are a family of AMP's with low molecular weight (about 5 kDa), rich in arginine, lysine, and cysteine residues. Their structure includes two antiparallel α-helices and an antiparallel double-stranded β-sheet with three or four conserved disulfide linkages (Nawrot *et al.*, 2014). They are positively charged at neutral pH. The groove between the α-helices and β-sheets possess the Tyr 13 residue, the membrane interactions of which may be associated with cell leakage (Majewski and Stec, 2010).

Thionins are divided into 3 groups; α-thionins, β-thionins and γ-thionins. The α-thionins and β-thionins are further subdivided into 5 sub-groups. Type I thionins are found in the endosperm of grains. They are highly cationic and cysteine rich and are composed of 45 amino acids. Type II thionins are slightly less cationic and consist of 46-47 amino acid residues. These were first isolated from the nuts of *P. pubera*. (Vernon, 1992). Type III thionins are composed of 45-46 amino acids and 3 disulfide bridges. They were isolated from the leaves and stems of *Viscum album* (visotoxin A1), *Phoradendron tomentosum* (phoratoxin A) and *Phoradendron liga* (ligatoxin A) (Samuelsson and Pettersson, 1977, Samuelsson and Pettersson, 1970, Thunberg and Samuelsson, 1982). Type IV thionins, also known as crambins, consist of 46 amino acids with three disulfide bridges. They carry no charge at neutral pH and have significant hydrophobic character. They were isolated from the seeds of *Crambe abyssinica* (Schrader-Fischer and Apel, 1994).

Thionins are known to have antimicrobial activity against a range of bacteria including *Pseudomonas* spp. and *Xanthomonas* spp. (Fernandez de Caleya *et al.*, 1972). They have also been found to have antimicrobial activity against fungi such as *Rhizoctonia solani* and *Sclerospora graminicola* (Oard *et al.*, 2004, Chandrashekhara *et al.*, 2010).

The cell wall associated peptides snakin-1 and snakin-2 were first isolated from potato tubers (Segura *et al.*, 1999). They are 63 amino acids long, are stabilised by 6 disulfide bonds and are highly cationic (Berrocal-Lobo *et al.*, 2002) (Fig. 1.5 A). Snakin-1 amino acid sequence alignments show similarity with members

of the tomato gibberellic acid stimulated transcript (GAST) family (gibberellic acid stimulated transcript) and *Arabidopsis* gibberellic acid stimulated in *Arabidopsis* (GASA) family and it was classified as a member of snakin/GASA family (Almasia *et al.*, 2010).

Not a lot is known about their mode of action but it is known that they possess antimicrobial activity against a range of fungi and bacteria such as *Botrytis cinerea, Clavibacter michiganensis* and *Ralstonia solanacearum* (Berrocal-Lobo *et al.*, 2002).

Cyclotides are a group of circular peptides which have been found in bacteria, plants and animals (Pelegrini *et al.*, 2007, Craik, 2010) (Fig. 1.5 B). The entire group have high sequence and structural similarities. Plant cyclotides are composed of 28 – 37 amino acids which are circularised head to tail and contain three intramolecular disulfide bonds arranged in a cysteine knot topology (Colgrave and Craik, 2004). Indeed it is this knot topology which is responsible for the high stability of the molecule as it forces the hydrophobic residues to be exposed at the molecular surface (Pranting *et al.*, 2010). Cyclotides are divided into 3 subfamilies based on structural properties; Mobius and bracelet which are based on the presence or absence of *cis*-proline, the third group, katata B8, is a hybrid of the other two.

Cyclotides have been isolated from the plants belonging to families *Violaceae, Rubiaceae, Cucurbitaceae* and *Poaceae* (Gruber, 2010). The first cyclotide to be isolated was kalata B1 from *Oldenlandia affinis* (Mylne *et al.*, 2010) (Fig. 1.5 B). Kalata B1 was used by women in Africa to accelerate labor and childbirth. These peptides have a diverse range of biological activities, including uterotonic, anti-HIV, antimicrobial, insecticidal, antihelmintic, and molluscidal properties (Craik, 2010). Although they have been shown to have antimicrobial activity against Gram negative bacteria, it should be noted that some cyclotides have been shown to be cytotoxic to human erythrocytes, a fact which may limit their suitability as food preservatives (Pelegrini *et al.*, 2007).

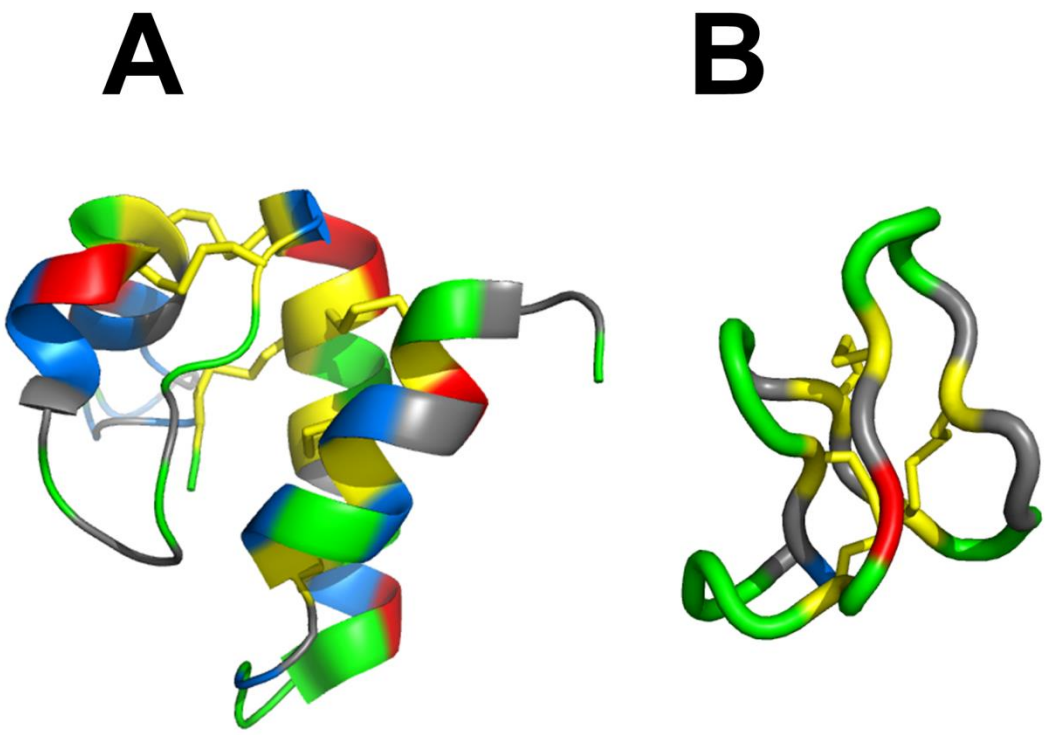


Fig 1.5 Examples of Plant Derived Antimicrobial Peptides. (A) Snakin-1 from *Solanum tuberosum* **(B)** .The cyclotide Kalata B1 from. *Oldenlandia affinis.* Cationic residues are coloured blue, anionic residues are coloured red, hydrophobic residues are coloured green, cysteine residues are displayed as sticks and are coloured yellow

Hevein is a small 4.7 kDa, cysteine rich, chitin binding peptide of 45 amino acids which is found in the lutoid bodies of the rubber tree *Hevea brasiliensis* latex (Van Parijs *et al.*, 1991). These peptides have also been found in a range of other plants (Koo *et al.*, 1998, Kiba *et al.*, 2003, Huang *et al.*, 2004).

All known chitin-binding proteins contain a common structural motif of 20–40 amino acids with several cysteine and glycine residues at conserved positions named the chitin-binding domain, which is responsible for binding the carbohydrate (Nawrot *et al.*, 2014). The hevein-like peptides differ in the number of disulfide bonds. Most of them possess eight cysteine residues forming four disulfide bonds; for example, the hevein homolog isolated from the seeds of *Pharabitis nil* and *Avena sativa* (Li and Claeson, 2003).

Their antifungal activity is generally mediated by their chitin binding ability but activity against non-chitin containing fungi has also been reported (Koo *et al.*, 1998). Antibacterial activity has also been reported against bacteria such as *E. coli, Agrobacterium* spp., *Pseudomonascichorii* and *Burkholderia plantarii* (Kiba *et al.*, 2003).

Another important class of plant derived AMP's are the knottin peptides. These peptides are small cationic AMP's with 6 conserved cysteine residues which form 3 disulphide bonds. These cysteine residues form an unusual linkage pattern; Cys1 – Cys4, Cys2 – Cys5 and Cys3 – Cys6. This linkage pattern forms a cysteine knot pattern within the molecule which confers exceptional structural stability and resistance to high temperatures, proteolysis as well as chemical chaotropic agents (Molesini *et al.*, 2017). Interestingly, this cysteine knot motif is also found in several human growth factors including transforming growth factor-β, nerve growth factor and vascular endothelial growth factors (Iyer and Acharya, 2011). Antimicrobial cysteine-knot proteins were identified in several plants including ginseng and poplar, whereas those belonging to 'toxins' have been described in some leguminous plants (Gracy *et al.*, 2008, Gelly *et al.*, 2004). The fact that this class of peptide contains known toxins, is structurally related to human growth factors and can withstand the harsh conditions of the human stomach means that adding them to food at high concentrations may not be a safe option for food preservation.

1.2.6 General Introduction to Defensins

Defensins are found in a wide range of organisms including plants, invertebrates and vertebrates. They are important effector molecules of the innate host immune system and possess a broad spectrum of antimicrobial activity against a wide variety of pathogens (Dhople *et al.*, 2006). They are divided into three subfamilies based on the organisation of their three disulfide bonds (α-defensins and β-defensins) and their cyclic nature (θ-defensins)(Fig. 1.6). They are cationic, range in size from 3-6 kDa, are amphipathic, possess hydrophobic domains and are ribosomally synthesised (Ouellette, 1999).

It is believed that all mammalian defensins evolved from an ancestral β-defensin. The α-defensins evolved from this ancestral gene and the θ-defensins, which are specific to primates, in turn evolved from the α-defensins after gene duplication (Xiao *et al.*, 2004).

Although the α-defensins and β-defensins are widespread throughout vertebrates, the θ-defensins are only found in non-human primates. It has been found that up to six θ-defensin pseudogenes with premature stop codons exist in the human genome (Seidel *et al.*, 2010). These defensins are the only known cyclic defensins in animals. They are active against a wide range of viruses including herpes simplex and human immunodeficiency virus (Cole *et al.*, 2002).

Invertebrate defensins are open ended cyclic peptides possessing three or four intramolecular disulfide bonds and includes the cecropins (Jenssen *et al.*, 2006). The cecropins are 29-42 amino acids in length and have a tryptophan at positions 1 or 2 and a C-terminal amidated residue (Bulet *et al.*, 2004). Cecropin

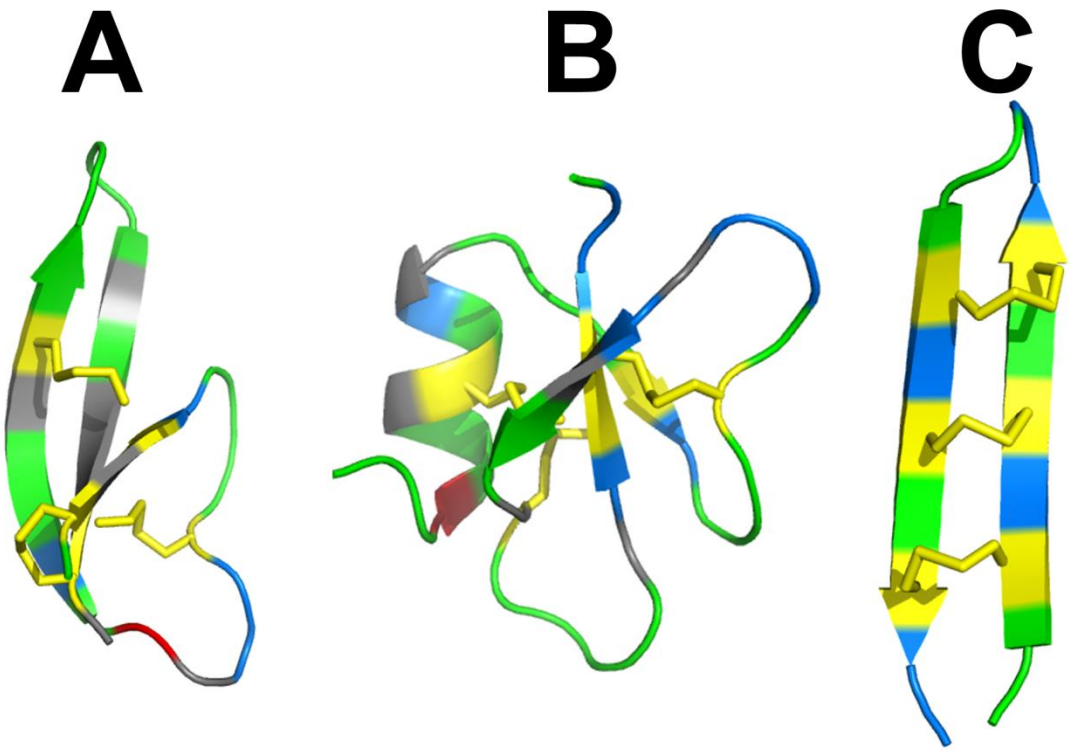


Fig 1.6 Tertiary Structure of Prototypic Defensins. (A) Human α-Defensin 4 **(B)** Human β-Defensin 2 **(C)** Baboon θ-Defensin 2. Cationic residues are coloured blue, anionic residues are coloured red, hydrophobic residues are coloured green, cysteine residues are displayed as sticks and are coloured yellow

An α-helical defensin, was originally isolated from *Hyalophora cecropia* (Steiner *et al.*, 1981). It possesses antimicrobial activity against bacteria *via* a membrane permeabilisation mechanism (Gazit *et al.*, 1996).

The most widely investigated defensins from vertebrates are the human defensins. There are currently known to be 17 defensin genes in the human genome (Jarczak *et al.*, 2013). The human defensin genes studied so far are all clustered on chromosome eight (Cederlund *et al.*, 2011). They are divided into two groups based on the arrangement of the intramolecular disulfide bridges; namely α-defensins and β-defensins (Dhople *et al.*, 2006).

Until recently, the amphipathic nature of defensins was thought to be the most important factor in their cell-inhibiting ability. This was thought because AMP's are rich in both cationic and hydrophobic residues making them well-suited to interactions with and integration into cell membranes, which are also made of amphipathic phospholipids (Ganz, 2003).

The α-defensins, due to their much later evolution in comparison to β-defensin progenitor, are only found in mammals and marsupials (Lynn and Bradley, 2007). Human defensins are produced by a number of different immune cells including leukocytes, neutrophils and several types of epithelial cells.

There are six α-defensins described in humans, designated as DEFA 1–6. DEFA 1–4 are also called Human Neutrophil Peptides 1–4 as they are most often expressed by neutrophils, commonly making up as much as 50% of the total protein content in these cells (Date *et al.*, 1994, Faurschou and Borregaard, 2003). These peptides are thought to be involved in the cell-killing activity of cells of the innate immune system, and are key players in systemic innate immunity (Khine *et al.*, 2006). DEFA 5 and 6, also known as Human Defensin 5 (HD5) and Human Defensin 6 (HD6), are produced only by Paneth cells of the small intestine (Lisitsyn *et al.*, 2012).

These short peptides (29-35 residues) are cationic and contain three conserved disulfide bond linkage patterns; namely Cys1-Cys6, Cys2-Cys4 and Cys3-Cys5. The tertiary structure is composed of a three stranded antiparallel beta sheet with a large unstructured loop between strands one and two. They are

synthesised as pre-proteins which possess no antimicrobial activity (Cederlund *et al.*, 2011, De Smet and Contreras, 2005).

These peptides play important roles in the human immune system and their antimicrobial activity is mainly due to cell permeabilisation (De Smet and Contreras, 2005). However it must be noted that, at least in the case of HNP-1, antimicrobial activity can also be due to inhibition of nucleic acid synthesis (Lehrer *et al.*, 1989). HNP-1 to -4 contribute to the oxygen independent killing of phagocytosed microorganisms (De Smet and Contreras, 2005).

While much effort is devoted to understanding the function of defensins in relation to bacteria and other living organisms, defensins have also been shown to exhibit direct antiviral activity and may play a key role in protecting hosts from enteric viral infections (Wilson *et al.*, 2016). As such, many examples of the antiviral properties have been uncovered in recent research. One example of this lies with JC polyomavirus, the pathogen responsible for progressive multifocal leukoencephalopathy. It was found that HD5 blocks infection with JC polyomavirus by stabilizing the viral capsid after infiltration of host cells, preventing release of the viral genome (Zins *et al.*, 2014). It should also be noted that disregulation of α-defensins may play important roles in human diseases such as Crohn's and coeliac disease where patients display decreased and increased levels respectively (Wehkamp *et al.*, 2004, Wehkamp *et al.*, 2005, Frye *et al.*, 2000).

The first human β-defensin (HBD1) was discovered in 1995 when it was extracted from the blood of renal patients (Bensch *et al.*, 1995). It is known that there are 31 β-defensin genes in the human genome of which six peptides have been isolated (Pazgier *et al.*, 2006). The best characterised of these peptides are HβD-1 to -4 which are encoded by DEFB1, DEFB2, DEFB103 and DEFB104 respectively (Dunsche *et al.*, 2001, Harder *et al.*, 2004).

The pattern of human β-defensin genes shows a common organization. The genomic structure consists of two exons and one intron. The only exception is the gene DEFB105, which contains three exons and two introns (De Smet and Contreras, 2005). In all but the DEFB1 gene, the first exon encodes the signal peptide and the second carries information about the sequence of mature

peptide preceded by a short anionic pro-peptide (Lehrer and Ganz, 2002b, Garcia *et al.*, 2001, Liu *et al.*, 1997, Michaelson *et al.*, 1992).

HBD1 is primarily expressed by epithelial cells of the respiratory and urinary tracts (McCray and Bentley, 1997, Valore *et al.*, 1998). HBD2 is mainly expressed by skin, gastrointestinal and respiratory tract cells (Bals *et al.*, 1998, Tsutsumi-Ishii and Nagaoka, 2003). HDB3 is expressed by epithelial cells and non-epithelial cells of the heart, liver, fetal thymus and placenta cells (Dunsche *et al.*, 2002, Garcia *et al.*, 2001, Hattenbach *et al.*, 1998). The primary sites of HBD4 expression are not currently known but they have been detected in various epithelial cells (Selsted and Ouellette, 2005).

As with the α-defensins, the β-defensins are characterised by a conserved linkage pattern between their cysteine residues; namely Cys1-Cys5, Cys2-Cys4 and Cys3-Cys6 (Tang and Selsted, 1993). Although they are quite variable at the primary amino acid sequence level, they possess the classic β-defensin tertiary structural fold; an α-helix and a three stranded antiparallel β-sheet (Schibli *et al.*, 2002). These peptides are highly cationic due to the presence of multiple lysine and arginine residues and possess charges ranging from +4 to +11 (Schroder and Harder, 1999).

Like other defensins, β-defensins are heavily involved in innate immunity where they provide both direct and indirect antimicrobial activity; indirect activity involves chemoattraction or stimulation of different host immune cells (Ganz, 1999, Pazgier *et al.*, 2007, Yang *et al.*, 2002, Yang *et al.*, 1999). HBD1 and HBD2 have been shown to be very effective at inhibiting Gram positive bacteria and some fungi but are significantly less effective at controlling Gram negative bacteria (Pazgier *et al.*, 2006). HBD3 has been shown to be a potent antimicrobial peptide with broad spectrum activity against a range of fungi, Gram positive and Gram negative bacteria including the vancomycin resistant *Enterococcus faecium* (Garcia *et al.*, 2001, Aoki and Ueda, 2013). It has also been reported that human β-defensins may contribute to the control of HIV-1 replication *In Vivo* (Sun *et al.*, 2005). A number of reports showed that the microbicidal activity of β-defensins, with the exception of HBD3, was strongly

inhibited by sodium chloride at physiological concentrations or by various divalent cations (Pazgier *et al.*, 2006).

The exact mechanism by which the β-defensins exert their antimicrobial activity is not currently known but there is evidence that HBD2 and HBD3 interact with microbial cell membranes as cationic β-sheet platforms which present the amphipathic helices for insertion into the lipid bilayer (Morgera *et al.*, 2008). Due to the fact that this process is not directed towards a specific target, bacterial resistance is rare, but not unachievable. Indeed it has been reported that HBD3 activity is inhibited by the 31 kDa protein streptococcal inhibitor of complement which is secreted by M1 strains of group A *Streptococci* (Yu *et al.*, 2008, Dhople *et al.*, 2006).

Plant defensins are generally 45 – 54 amino acids in length and have been isolated multiple different taxa, and, are most likely ubiquitous in the plant kingdom (Broekaert *et al.*, 1997). All known members of the plant defensin group have eight cysteine residues forming four disulfide bonds. The three-dimensional structure of plant defensins consists of a triple stranded antiparallel β-sheet and one α-helix that is stabilized by disulfide bonds (Broekaert *et al.*, 1997). Most of the plant defensins possess a signal peptide and lack a C-terminal propeptide with the exception of two cases in which possess a 30 residue C-terminal propeptide similar to that of the thionins (Karunanandaa *et al.*, 1994, Chiang and Hadwiger, 1991, Gu *et al.*, 1992, Milligan and Gasser, 1995). Generally, plant defensins are composed by one subunit, being found in monomeric forms. On the other hand, the defensins from *Pachyrrhizus erosus* and other from *Vigna unguiculata* showed the ability to dimerize (Pelegrini and Franco, 2005).

The production of defensins is widely distributed throughout the plant and they have been isolated from leaves, tubers, flowers, pods and seeds (Kragh *et al.*, 1995, Moreno *et al.*, 1994). Defensins have been isolated from *Heuchera sanguinea* (Hs-AFp1), *Raphanus sativus* (Rs-AFP1), *Aesculus hippocastanum* (Ah-AMP1), *Dahlia merckii* (Dm-AMP1), and *Clitoria ternatea* (Ct-AMP1) to name but a few (De Lucca *et al.*, 2005). In arabidopsis there are at least 5 different defensins whose genes are expressed in a tissue-specific manner

(Epple *et al.*, 1997). Plant defensins are preferentially located in peripheral cell layers and have also been reported in the xylem, in stomatal cells and in cells that line the substomatal cavity, all of which are locations where first contact and entry of pathogens take place (Moreno *et al.*, 1994, Broekaert *et al.*, 1997).

Expression of some defensin genes is developmentally regulated in a strict manner, whereas the expression of others is influenced by external stimuli (Epple *et al.*, 1997, Broekaert *et al.*, 1997, Kragh *et al.*, 1995). Thus, defensin genes induced upon pathogen infection have been identified in pea, tobacco, radish, and arabidopis (Chiang and Hadwiger, 1991, Gu *et al.*, 2002, Broekaert *et al.*, 1997). It has also been reported that in arabidopsis seedlings there is a defensin gene which is inducible by pathogenesis by *Fusarium oxysporum* (Broekaert *et al.*, 1997).

There are four distinct classes of plant defensins. Defensins of group I cause inhibition of Gram-positive bacteria and fungi, and the fungal inhibition occurs with marked morphological distortions of hyphae (branching); those of group II are active against fungi, without induction of hyphal branching, and inactive against bacteria; those of group III are active against Gram-positive and Gram-negative bacteria, but inactive against fungi; and those of group IV are active against Gram positive and Gram negative bacteria, and against fungi, without causing hyphal branching (Broekaert *et al.*, 1997, Hegedus and Marx, 2013).

The mechanism of action of antimicrobial activity has been extensively studied and is known to involve the electrostatically mediated interaction between the cationic peptide and the negatively charged cell membrane. This initial interaction is followed by the, hydrophobic residue mediated, insertion of the peptide into the membrane. The ultimate result of this process is membrane disruption or permeabilization, activation of cell wall lytic enzymes, disruption of membrane bound multienzyme complexes or other intracellular affects (Nuding *et al.*, 2009).

Although this process has been well studied, the exact mechanism of action is currently unknown but there are three major theories about how the events occur. The first model is known as the “Carpet Model”, where, the peptides lawn themselves over the surface of the microbe parallel to the surface of the

membrane (Chang *et al.*, 2008) (Fig. 1.7 A). The other two models, “The Barrel-Stave Model” (Fig. 1.7 B) and “Toridal Pore Model” (Fig. 1.7 C), suggest that the peptide insertion into the membrane mediates the formation of pores, through which, the intracellular contents can leak, causing cell death (van der Weerden *et al.*, 2010).

The antifungal activity of plant defensins is antagonized by increasing the ionic strength and, most notably, by divalent cations (Osborn *et al.*, 1995). The mode of action of antimicrobial activity is unknown but studies have shown that the antifungal activity is not related to membrane permeablisation (Thevissen *et al.*, 1996).

Given the broad range of antimicrobial activity of defensins in general, coupled with their wide distribution in nature and the fact that they are already present in large quantities in many of the food products we consume today, defensins offer a novel and likely safe alternative as food preservatives than many that have been discussed thus far. These characteristics coupled with the fact that defensins are ribosomally synthesised means that they possess great potential to be produced *in situ* by *Saccharomyces* with a view to preservation of yeast based foods.

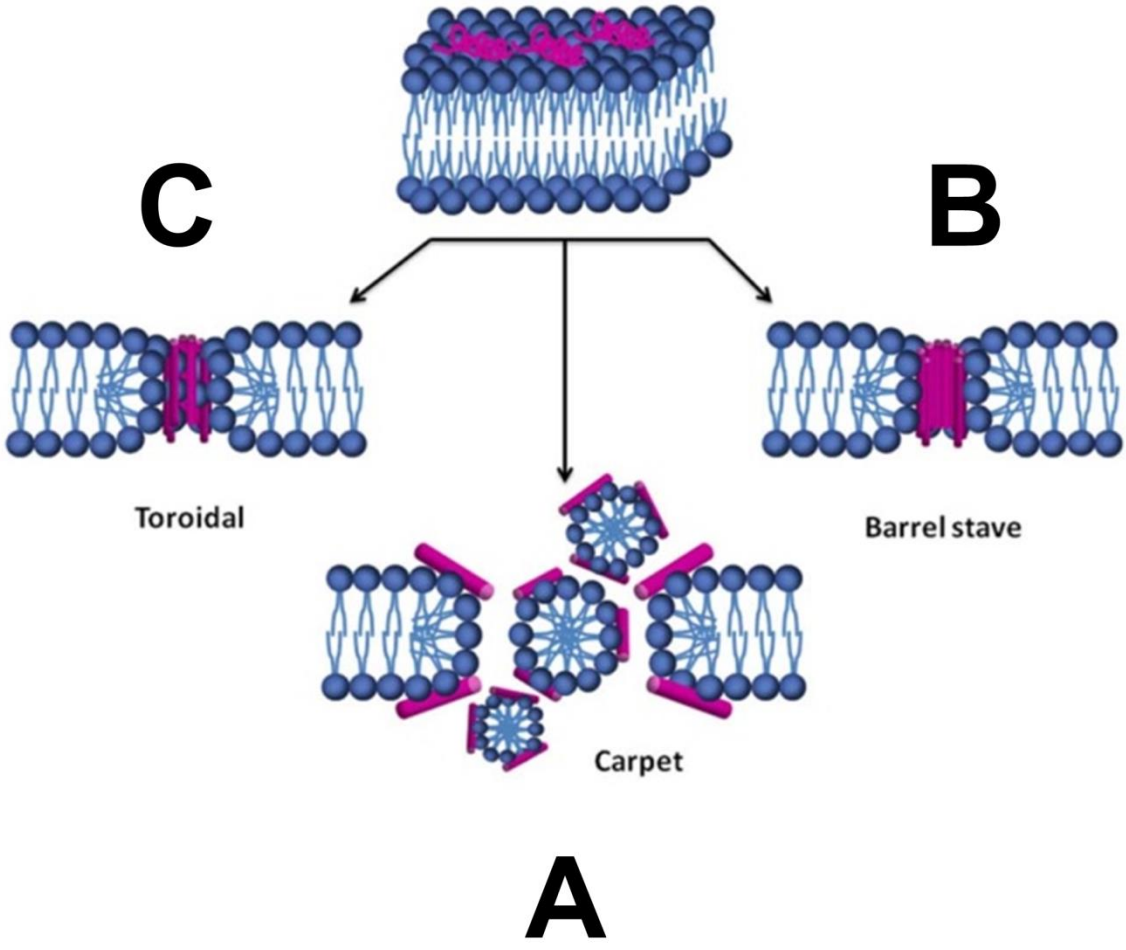


Fig 1.7 Proposed Models for the Mode of Antimicrobial Activity of Defensins (A) Carpet Model **(B)** Barrel-Stave Model**(C)** Torodial Pore Model. (Adapted from Silva et al., 2014)

1.2.7 Discovery and Production of New AMP's

Many of the original antimicrobials were discovered completely serendipitously. Indeed the very first antibiotic, penicillin, was discovered in 1928 when Alexander Fleming noticed that the growth of *S. aureus* on plates contaminated with the mold *Penicillium nota* was inhibited. It was not until 11 years later that the molecule was isolated and characterised by Dr. Howard Florey (Kyle *et al.*, 2015). This style of discovery pipeline consisting of producer screening, molecule isolation and characterisation, continued from many years after these initial discoveries.

In many cases these screening studies involved vast random libraries of organisms and molecules (Farha and Brown, 2016). One example of this was a study carried out in 2013 when 12,000 plantaricin derivatives were expressed from bacteria as an AMP-encoding library and tested for antimicrobial activity against the Gram positive bacteria *Listeria* innocua (Guralp *et al.*, 2013) (Fig. 1.8). Another example of this approach was used to screen for hexapeptides with antifungal activity against the aetiological agent of rice blast; the fungi *Magnaporthe oryzae*. The goal of this study was to find antifungal compounds which possessed activity against a specific part of the fungal life cycle. This study used a random, non-defined, library of synthetic peptides based on the sequence of a previously discovery peptide PAF104 in an effort to improve the antifungal activity of this peptide. This screening process uncovered 29 potential candidate peptides three of which possessed antifungal activity specific to the target organism (Rebollar *et al.*, 2014). One advantage of this random library screening approach is that novel peptide sequences never previously known to have antimicrobial activity may be discovered. Due to the low discovery rates in comparison to cost of this discovery process, researchers sought to find cheaper strategies with higher rates of success.

A further problem with the random screening of natural compounds from the native source approach is that in some cases the genes for the production of the antimicrobial remain silent until some environmental signal activates their transcription. This was found to be the case with some of the actinomycetes which do not produce antimicrobials until they are co-cultured with competing bacteria or until activators such as goadsporin are present in the environment

(Onaka, 2017). To overcome this problem, bioinformatic screening of genomes has been employed to discover these silent genes (Zarins-Tutt *et al.*, 2016). This approach was employed by Vila-Farres *et al* to screen bacterial genomes for silent genes. The products of these genes were then chemically synthesised and characterised *In vitro*; this approach allowed the culture steps to be by-passed (Vila-Farres *et al.*, 2017).

Many of the AMP classes discussed already are highly diverse at the primary sequence level; this hampers their discovery by simple genome mining. One particular class of AMP with which this approach has yielded positive results are the cathelicidins. These peptides contain a highly conserved N-terminal domain. Genome mining using this motif has been used to discover a range of novel cathelicidins from sources including reptiles, bats and birds (Bals and Wilson, 2003, Zanetti, 2004, Lehrer and Ganz, 2002a).

In addition to AMP discovery, advances in machine learning have meant that bioinformatics can also be used to predict the antimicrobial activity of discovered molecules (Lee *et al.*, 2017). For example, the online tool APD3 can be used to calculate the possible antimicrobial activity of AMP's based on characteristic such net charge, length, percentage of hydrophobic residues and peptide helicity (Wang *et al.*, 2016a). Another tool, AMPA, can predict antimicrobial regions on any protein by scoring each residue of the protein using an antimicrobial index (Torrent *et al.*, 2012). Machine learning based evaluation of structure-function antimicrobial activity relationships have also been used to predict the activity of membrane active AMP's based on their tertiary structure (Lee *et al.*, 2018).

As has been discussed already, many of the classes of AMP's are produced as inactive pre-pro-peptides and are only active upon cleavage of the pre-pro amino acid sequence. Many of these peptides are already found in our foods in the inactive forms. There are also many proteins in our food which are not primarily antimicrobial in nature but which contain amino acid sequences buried within their sequence which if released may then become antimicrobial (Moller *et al.*, 2008).

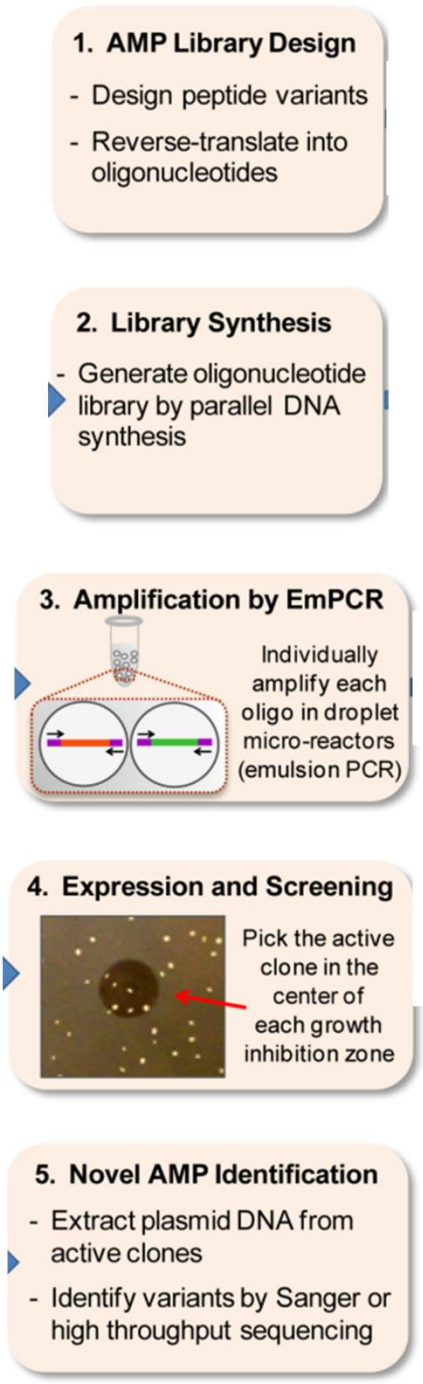


Fig 1.8 Oligonucleotide Library based Discovery of Antimicrobial Peptides. (Adapted from Guralp et al., 2013)

An exciting area of active research today surrounds releasing these bioactive components of the foods we already consume. This is achieved by digesting these foods with various proteinaceous enzymes to produce hydrolysates with antimicrobial activity. Initial research used various enzymes and enzyme combinations to hydrolyse the starting material after which the antimicrobial activity of the resulting hydrolysate was investigated (Tan *et al.*, 2013). These initial studies knew little about potential protein sequences with the starting material and their potential as antimicrobial compounds and indeed these studies were reminiscent of the original large scale screening approaches discussed earlier. Recently a more directed bioinformatic based approached to discover potential antimicrobial sequences in the proteome of these foods has been used (Ji *et al.*, 2017). This approach also allows streamlining of hydrolysate production as it also predicts which digestive enzymes will release the sequence of interest.

The most directed approach for the development of efficacious AMP's is a combined bioinformatic and rational design based approach to synthetic AMP analogues which do not exist in nature. This strategy relies on using known characteristics which determine antimicrobial activity of AMP's, such as net charge, hydrophobicity and helicity, to modify existing AMP's with a view to increasing their activity (Andra *et al.*, 2007). It should be noted however that early studies showed that in many cases increasing hydrophobicity or helicity also increased cytotoxicity (Giangaspero *et al.*, 2001, Dathe *et al.*, 2001, Zelezetsky *et al.*, 2005). The ultimate aim of the rational design process is to design AMP's with increased antimicrobial activity and no cytotoxicity.

Many of the original studies into rational design of AMP's began by identifying the motifs responsible for the antimicrobial activity of known efficacious molecules and created chimeras with AMP's of low activity in an effort to increase the antimicrobial activity.

Examples of this approach were the chimeras created using HBD3 and HBD2 (Jung *et al.*, 2011). It was known that HBD3 killed *S. aureus* with 4-8 fold higher efficiency than HBD2 but that they killed *E. coli* with the same efficiency. Chimeras created from a combination of these two parental peptides indicated that the N-terminal region of HBD3 was the most important region for

antimicrobial activity and that that this activity was not correlated to the net charge of the chimera. It also found that the internal portion of HBD2 was responsible for its antimicrobial activity. Chimeras created using these two motifs had increased activity against both *S. aureus* and *E.coli*.

Another key finding from some of these initial studies was that positively charged side chains with long aliphatic side chains such as lysine and arginine allow the AMP to integrate into bacterial membranes while the charged groups were still interacting with the anionic head groups of the bacterial membrane (Zelezetsky *et al.*, 2005).

The genome mining approach was used to discover a putative antifungal AMP gene *afp*B from *P. digitatum* however efforts to isolate the AfpB protein were unsuccessful despite the detection of high levels of the *afp*B mRNA (Garrigues *et al.*, 2016). A further study modelled the structure of this AMP *in silico* and used this modelled tertiary structure to rationally design four cysteine containing peptides. Two of these analogue peptides showed good antifungal activity against *P. digitatum* and *Botrytis cinerea* (Garrigues *et al.*, 2017).

Recently, an approach which used bioinformatic screening of sequence databases in combination with *de novo* design and protein engineering successfully predicted a small AMP with specific activity against *Mycobacterium tuberculosis* (Pearson *et al.*, 2016). Another study used *in silico* peptide evolution to mimic natural evolution to evolve a peptide with extremely potent antimicrobial activity against bacteria (Porto *et al.*, 2018). This study started with a lead peptide Pg-AMP1 from *Psidium guagava*, a glycine rich AMP which possess no antimicrobial activity against *P. aeruginosa*. The primary sequence of this peptide was passed through an algorithm which used two peptide characteristics, hydrophobic moment and α-helix propensity, to evolve the sequence *in silico* into a putative AMP with good activity known as guavanin-2 (Fig. 1.9). It was subsequently found that this peptide possessed excellent antimicrobial activity against Gram-negative bacteria, particularly *Escherichia coli*, *Acinetobacter baumannii*, and exhibited moderate activity towards *Klebsiella pneumoniae*.

These studies show the power of bioinformatics for the discovery or design of highly effective AMP's. This is only half the battle however. Once active peptides are discovered or designed they must next be produced on an industrial scale if they are to be used as food preservatives. Chemical synthesis of these peptides would be cost prohibitive. The use of hydrolysates that have been described has a number of limitations; enzymatic digestion on an industrial scale is also cost prohibitive and may also lead to undesired off

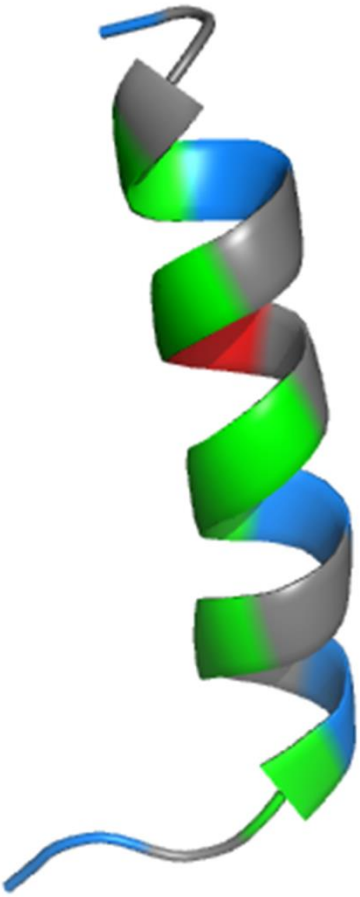


Fig 1.9 Tertiary Structure of the *In Silico* Evolved Peptide Guavanin-2. Peptide was evolved *in silico* from Pg-AMP1 from *Psidium guajava*

flavours or aromas. The use of microorganisms as bio-factories would provide a cost effective alternative to these problems (de Souza *et al.*, 2018).

There are two ways this could be achieved; use the source organism or engineer another organism to produce the AMP. The first method relies on the AMP of interest being naturally produced by an organism in nature, if this is the case then it can either be extracted from the source or the organism can be directly added to food (Kong and Lu, 2014, Cotter *et al.*, 2005). Indeed, as has been described in a previous section of this introduction, natural AMP producing members of lactic acid bacteria have been used to prevent food spoilage. One drawback of using this approach is that in many cases the levels of AMP produced by the natural source is too low to provide efficient antimicrobial activity (Cotter *et al.*, 2005).

In an effort to circumvent this, research has been conducted to try to increase the level of production by the natural source. One way this can be achieved is through strict control of growth conditions, as an example, it was found nisin production by *Lactococcus lactis* was increased in the presence of skimmed milk (Dussault *et al.*, 2016). Another solution to the problem is to engineer overproducing strains of the source microorganism. In one study, multiple copies of genes involved in the biosynthesis of nisin were introduced into *L. lactis* which greatly increased the production of the AMP (Cheigh *et al.*, 2005). There are limitations to this solution however. It has been shown that at high concentrations, several AMP's can actually be toxic to the producing organism (Boto *et al.*, 2018).

A solution to this problem would be to express the recombinant AMP in a resistant microorganism. This has been achieved with industrial scale production of plectasin. This peptide is naturally produced by the fungus *Pseudoplectania nigrella*. The fungus *Aspergillus oryzae* has successfully been used to produce plectasin on an industrial scale as it, being a eukaryotic, is naturally resistant to the AMP's effects (Mygind *et al.*, 2005). *Penicilliuum chrysogenum* has also been successfully used to express analogues of the small, cysteine rich antifungal peptides NFAP from *Neosartorya fischeri* and PAF from *P. chrysogenum* (Sonderegger *et al.*, 2016). Although this expression system was developed for laboratory scale purification of the peptides for

structural analytical purposes, there is no reason why this system could not be scaled up to industrial scale.

Another popular microorganism for recombinant AMP production is *E. coli*. There are numerous cases where this bacterium has been used for the heterologous production of AMP's on an industrial scale; indeed the HBD3 peptide standards used in many parts of the study described in this thesis were recombinantly produced in *E. coli* (Tanhaiean *et al.,* 2018). Another good example is the production of correctly folded disulfide bond containing Snakin-2 in *E. coli* (Herbel *et al.*, 2015).

Although recombinant production of peptides in bacteria can be carried out relatively cheaply on industrial scales there are some limitations to the use of this technology for AMP production for food use. In order to be used in food, the peptides would need to be extracted, either from bacterial lysates or from the extracellular environment, from the bio-refineries. This process would add another layer of cost to the process. Another problem may be the presence of toxins or immune responsive molecules such as lipopolysaccharide.

An excellent solution to this problem is to use *Saccharomyces* spp. to produce the recombinant AMP's. There are a number of reasons that this yeast is a particularly appropriate yeast for this purpose; it is a generally regarded as safe (GRAS) organism and as such is safe for use in food, it is also very amenable to genetic manipulation and, most importantly for the purposes of this thesis, it is already used to produce the food which this work is interested in (James *et al.*, 2014).

Synthetically engineered yeasts have already been used to produce some economically important food additive products. For example the biosynthetic pathway for, one the world's most important flavour compounds, vanillin was engineered into the fission yeast *Schizosaccharomyces pombe* and it was seen that the yeast was able to produce up to 65 mg/L of the compound (Hansen *et al.*, 2009). *S. cerevisiae* strains have also been engineered to express recombinant AMP's. The first such example was expression of the pediocin PA-1, derived from *Pediococcus acidilactici*. It was shown that the peptide was efficiently expressed when driven by the *Saccharomyces* ADH1 promoter and

that the peptide was secreted into the extracellular environment due to the fusion of the peptide to the *Saccharomyces* alpha factor secretion signal (Schoeman *et al.*, 1999). It has been shown that *Saccharomyces* strains expressing and secreting the human AMP HBD3, could effectively limit the growth of the beer spoiling microorganism *L. brevis* in a pilot scale setting (James *et al.*, 2014). These proof of principle studies suggest that the use of *Saccharomyces* is the most appropriate choice for the work reported in this study.

1.3 Specific Aims of this Project

The identification and characterization of new AMP's offers many novel avenues for the control of food borne pathogens and spoilage organisms. The development of novel therapeutics such as these would provide a *via*ble alternative to conventional preservatives such as chemical additives and high energy treatments, the use of which has become cost prohibitive and unethical due to public health concerns. Expanding the repertoire of available safe food additives is of global economic significance. Previous work conducted by this lab had shown that human beta-defensin-3 (HBD3) was an effective treatment measure for the control of food spoilage organisms such as the lactic acid bacterium *Lactobacillus brevis* (James *et al.*, 2014).

The use of a biotechnology approach for the production of recombinant peptides, such as HBD3, *in situ* from *Saccharomyces* represents a highly efficacious and cost effective delivery system for these novel therapeutics. Given that in many cases the spent yeast after fermentation is used as animal feed, this technology could also provide a secondary, indirect, benefit to the food industry by reducing the need for prophylactic additives in animal feeds. Indeed, EU regulation 1831/2003/EC banned the use of antibiotics, due to the global prevalence and increase of antibiotic resistance, as growth promoters in animal feeds in 2006 and since then the industry has been searching for *via*ble, safe and cost effective replacements; this technology has the potential to provide that replacement. The exogenous expression of a human derived peptide in products intended for human consumption may however be

unpalatable to consumers given the growing public scepticism of genetically modified organisms, particularly organisms expressing human derived compounds.

In an effort to alle*via*te some of these concerns, this study sought to identify plant based peptides which were ribosomally synthesised and could provide a similar level of *In Vivo* antimicrobial activity . It was felt that this approach would provide a more attractive alternative because the public have long accepted plant based additives in food products. As it had been previously shown that HBD3 had effective antimicrobial activity in the past it was decided that this peptide was a good benchmark for use in the discovery and screening process. The result of this discovery approach would then be used to rationally design a set of peptides with increased antimicrobial activity.

The specific aims of this thesis and methodologies employed to achieve them were:

1. To use a bioinformatics based approach to discover plant derived peptides, based on structural and biochemical comparisons to that of HBD3, which possess antimicrobial activity.
    a. Mine peptide amino acid sequences from PhytAMP database.
    b. Retrieve or model peptide structures.
    c. Filter peptides which possess unwanted structural motifs.
    d. Rank peptides structural similarity to HBD3 and carry top three peptides forward for analysis.
    e. Analyse peptide antimicrobial activity against *Lactobacillus brevis* under various conditions such as high/low salt or pH.
    f. Analyse the importance of tertiary structure for antimicrobial activity.
    g. Analyse the thermostability of peptide antimicrobial activity.

2. To identify the structural or biochemical characteristics responsible for antimicrobial activity from these discovered peptides.
    a. Rank candidate peptides by antimicrobial activity.

b. Identify correlations between antimicrobial activity and physiochemical characteristics such as charge/hydrophobicity or structural characteristics.

3. To use those identified characteristics to rationally design a set of peptides with increased antimicrobial activity.
    a. Modify the amino acid sequence of the least active peptide to include the characteristics identified in aim 3.
    b. Model the peptide structure to ensure the modifications do not significantly alter the peptide tertiary structure.
    c. Test the analogue peptides for antimicrobial activity under the same conditions used in aim 1.

4. To develop a synthetic and modular peptide expression platform which is stable within *Saccharomyces,* without the need for selective pressure.
    a. Modify the yeast artificial chromosome pYAC4 to include the antibiotic resistance cassette KanMX for maintenance under selective pressure within *Saccharomyces pastorianus*.
    b. Integrate 1, 2 or 3 copies of a previously constructed rHBD3 expression cassette into the newly constructed expression platform in a modular fashion.
    c. Assess the intracellular stability of the expression platforms with and without selective pressure in both circular and linear forms in *S pastorianus*.

5. To examine the possible linear relationship between recombinant gene copy number and the level of recombinant peptide produced *In Vivo*.
    a. Using the expression platforms from aim 4, and a high-copy number pRS42H-HBD3 plasmid, protein fractions harvested from supernatant, cell surface and intracellular locations.
    b. Level of rHBD3 production and localisation examined *via* α-HBD3 ELISA.

6. Develop expression system for peptides discovered in aim 1 or rationally designed in aim 3.
    a. Reverse translate peptide amino acid sequences into *Saccharomyces* sp. codon-biased nucleotide sequence.
    b. Construct genes for peptides using polymerase chain assembly.
    c. Integrate constructed genes into pYAC4 based vector and high-copy pRS42H plasmid.
    d. Insert expression systems into *S. pastorianus*.

7. To examine the level of *In Vivo* antimicrobial control produced by all expression platforms in *S. pastorianus*.
    a. Co-culture *S. pastorianus* harbouring the expression systems with a defined number *L. brevis* bacteria in laboratory YEPD medium at 30°C and 10°C for 24h and assess bacterial survival.
    b. Co-culture *S. pastorianus* harbouring the expression systems with a defined number *L. brevis* bacteria in brewers' wort at 30°C and 10°C for 24h and assess bacterial survival.

Chapter 2:

Materials and Methods

2.1 Reagents, Strains, Plasmids, Media and Growth Conditions

All chemicals and reagents were of analytical grade and unless otherwise stated were purchased from Sigma-Aldrich (Dublin, Ireland). The water used throughout this study was sterile distilled water.

2.1.1 Strains

Saccharomyces cerevisiae BY4741 (Bond Research Group) (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) is a haploid laboratory yeast strain. *Saccharomyces pastorianus* CMBS33 is a polyploid yeast strain (Bond Research Group). *Lactobacillus brevis* L1033 is a Gram-positive lactic acid bacteria (Bond Research Group). All plasmid propagation was carried out in *E. coli* DH5α (Bond Research Group) (F^-, Φ80dlacZ△M15, △ (lacZYA-argF)U169, deoR, recA1, endA1, hsdR17 (rk^-,mk^+), phoA, supE44, λ^-, thi-1, gyrA96, relA1).

2.1.2 Plasmids

All plasmids used throughout this study are listed in Table 2.1. Where plasmids were propagated in *E. coli* the vectors were maintained using the antibiotic carbenicillin at a final concentration of 100 μg/ml. Auxotrophic selection in *Saccharomyces* was carried out using minimal medium lacking uracil. Antibiotic selection in *Saccharomyces* was carried out using Hygromycin B at 200 μg/mL or G418 disulphate (geneticin) (Melford, Suffold, UK) at a final concentration of 200 μg/mL.

Table 2.1 Plasmids used throughout this study

Plasmid Name	Yeast Selective Agent	Bacterial Selective Agent	Source
pYAC4	Ura^-	Carb	Bond Lab
pRS42K	G418	Carb	Bond Lab
pRS42H-HBD3	Hyg	Carb	Bond Lab
pRS42H-Fabatin	Hyg	Carb	This Study
pRS42H-Cp-thionin	Hyg	Carb	This Study
pRS42H-3Arg Analogue	Hyg	Carb	This Study
pYAC-Kan Circular	Ura^-/G418	Carb	This Study
pYAC-Kan Linear	Ura^-/G418	None	This Study
pYAC-Kan 1 x HBD3 Circular	Ura^-/G418	Carb	This Study
pYAC-Kan 1 x HBD3 Linear	Ura^-/G418	None	This Study
pYAC-Kan 2 x HBD3 Circular	Ura^-/G418	Carb	This Study
pYAC-Kan 2 x HBD3 Linear	Ura^-/G418	None	This Study
pYAC-Kan 3 x HBD3 Circular	Ura^-/G418	Carb	This Study
pYAC-Kan 3 x HBD3 Linear	Ura^-/G418	None	This Study

Hyg = Hygromycin B

Amp = Ampicillin

Ura^- = Auxotrophic Selection with URA^- *Saccharomyces* strains

2.1.3 Yeast Media and Growth Conditions

Unless otherwise stated, all yeast strains were grown at 30°C for 48 h. The standard growth medium for yeast was YEPD (10g/L yeast extract, 20g/L tryptone, 20g/L glucose). Yeast auxotrophic selection medium was Complete Supplement Mixture(CSM) lacking uracil (1.7g/L yeast nitrogen base, 5g/L ammonium sulfate, 600mg/L Synthetic Dropout Powder; Leu^-/Ura^- (Formedium), 80mg/L leucine, 20g/L glucose). In all cases where solid media were used, the above media compositions were supplemented with 20 g/L agar. Where plasmids were maintained *via* antibiotic selection, the above media compositions were supplemented with the appropriate antibiotic at the concentrations listed in section 2.1.2.

2.1.4 Bacterial Media and Growth Conditions

Growth of *L. brevis* was carried out at 30°C for 48h in MRS broth (LabM) supplemented with Tween-80 at a final concentration of 0.1% (v/v) unless otherwise stated. Growth of *E. coli* DH5α was carried out at 37°C in Lennox broth for 18h – 24h unless otherwise stated. Where solid media were used, the above compositions were supplemented with 20 g/L agar. Where antibiotic selection was used, the above media were supplemented with the appropriate antibiotic at the concentrations listed in section 2.1.2

2.1.5 Monitoring Growth of Yeast and Bacterial Cultures

The growth of yeast and bacterial cultures were monitored by measuring the optical density of the cultures at a wavelength of 600nm. 1 mL of culture was aliquoted into a plastic cuvette and the OD_{600} value was measured using an Eppendorf BioPhotometer V1.35.

2.2 *In silico* Peptide Screening and Bioinformatic Analysis

2.2.1 Database Screening

Potential candidate plant AMP's were mined from the PhytAMP database (http://phytamp.pfba-lab-tun.org/main.php). The entire database was filtered to return only AMPs from the defensin family. Peptide sequences for the identified candidate peptides were retrieved from the UniProt database (http://www.uniprot.org/) and any containing the "Knottin" peptide motif and scorpion toxin-like motifs were rejected. Where structures were available, they were retrieved from the Protein Data Bank (PDB) server (http://www.rcsb.org/pdb/home/home.do).

2.2.2 Molecular Modelling of AMP Tertiary Structure

The primary amino acid sequences of all peptides used in this study are listed in Table 2.2. Where structures for candidate peptides were available, they were retrieved from the Protein Data Bank (PDB) server (http://www.rcsb.org/pdb/home/home.do). Where experimentally determined structures were not available, the primary amino acid sequence was used for homology modelling in intensive mode with the webtool "Phyre2" (http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index).

Both the retrieved PDB structures and homology modelled structure were next passed to the molecular modelling software suite "GroMacs". Molecular modelling was carried out as described in http://www.mdtutorials.com/gmx/lysozyme/index.html. After energy minimisation, the structures were modelled in a, charge balanced, water based environment over the course 1 ns with energy measurements and structural poses being recorded every 10 ps. Radius of gyration data was also collected in 10 ns increments over the course of the simulation.

The resulting tertiary structure models were visualised using the molecular visualisation software suite "PyMol". This suite was also used for all interatomic measurements and calculations of root mean square de*via*tion (RMSD) between structurally aligned pairs of peptides.

Table 2.2 Primary Amino Acid Sequences of All Peptides used in this Study

Peptide Name	Peptide Sequence
HBD3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK
Mungbean Defensin	KTCENLANTYRGPCFTTGSCDDHCKNKEHLRSGRCRDDFRCWCTRNC
Fabatin	LLGRCKVKSNRFNGPCLTDTHCSTVCRGEGYKGGDCHGLRRRCMCLC
Cp-Thionin	KTCMTKKEGWGRCLIDTTCAHSCRKYGYMGGKCQGITRRCYCLLNC
3-Arg Analogue	KTCRNLANTYRGPCFTTGSCRRHCKNKEHLRSGRCRDDFRCWCTRNC
5-Arg Analogue	KTCRNLANTYRGPCFTTGSCRRHCKNKEHLRSGRCRRRFRCWCTRNC
All Cysteine Knockout Analogue	KTARNLANTYRGPAFTTGSARRHAKNKEHLRSGRARRRFRAWATRNA
Internal Cysteine Knockout Analogue	KTCRNLANTYRGPAFTTGSARRHAKNKEHLRSGRARRRFRAWATRNC
N-terminal Fluid Analogue	FTTGSCRRHCKNKEHLRSGRCRRRFRCWCTRNCGGKTCRNLANTYRGPC
1-Strand Deletion Analogue	FTTGSCRRHCKNKEHLRSGRARRRFRCWCTRNA
2-Strand Deletion Analogue	FTTGSCRRHCKNKRHLRCGRARRRFR

2.2.3 Prediction of Peptide Physiochemical Characteristics

Predictions for the peptides isoelectric point and molecular weight were carried out using the online tool “PepCalc” with a C-terminal amidation option set to true (https://pepcalc.com/). Peptide average hydropathy was predicted using the webtool “GRAVY” with default parameters (http://www.gravy-calculator.de/). Predictions of peptide net charge at variable pH values was conducted using the webtool Protein Calculator v3.4 (http://protcalc.sourceforge.net/). Predictions were carried out in pH increments of 0.5 between pH4 and pH8. All of the above predictions used the primary amino acid sequence of the candidate peptides as the input.

Global scoring of candidate peptide tertiary structural similarity to the tertiary structure of HBD3 was carried using the webtool “TM-score” (https://zhanglab.ccmb.med.umich.edu/TM-score/). The analysis was carried out with default parameters using the structures generated using GroMacs.

2.3 *In vitro* Analysis of Antimicrobial Activity

2.3.1 Synthesis of Candidate AMP’s

All peptides synthesised for this study are listed in Table 2.2. Synthetic AMP’s were chemically synthesised at a purity of >70% (GL Biochem Ltd , Shanghai, PRC.). Peptides were synthesised with an amide protecting group on the carboxy terminus. The peptides were dissolved in 100mM acetic acid at 50mg/ml and stored at -70°C.

2.3.2 *In vitro* Antimicrobial Assay

To accurately determine the inhibitory concentrations (IC) of each peptide, a spreadplate based assay was employed. Briefly, *L. brevis* cultures were grown for overnight as previously described. The bacterial cultures were diluted 1:50 and grown to mid exponential phase (OD_{600}=0.5). 100µl of AMPs, at varying concentrations, were mixed with 100µl of *L. brevis* (final concentration 2 $x10^3$ cell/mL) in a 96-well microtitre plate. Following incubation for 1.5 hour at 30°C, 100µl from each well was spread onto an MRS agar plate. Surviving colonies

were counted after 48h incubation at 30°C. Bacterial survival following AMP treatment was assessed relative to untreated control samples and expressed as a percentage of the control. Assay was conducted using two biological replicates with three technical replicates each. The concentration of peptide required to inhibit 50% and 90% of bacteria, IC_{50} and IC_{90}, respectively, under the experimental conditions was determined.

The buffers used to perform these were phosphate buffered saline (PBS)(137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 2mM KH_2PO_4) and phosphate buffer (PB) (2.7mM KCl, 10mM Na_2HPO_4 and 2mM KH_2PO_4). These assays were carried out at pH4, pH7 and pH8.

2.3.3 *In vitro* Antimicrobial Assay to Determine Importance of Disulfide Bonds

To determine the importance of correct disulfide bond formation the assay was carried out as in section 2.3.2 in PB at pH7 using the peptides at a final concentration equal to the IC_{50} concentration as determined in section 2.3.2 in PB at pH7. The assay was conducted in the presence and absence of the reducing agent dithiothreitol (DTT) at a final concentration of 5mM. The results of the assay were represented as a % of a set of samples not containing DTT. The assay was conducted using two biological replicates with three technical replicates each.

2.3.4 *In vitro* Antimicrobial Assay to Determine Peptide Thermostability

To determine the importance of correct disulfide bond formation the assay was carried out as in section 2.3.2 in PB at pH7 using the peptides at a final concentration equal to the IC_{50} concentration as determined in section 2.3.2 in PB at pH7. Before mixing the peptides with the bacteria, the peptides were first heated to 90°C for 10min before being cooled to 20°C over 5 min. The results were expressed as a % of the activity of a set of peptides which did not undergo the thermal pre-treatment. The assay was conducted using two biological replicates with three technical replicates each.

2.4 Strain Construction

2.4.1 Determination of DNA Concentration

DNA concentration was determined by measuring the absorbance at 260nm using a Nanodrop Spectrophotometer ND-1000.

2.4.2 *Saccharomyces* DNA Isolation

DNA isolation from yeast was carried out using the Zymolyase – Alkaline Lysis method. Yeast cells were grown for 16-18h as described previously. 200µl packed cell volume was harvested and washed in SCE buffer (1M Sorbitol, 100mM Sodium Acetate, 60mM EDTA). Cells were resuspended in 2ml Zymolylase solution (1xSCE buffer, 1.2mg/ml Zymolyase 20T, 0.002% 2-β-Mercaptoethanol). Cells were incubated at 37°C for 1h. Spheroplasts were harvested and resuspended in 500µl uLPK buffer (100mM EDTA, 0.2% Sodium Deoxycholate, 1% Sodium N-Lauroylsarcosine, 100U Proteinase-K). Spheroplasts were incubated at 55°C for 30min. 500µl Lysis buffer (200mM NaOH, 1% SDS) was added. Cells were incubated with rocking at RT for 5min. 1ml Neutralisation buffer (4.2M Guanidine Hydrochloride, 900mM Potassium Acetate, pH4.8) was added. Supernatant was harvested by centrifugation at 4°C. 0.7vol 2-propanol was added and precipitation was carried out at RT for 15min. DNA pellet was harvested by centrifugation and washed twice with 70% EtOH. DNA pellet was dried at 55°C for 15min and resuspended in sterile distilled water. DNA concentration was determined as described in section 2.4.1.

2.4.3 *E. coli* DNA Isolation

DNA isolation from *E. coli* was carried out using QIAprep Spin Miniprep Kit (QIAgen) as per manufacturer's instructions. DNA concentration was determined by agarose gel electrophoresis.

2.4.4 Ethanol Precipitation of DNA

To the crude DNA preparation was added 0.1 vol 3M Sodium Acetate and 3vol 100% EtOH. Precipitation was carried out at -70°C for 1h. The DNA pellet was harvested by centrifugation at 4°C. The DNA pellet was washed once using 70% EtOH and dried at 55°C for 15min. The dried DNA pellet was resuspended in sterile distilled water. DNA concentration was determined by agarose gel electrophoresis.

2.4.5 DNA Restriction Enzyme Digestion

Unless otherwise stated, all restriction digests were carried out using NEB buffer #2 (NewEngland Bioscience). Restriction digests were carried out in 50µl reactions containing 1x restriction enzyme buffer, 100µg/ml BSA, 10U restriction enzyme, 1µg template DNA. Digestion was carried out at 37°C for 1h. Digestion product was treated with 2U shrimp alkaline phosphatase (Roche) for 1h at 37°C. Enzymes were heat inactivated at 65°C for 20min. Digested DNA was recovered by ethanol precipitation as previously described. DNA concentration was determined as previously described.

2.4.6 Template Preparation for Colony Polymerase Chain Reaction

A single isolated colony was added to 20µl 20mM NaOH. The colony was lysed at 95°C for 10min with a hold at 4°C until use.

2.4.7 Polymerase Chain Reaction

Standard polymerase chain reactions (PCR) were carried out in 25µl reactions containing 100µM dNTPs, 0.4nM of each oligonucleotide primer, 1X *Taq* DNA polymerase buffer (10 mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, pH 8.3), 1U *Taq* DNA polymerase (YorkBio), 200ng template DNA or 1 µl of colony template described previously and sterile water added to 25µl. High fidelity PCR were carried out in 50µl reactions using 2U AccuTaq DNA polymerase. All other components were the same as the standard PCR reaction. Cycling

conditions used for PCR reactions were as follows; 95°C for 5 min followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 1 min followed by 1 cycle of 72°C for 5 min. All primers used in this study are listed in Table 2.3.

Table 2.3 Primers Used for this Study

Primer	DNA Sequence
YAC-F1	GGTTTAAGGCGCAAGACTTTAATTTATCACTACGGCCAAGAATTACTCGTGAGTAAGGAA
YAC-F2	TATAATCTTGGCGAACGGGCTTCTACGATAGCGAGAGATGCCAAGAATTACTCGTGAGTAAGGAA
YAC-F3	AACTGACAGGAAGGCGATCCAAGAGTAGTGAAGCGACCTCCCAAGAATTACTCGTGAGTAAGGAA
Seq-YAC NotI R1	GGCGATCGAACGCCCGATCTCAAGATTACGGAATTGCGGCCGCCATCTCTCGCTATCGTAGAAGCCCGTTCGCCAAGATTATAATATTACCCTGTTATCCCTAGCG
Seq-YAC NotI R2	GGCGATCGAACGCCCGATCTCAAGATTACGGAATTGCGGCCGCGAGGTCGCTTCACTACTCTTGGATCGCCTTCCTGTCAGTTATATTACCCTGTTATCCCTAGCG
Seq-YAC NotI R3	GGCGATCGAACGCCCGATCTCAAGATTACGGAATTGCGGCCGCGAGGACCCTTCAAGCTATGTGGACACGGTGGTCTACATCTATATTACCCTGTTATCCCTAGCG
YAC-Chk-F1	GGTTTAAGGCGCAAGACTTT
YAC-Chk-R1	CTATCGTAGAAGCCCGTTCG
YAC-Chk-F2	CGAACGGGCTTCTACGATA
YAC-Chk-R2	GATCGCCTTCCTGTCAGTTG
YAC-Chk-F3	TAGTGAAGCGACCTCCCAAG
YAC-Chk-R3	GGACCCTTCAAGCTATGTGG
TDH3 F	CTGTCAAGTTGAACAAGGAAACCAC
TDH3 R	CAACGTGTTCAACCAAGTCGACAA
Amp F	GCTCGTCGTTTGGTATGGCTTCATTAG
Amp R	TTCTGCTATGTGGCGCGTATTATCCC
Fabatin F	CTGCTGTTTTGTTCGCTGCTTCTTCTGCTTTGGCTTGTTTGTGTATGTGTAGAAGAAGATTGGG
Fabatin R	ATCATAAGAAATTCGCTTATTTAGAAGTGGCGCGCCTTACAACAAACCTCTACACTTAAC
Cp-Thionin F	CTGCTGTTTTGTTCGCTGCTTCTTCTGCTTTGGCTTGTAACTTGTTGTGTTACTGTAGAAGAACCATCGG
Cp-Thionin R	ATCATAAGAAATTCGCTTATTTAGAAGTGGCGCGCCTTACTTGGTACACATGGTCTTCTTTTCAC
3-Arg F	CTGCTGTTTTGTTCGCTGCTTCTTCTGCTTTGGCTAAGACCTGTAGAAACTTGGC
3-Arg R	ATCATAAGAAATTCGCTTATTTAGAAGTGGCGCGCCTTATTAACAGTTTCTGGTACACC
Eco Chk F	GACAGCATCGCCAGTCACTA
Eco Chk R	AGTAGGTTGAGGCCGTTGAG
Yac-Kan F	CATTGGTGACTATTGAGCACGTGAGTATACGTGATTCTCGACGTTTTCGACACTGGATG
Yac-Kan R	GTCTCCACACCTCCGCTTACATCAACACCAATAACGTCTAGAGATCTGTTTAGCTTGCCTCG

2.4.8 Design of Oligonucleotides for Polymerase Chain Assembly

All steps in the design of PCA oligonucleotides were carried out using the "GeneDesign" webtool (http://54.235.254.95/gd/). The amino acid sequences of candidate peptides were reverse translated using the *Saccharomyces* codon bias. These *Saccharomyces* optimized nucleotide sequences were divided into a set of overlapping oligonucleotides using the "Building Block Design; constant length overlap" feature of the webtool. All oligonucleotides were synthesised by Sigma-Aldrich. All oligonucleotides used in this study are listed in Table 2.4

2.4.9 Polymerase Chain Assembly

Polymerase chain assemble (PCA) reactions were carried out in 25µl reactions using 1x *Taq* DNA polymerase buffer (10 mM Tris-HCl, 50mM KCl,1.5mM $MgCl_2$, pH 8.3), 100µM dNTP's, 1U AccuTaq DNA polymerase, template primer mix (300nM final concentration each PCA oligonucleotide). All oligonucleotides used to carry out PCA are listed in Table 2.4

Template primer mix for the Fabatin, Cp-Thionin and 3-Arg genes were composed of oligonucleotides Fabatin.O1-Fabatin.O6, Cp-Thionin.O1-Cp-Thionin.O6 and 3-Arg.O1-3-Arg.O6 respectively. Cycling conditions were as follows; 95°C for 15min, 55°C for 30s, 72°C for 1min, then 25 cycles of; 95°C for 30s, 55°C for 30s, 72°C for 1min. The PCA product concentration was determined as previously described.

Table 2.4 Oligonucleotides used throughout this study

Oligonucleotide	Oligonucleotide DNA Sequence
Fabatin.O1	TTGTTGGGTAGATGTAAGGTTAAGTCTAACAGATTCAACGGTCCATG
Fabatin.O2	GTGGGTGTCGGTCAAACATGGACCGTTGAATCTGTTAGACTT
Fabatin.O3	CCATGTTTGACCGACACCCACTGTTCTACCGTTTGTAGAGGTGAAG
Fabatin.O4	GACAGTCACCACCCTTGTAACCTTCACCTCTACAAACGGTAGAAC
Fabatin.O5	AGGTTACAAGGGTGGTGACTGTCACGGTTTGAGAAGAAGATGTATGTG
Fabatin.O6	TTAACACAAACACATACATCTTCTTCTCAAACCGTG
Cp-Thionin.O1	AAGACCTGTATGACCAAGAAGGAAGGTTGGGGTAGATGTTTGATC
Cp-Thionin.O2	GTGAGCACAGGTGGTGTCGATCAAACATCTACCCCAACCTTC
Cp-Thionin.O3	ATCGACACCACCTGTGCTCACTCTTGTAGAAAGTACGGTTACATGGG
Cp-Thionin.O4	GATACCTTGACACTTACCACCCATGTAACCGTACTTTCTACAAGA

Table 2.4 Continued

Oligonucleotide	Oligonucleotide DNA Sequence
Cp-Thionin.O6	TTAACAGTTCAACAAACAGTAACATCTTCTGGTGAT
3-Arg.O1	AAGACCTGTAGAAACTTGGCTAACACCTACAGAGGTCCATGTTTC
3-Arg.O2	TCTTCTACAAGAACCGGTGGTGAAACATGGACCTCTGTAGGTGTT
3-Arg.O3	TTCACCACCGGTTCTTGTAGAAGACACTGTAAGAACAAGGAACACTTG
3-Arg.O4	GTCTCTACATCTACCAGATCTCAAGTGTTCCTTGTTCTTACAGTGTC
3-Arg.O5	CTTGAGATCTGGTAGATGTAGAGACGACTTCAGATGTTGGTGTACC
3-Arg.O6	TTAACAGTTTCTGGTACACCAACATCTGAAGTCGTC

2.4.10 DNA Agarose Gel Electrophoresis

Unless otherwise stated all electrophoresis was carried out using 1% Agarose gels. Pre-stained agarose gels were made using 1xTAE buffer (40mM Tris, 20mM Acetic Acid, 1mM EDTA) plus 1.25µg/ml ethidium bromide. DNA was mixed with 10x loading dye (50% Glycerol, 0.1% Bromophenol Blue, 0.1% xylene cyanol) before loading onto gel. Electrophoresis was carried out in 1xTAE buffer at 80V for 1h. Gels were imaged using a UV imaging system. Unless otherwise stated the Q-Step 4 (Yorkshire Bioscience) molecular size marker was used as per manufacturer's instructions to calculate the DNA size.

2.4.11 DNA Sequencing

DNA sequencing was carried out by GATC Biotech (Constance, Germany) with the Sanger method using fragment specific primers. The resulting DNA sequences were compared to the expected sequence using the computer program Serial Cloner.

2.4.12 Transformation of *Saccharomyces* strains

Saccharomyces strains were grown for 16-18h, as per growth conditions listed previously. 50µl packed cell volume was harvested and washed with 100mM LiAc. The cell pellet was mixed with transformation solution; 33% PEG-4000, 100mM LiAc, 275µg/ml denatured single stranded carrier DNA and 0.25pmol transforming DNA. Where homologous recombination was being used to create a vector, a 5 fold molar excess of each integrating DNA fragment was also added. Cells were heat shocked at 42°C for 1h. Cells were washed and recovered for 18h at 30°C in YEPD broth. Cells were washed and plated onto selective plates as per Table 2.1. Positive and negative controls were included in all cases.

2.4.13 Transformation of *E. coli* DH5α

100µl of chemically competent *E. coli* DH5α cells were mixed with ~100ng of transforming DNA. Cells were incubated on ice for 1h. Cells were heat shocked

at 42°C for 90s. Cells were incubated on ice for 2min. 500µl Lennox broth, pre-warmed to 37°C, was added to the cells. Cells were recovered for 1h at 37°C and plated onto selective medium as per Table 2.1.

2.4.14 Construction of Plasmid pYAC-Kan Circular

The donor vector pYAC4 was isolated from *S. cerevisiae* BY4741 as previously described. 100 ng of pYAC4 DNA was transformed into *E. coli* DH5α as described previously to propagate the plasmid. The plasmid was isolated from the bacteria as previously described. 1 µg of isolated pYAC4 vector was digested with the restriction enzyme XbaI which linearised the vector within the TRP1 gene. The KanMX gene was amplified by high fidelity PCR using the primer pair Yac-Kan F and Yac-Kan R (Table 2.3) using the donor vector pRS42K which had been isolated from *S. cerevisiae* BY4741 pRS42K as a template. The linearised pYAC4 DNA and 5-fold molar excess of the amplified KanMX cassette were used to transform *S. cerevisiae* BY4741 as previously described. The yeast cells were recovered overnight before being plated onto YEPD agar supplemented with 200 µg/mL G418. Confirmation of the integration was confirmed by colony PCR using the primer pair Yac-Kan F and Yac-Kan R. DNA sequencing confirmed the correct integration of the KanMX cassette. The vector was isolated from *S. cerevisiae* and 100 ng of the vector was used to transform *E. coli* DH5α. The vector was isolated from the bacteria and 1 µg of the isolated vector was used to transform *S. pastorianus* CMBS33 to create the strain *S. pastorianus* pYAC-Kan.

2.4.15 Construction of Plasmid pYAC-Kan Linear

1 µg of isolated pYAC-Kan circular DNA was digested with the restriction enzyme BamHI as previously described which linearised the vector by excising the HIS3 gene and exposing the telomere sequences. The digested product was transformed in *S. pastorianus* CMBS33 to create the strain *S. pastorianus* pYAC-Kan linear.

2.4.16 Construction of Plasmid pYAC-Kan 1xHBD3 Circular

The HBD3 expression cassette used as the donor cassette for PCR is composed of a rHBD3 gene translationally fused to an alpha-factor secretion signal which mediated secretion into the extracellular supernatant. Transcription of the gene is driven by the phosphoglycerate kinase (PGK) promoter and terminated by an alcohol dehydrogenase (ADH) terminator. High fidelity PCR amplification of the expression cassette was carried out with template isolated from *S. pastorianus* pRS42H-HBD3 using the primer pair YAC-F1, which contained a 5' tail homologous to the pYAC-Kan vector upstream of the intended insertion site and Seq-YAC NotI R1 which had a 3' tail homologous to the Yac-Kan vector downstream of the intended insertion site and a unique barcode sequence which would allow for confirmation of insertion *via* PCR using a checking primer designed for this barcode. This tail also added a NotI restriction enzyme recognition site between the barcode sequence and the pYAC-Kan vector backbone.

1 μg of isolated pYAC-Kan circular DNA was digested with the restriction enzyme EcoRI as previously described which linearised the vector at the intended site of cassette insertion; the SUP4-o gene. The digested vector along with a 5-fold molar excess of the amplified cassette were co-transformed into *S. cerevisiae* BY4741. The cells were recovered overnight at 30°C in YEPD before being plated onto YEPD agar supplemented with 200 μg/mL G418. Colony PCR using the primer set YAC-Chk-F1 and YAC-Chk-F2 was used to identify potential clones with the correct insert. Subsequent DNA sequencing confirmed the correct insertion of the expression cassette. The vector was propagated within and isolated from *E. coli* DH5α as described previously and 1 μg of isolated vector was used to transform *S. pastorianus* CMBS33 to create the strain *S. pastorianus* pYAC-Kan circular.

2.4.17 Construction of Plasmid pYAC-Kan 1xHBD3 Linear

The circular pYAC-Kan 1xHBD3 was linearised as in section 2.4.14 and transformed into yeast to create the strain *S. pastorianus* pYAC-Kan 1xHBD3 Linear.

2.4.18 Construction of Plasmid pYAC-Kan 2xHBD3 Circular

The donor HBD3 expression cassette was amplified with the primer pair YAC-F2, which contained a 5' tail with homology to the unique barcode sequence from the vector pYAC-Kan 1xHBD3, and Seq-YAC NotI R2 which contained a 3' tail homologous to the pYAC-Kan vector backbone, a unique barcode sequence and a NotI restriction between these.

1 μg of isolated pYAC-Kan 1xHBD3 circular DNA was digested with the restriction enzyme NotI as previously described. This linearised the vector between the vector backbone and the first integrated HBD3 expression cassette. The linearised vector was co-transformed with the amplified expression cassette into *S. cerevisiae* and recovered as previously described. Colony PCR using the primer pair YAC-Chk-F2 and YAC-Chk-F2 was used to identify potentially successful insertions while the primer pair YAC-Chk-F1 and YAC-Chk-F1 was used to ensure that the first integrated cassette was still present. Subsequent DNA sequencing using both of these primer pairs was used to confirm the correct insertion of the second rHBD3 expression cassette downstream of the first.

The newly constructed vector was isolated for yeast and propagated in *E. coli* as previously described before subsequent isolation and transformation into yeast to create the strain *S. pastorianus* pYAC-Kan 2xHBD3.

2.4.19 Construction of Plasmid pYAC-Kan 1xHBD3 Linear

The circular pYAC-Kan 2xHBD3 was linearised as in section 2.4.14 and transformed into yeast to create the strain *S. pastorianus* pYAC-Kan 2xHBD3 Linear.

2.4.20 Construction of Plasmid pYAC-Kan 3xHBD3 Circular

The donor HBD3 expression cassette was amplified with the primer pair YAC-F3, which contained a 5' tail with homology to the unique barcode sequence from the vector pYAC-Kan 2xHBD3, and Seq-YAC NotI R3 which contained a 3' tail homologous to the pYAC-Kan vector backbone, a unique barcode sequence and again a NotI restriction between these.

1 µg of isolated pYAC-Kan 2xHBD3 circular DNA was digested with the restriction enzyme NotI as previously described. This linearised the vector between the vector backbone and the second integrated HBD3 expression cassette. The linearised vector was co-transformed with the amplified expression cassette into *S. cerevisiae* and recovered as previously described. Colony PCR using the primer pair YAC-Chk-F3 and YAC-Chk-F3 was used to identify potentially successful insertions while the primer pairs YAC-Chk-F1 and YAC-Chk-F1, and, YAC-Chk-F2 and YAC-Chk-F2 were used to ensure that the first and second integrated cassettes were still present. Subsequent DNA sequencing using all three of these primer pairs was used to confirm the correct insertion of the third rHBD3 expression cassette downstream of the first and second.

2.4.21 Construction of Plasmid pYAC-Kan 3xHBD3 Circular

The circular pYAC-Kan 3xHBD3 was linearised as in section 2.4.14 and transformed into yeast to create the strain *S. pastorianus* pYAC-Kan 3xHBD3 Linear.

2.4.22 Construction of Plasmid pRS42H-Fabatin

The donor vector pRS42H-HBD3 was digested using the restriction enzyme AscI (ThermoFisher) in 1 x fast digest buffer, which linearised the vector within the rHBD3 ORF at the intended site of cassette integration. The digested product vector was purified by ethanol precipitation as previously described.

The recombinant Fabatin ORF was constructed using polymerase chain assembly as described previously described. 100 ng of PCA product was amplified by PCR using the primer pair Fabatin-F, which included a 5' tail with homology to the alpha factor secretion signal of the donor vector and Fabatin-R which included a 3' tail possessing homology to the ADH terminator of the donor vector.

The digested donor vector and a 5-fold molar excess of the PCR product were used to co-transform *S. cerevisiae* BY4741. The yeast was recovered overnight at 30°C before being plated onto YEPD agar supplemented with 200 μg/mL Hygromycin B. Colony PCR using the primer pair Fabatin-F and Fabatin-R were used to screen for potentially successful integrants. Subsequent DNA sequencing using the primer pair YAC-F1 and Seq-YAC NotI R1 confirmed that the recombinant gene had been correctly inserted into the vector.

The vector was isolated and propagated in *E. coli* DH5α as previously described. The isolated DNA was used to transform *S. pastorianus* CMBS33 to create the strain *S. pastorianus* pRS42H-Fabatin.

2.4.23 Construction of Plasmid pRS42H-Cp-Thionin

The pRS42H-HBD3 donor vector was linearised at the site of integration as described in section 2.4.20. The recombinant Cp-Thionin ORF was prepared as in section 2.4.20 using the primer pair Cp-Thionin-F and Cp-Thionin-R which possessed the same homologies described in that section.

The linearised donor vector and the PCR product were used to co-transform *S. cerevisiae* BY4741 as described in section 2.4.20. Colony PCR using the primer pair Cp-Thionin-F and Cp-Thionin-R were used to screen for potentially successful integrants. Subsequent DNA sequencing using the primer pair YAC-

F1 and Seq-YAC NotI R1 confirmed that the recombinant gene had been correctly inserted into the vector.

2.4.24 Construction of Plasmid pRS42H-3-Arg

The pRS42H-HBD3 donor vector was linearised at the site of integration as described in section 2.4.20. The recombinant 3-Arg ORF was prepared as in section 2.4.20 using the primer pair 3-Arg-F and 3-Arg-R which possessed the same homologies described in that section.

The linearised donor vector and the PCR product were used to co-transform *S. cerevisiae* BY4741 as described in section 2.4.20. Colony PCR using the primer pair 3-Arg-F and 3-Arg-R were used to screen for potentially successful integrants. Subsequent DNA sequencing using the primer pair YAC-F1 and Seq-YAC NotI R1 confirmed that the recombinant gene had been correctly inserted into the vector.

2.5 Characterisation of the *In Vivo* Expression from Recombinant Expression Systems

2.5.1 Determination of Protein Concentration by Bradford Assay

Bradford reagent (100 mg/L Coomassie Brilliant Blue G-250, 4.75% (v/v) ethanol and 8.5% phosphoric acid) was brought to room temperature. Total protein was diluted 1:10 and 1:100. 100 μL of each dilution of total protein and concentrated protein were added in duplicate to a 96 well microtitre plate. Bovine serum albumin (BSA) standards ranging from 0.05 mg/mL to 2.0 mg/mL were also added in duplicate to the 96 well plate in 100 μL volumes. 200 μL of Bradford reagent was added and the plate was incubated for 10 min at room temperature to allow colour development. Plate was read at 595nm using a SynergyH1 microtitre plate reader (BioTek). A standard curve was generated based on the BSA standards. Total protein dilutions falling within the linear range of the standard curve were used to calculate protein concentration.

2.5.2 Harvesting *In Situ* Produced rHBD3

2.5.2.1 Growth Conditions

S. pastorianus strains harbouring the vectors pRS42H-HBD3, pYAC-Kan 1xHBD3 Linear, pYAC-Kan 2xHBD3 Linear and pYAC-Kan 3xHBD3 were grown overnight at 30°C in YEPD broth supplemented with the appropriate selective antibiotic as described in Table 2.1. Cells were harvested by centrifugation at 14,000 x g for 20 min using a Sorval RC 5C Plus centrifuge. Cells were washed twice to remove all traces of YEPD. Cells were seeded into 1 L cultures of complete synthetic medium (CSM) supplemented with 2% (w/v) maltose and appropriate selective antibiotics at a cell density of 2 x 10^5 cfu/mL, as determined by haemocytometer counting. Cells were grown for 36h at 30°C.

2.5.2.2 Harvesting Extracellular rHBD3

Extracellular supernatant was harvested by centrifugation at 14,000 x g for 20 min using a Sorval RC 5C Plus centrifuge. Harvested cell pellet was retained for further processing. Harvested supernatant was filtered through Whatman #1 filter paper. The harvested supernatant was concentrated by freeze drying before being resuspended in 1 mL sterile distilled water. Concentrated supernatant was filtered through a 0.22 μm PVDF filter (VWR International). Protein concentration was determined using a Bradford assay. Concentrated supernatant was stored at -80°C until use.

2.5.2.3 Harvesting Cell Surface rHBD3

The previously harvested cell pellet was washed twice using 10 mL 10% (v/v) acetic acid with gently shaking for 10 min each. Cell pellet was recovered between and after washing steps by centrifugation at 14,000 x g. Acid wash fractions were pooled and concentrated by freeze drying before being resuspended in 1 mL sterile distilled water. Protein concentration was determined by Bradford assay and the cell surface protein fraction was stored at -80°C until use.

2.5.2.4 Harvesting Intracellular rHBD3

Previously harvested cell pellet was suspended in an equal volume of CellLytic™-Y lysis buffer. An equal volume of acid washed glass beads were added. Cells were mechanically lysed until 90% lysis had occurred using a FastPrep-24 5G homogeniser (MpBio). The cell lysate was cleared by centrifugation at 14,000 x g and stored at -80°C until use.

2.5.3 Anti-HBD3 ELISA

All ELISA's were carried out in 96-well Microlon flat bottom high binding ELISA plates (Greiner Bio-one). Unless stated otherwise, all additions to the plate were in 60µl volumes. All washing steps were carried out with 300µl of washing buffer (0.05% Tween-20 in PBS) per well. All supernatant and cell surface wash samples were assayed neat. Intracellular lysates were assayed using 10^{-1} and 10^{-2} dilutions. Unless otherwise stated, all reagents were components of the Human βD-3 ELISA Development Kit (Peprotech). Serial dilutions of synthetic HBD3, ranging from 0-2µg diluted in ELISA diluent (0.05% Tween-20, 0.1%BSA, PBS), were used to compute a standard curve, from which, all HBD3 sample concentrations were determined. Standards and samples were assayed in duplicate. Anti-HBD3 capture antibody (2.5µg/ml in PBS) was added to each well. Plate was sealed and incubated, with shaking, for 24h at 20°C to immobilize the antibody to the plate. Wells were washed 4 times. 300µl blocking buffer (1% BSA in PBS) was added to each well to block remaining binding sites in each well. Plate was incubated for 2h at room temperature. Wells were washed 4 times. Standards and samples were added to the plate. Plate was incubated for 24h at 4°C to allow binding to the immobilized capture antibody. Wells were washed 4 times. Biotinylated detection antibody (0.25µg/ml in PBS) was added to each well. Plate was incubated with shaking for 2h at room temperature. Wells were washed 4 times. Avidin-HRP conjugate was diluted 1:2000 in ELISA diluent and added to each well. Plate was incubated at room temperature for 30min. Wells were washed 6 times. TMB (3,3',5,5-Tetramethylbenzidine) Liquid Substrate System (Sigma) was added to each well. Plate was incubated, in the dark, for 30min at room temperature.

Color development was stopped with the addition of 0.1M sulfuric acid. Plate was read at 450nm with wavelength correction at 650nm using a SynergyH1 microtitre plate reader (BioTek). A standard curve was generated based on the HBD3 standards. Analyte HBD3 dilutions falling within the linear range of the standard curve were used to calculate analyte HBD3 concentrations. Assay was conducted using two biological replicates with three technical replicates each.

2.5.4 Determination of Vector Copy Number by Quantitative Polymerase Chain Reaction (qPCR)

Whole genome DNA was extracted from *S. pastorianus* strains harbouring the vectors pRS42H, pRS42H-HBD3, pYAC-Kan Circular, pYAC-Kan 1xHBD3 Circular, pYAC-Kan 2xHBD3 Circular and pYAC-Kan 3xHBD3 Circular as previously described. The qPCR reaction was carried out using 300nM of the primer pairs Amp-F and Amp-R, to amplify the Amp^r gene contained on all of the vectors and TDH3-F / TDH3-R to amplify the chromosomal Glyceraldehyde-3-phosphate dehydrogenase gene. 10 ng of total genomic DNA was used as template. The reaction was carried out using 1 x SYBR Green mastermix in a 20 μL volume using the following conditions; 95°C for 5 min followed by 35 cycles of 95°C for 30s, 55°C for 30s and 72°C for 1 min followed by 1 cycle of 72°C for 5 min. Vector copy number was expressed as copy number relative to pYAC-Kan Circular using the ΔΔCt method. The assay was conducted using two biological replicates with three technical replicates each.

2.5.5 Analysis of the Stability of Circular *vs.* Linear pYAC-Kan expression systems under Non-selective Growth Conditions

To assess the intracellular stability of the pYAC-Kan expression platforms *S. pastorianus* strains harbouring the vectors pYAC-Kan Circular, pYAC-Kan Linear, pYAC-Kan 1xHBD3 Circular, pYAC-Kan 1xHBD3 Linear, pYAC-Kan 2xHBD3 Circular, pYAC-Kan 2xHBD3 Linear, pYAC-Kan 3xHBD3 Circular, pYAC-Kan 3xHBD3 Linear were grown overnight at 30°C in YEPD broth supplemented with G418. The yeast were subcultured into YEPD broth with

and without G418 at a final cell density of 2 x 10^5 cfu/mL and grown for 48h at 30°C. Samples were harvested, diluted appropriately and plated onto YEPD agar plates with and without G418. The data for each vector were represented as a % of the plasmid retained in comparison to the total colony counts on the non-selective YEPD agar plates.

2.5.6 Analysis of *In Vivo* Peptide Production from Recombinant Fabatin, Cp-Thionin and 3-Arg Analogue High Copy Plasmid Systems

2.5.6.1 Growth and Total Protein Extraction

S. pastorianus strains harbouring the vectors pRS42H, pRS42H-Fabatin, pRS42H-Cp-Thionin and pRS42H-3Arg were as described in section 2.5.2.1 in CSM broth supplemented with Hygromycin B and Maltose.

Extracellular protein was harvested as described in section 2.5.2.2. Cell surface protein was harvested as described in section 2.5.2.3. Intracellular protein was harvested as described in section 2.5.2.4.

2.5.6.2 Sample Fractionation by Reverse Phase High Precision Liquid Chromatography

10 μg of synthetic Fabatin, Cp-Thionin and the 3-Arg analogue peptide were individually resuspended in 100 μL of sterile distilled water containing trifluroacetic acid (TFA) at a final concentration of 0.1% (v/v). Samples were applied to a C18 RP-HPLC column (Zorbax 300SB-C18-Agilent; 150 x 4.6mm) which had been equilibrated with 0.1 TFA in aqueous buffer. The samples were fractionated over 45 min using a gradient starting with 2% (v/v) acetonitrile and 0.1% TFA (v/v) at a flow rate of 1 mL/min. This allowed the elution profile of the analyte samples to be predicted. 100 μL of intracellular, cell surface and extracellular analyte samples were acidified with TFA at a final concentration of 0.1% (v/v). Fractions of each sample were taken for 3 min centered around the elution time of the synthetic variant of each target peptide. The 3 mL samples were concentrated by freeze drying and resuspended in 100 μL of sterile distilled before being stored at -80°C until use.

2.5.6.3 Trypsin Digestion of HPLC Fractions

~50 μg of HPLC fractions were resuspended in reaction buffer with a final concentration of 6M guanidine-HCl and 25mM ammonium bicarbonate pH8. Dithiothreitol was added to a final concentration 20mM and the mixture was incubated at 55°C for 30 min. The mixture was allowed to cool to room temperature. Iodoacetamide was added to a final concentration of 100mM and the mixture was incubated at room temperature in the dark for 1h.The total concentration of guanidine was reduced to 0.6M with the addition of 25mM ammonium bicarbonate pH8. 2 μg of sequencing grade modified Trypsin (Promega) was added and the mixture was incubated for 16h at 37°C. The trypsinised peptide fragments were desalted using ZipTips™ (Millipore) with C18 resin as per manufacturer's instructions before being resuspended in 50 μL 25mM ammonium bicarbonate pH8. The samples were stored at -80°C until use.

2.5.6.4 Detection of Peptide Fragments by Mass Spectrometry

50 μL of desalted trypsinised peptide fractions were analysed using a Thermo Fisher Q-Exactive hybrid quadrupole-orbitrap mass spectrometer coupled to a Dionex RSLC nano. Liquid chromatography gradients ran from 14% to 35% solution B (Solution A: 0.1% (v/v) formic acid, Solution B: 80% (v/v) acetonitrile, 0.1% (v/v) formic acid) over 2 h, and data was collected using a Top 15 method for MS/MS scans. The raw data were analysed with the Proteome Discoverer software suite using a custom library composed of the primary amino acid sequences of Cp-Thionin, Fabatin and the 3-Arg analogue peptide.

2.5.7 Analysis of *In Vivo* Antimicrobial Activity of High Copy Number Expression Systems for Fabatin, Cp-Thionin and the 3-Arg Analogue

S. pastorianus strains harbouring the vectors pRS42H, pRS42H-HBD3, pRS42H-Fabatin, pRS42H-Cp-Thionin and pRS42H-3Arg Analogue were grown overnight as previously described. *L. brevis* was grown over night as previously described. The cells were harvested by centrifugation at 14,000 x g for 20 min and washed twice in phosphate buffered saline to remove all traces of growth

media. The cell were co-inoculated into either YEPD broth or brewers wort to a final cell density of 2 x10^3 cfu/mL for *L. brevis* and 1 x 10^{10} cfu/mL for the *S. pastorianus* strains. The bacteria were challenged for 24h at either 10°C or 30°C before being adequately diluted and plated onto MRST agar plates supplemented with 80 μg/mL of the eukaryotic specific antibiotic cyclohexamide which allowed specific enumeration of the bacteria. Data were reported as % survival in comparison to pRS42H empty vector control. Assays were conducted using two biological replicates with three technical replicates each.

2.5.8 Statistical Analysis

Statistical analysis was conducted using a pairwise Students T-test of biological replicates only. Where analysis was conducted on survival curve data, analysis was carried out using pairwise data at specific concentrations with correction for multiple testing using the Bonferroni method. Values below 0.05 were considered statistically significant.

Chapter 3:

Bioinformatic Discovery and Comparative Analysis of Plant Based Defensin-like Antimicrobial Peptides

3.1 Introduction

The work discussed in this chapter sought to discover plant based defensin-like AMP's with effective antimicrobial activity against food spoilage bacteria using a bioinformatic based approach. This approach was based on tertiary structure comparisons to the prototypic defensin, human beta defensin 3 (HBD3), which was previously used as "Gold Standard" antimicrobial peptide in this lab (James *et al.*, 2014).

Microbial contamination of food products is a major concern for producers and consumers worldwide. Current methods to combat food spoilage such as heat treatment, chemical treatment, addition of preservatives or antibiotics represent less than ideal additional inputs to the food processing system (Dagnas and Membre, 2013, Casewell *et al.*, 2003). This study aimed to develop a safer, low cost and more palatable alternative to combat food spoilage. Although food spoiling organisms are from diverse kingdoms, this project will focus mainly on methods to combat bacterial food spoilage, particularly spoilage caused by the beer spoiling microorganism *Lactobacillus brevis*. This decision was taken because of the importance and persistent nature of contamination by lactic acid bacteria in breweries and the industrial financial implications of developing a novel method to control these bacteria (Bokulich and Bamforth, 2013).

AMP's (AMPs) are small molecular weight peptides with broad spectrum antimicrobial activity against bacteria, viruses, and fungi. These evolutionarily conserved peptides are usually positively charged and have amphipathic character which enables the molecule to be soluble, and active, in both aqueous and lipid-rich environments, such as biological membranes(Boman, 1991). There are many classes of AMP ranging in size, charge and structure. This study focused on the use of defensins and defensin-like molecules.

Defensins are small cysteine-rich cationic peptides forming a major part of the innate immune response of many higher eukaryotes. Although at the primary amino acid sequence level defensins are highly variable, they possess a conserved tertiary structure known as the "defensin fold" (Lehrer *et al.*, 1991). This consists of an amphipathic helix and a 2-3 stranded anti-parallel β-sheet, all of which are stabilized by 3-5 disulfide bridges.

Although defensins have been studied in great detail the exact mode of their antimicrobial activity remains elusive. It is widely believed however that they exert their antibacterial activity by forming pores in microbial membranes. A number of independent models supporting this theory have been proposed. Many of these models are linked by certain key steps in the process; first, the cationic regions of the defensins mediate the initial interaction with negatively charged regions of the microbial membrane. After a certain threshold concentration of defensin molecules have attached to the membrane, the hydrophobic character of the amphipathic helix allows its insertion into the lipid bilayer of the membrane (Arnett and Seveau, 2011). It should be noted however that many defensins also have secondary non-membrane targets particularly when exhibiting anti-fungal activity (Antcheva *et al.*, 2013).

Throughout this study the “Gold Standard” reference peptide, to which all other peptides were compared, was Human Beta-Defensin-3. Human Beta-Defensin-3 (HBD3) has previously been shown to be highly effective at controlling microbial contamination within the brewing process when expressed *in situ* from *Saccharomyces pastorianus* (James *et al.*, 2014). Although this system offers many advantages over conventional preservation treatments it is believed that this solution may be unpalatable to the consumer due to public misconceptions surrounding the safety of human derived peptides used as food additives. It is for this reason that this project sought to discover plant derived defensins and defensin-like molecules with effective antimicrobial activity. The rationale behind this stems from the fact that the public have been accustomed to using plant extracts as dietary supplements for many generations (Franz *et al.*, 2011).

Discovery of candidate AMP’s presents many challenges. The heterogeneity at the primary sequence level renders genome mining virtually impossible (Machado and Ottolini, 2015). The use of non-uniform assays and strains from publication to publication makes it difficult to compare and predict effective candidates based on literature searches and database mining.

This study used a combined discovery approach. Initially, a shortlist of potential candidates was chosen based on literature and database mining. This shortlist was then filtered in a two stage process. First the primary sequence was

scanned for obviously unwanted motifs such as the, scorpion toxin related, “Knottin” fold (Harrison *et al.*, 2014). The remaining candidates were filtered by comparing the structures to that of the HBD3 “benchmark”. Where no structure existed for a potential candidate, one was modeled using a combined fragment homology - *Ab initio* approach. *Via*ble candidates were synthesised and tested for efficacy *In vitro* against the model food spoilage bacterium *L. brevis*. These candidates were also screened for thermostability which would be a highly desirable trait for use in high heat processes such baking.

Overall this chapter sought to answer the questions, “Can the structural characteristics of peptides be used to discover AMP’s effective at controlling bacterial growth based on comparisons to AMP’s of known effective activity?” and “If structure is not the most important determinant of antimicrobial activity, what is?”.

3.2 Results

3.2.1 Bioinformatic Discovery of Candidate AMP's based on Structural Similarity to Human Beta Defensin 3

Potential candidate AMP's from plants were mined from the PhytAMP database (http://phytamp.pfba-lab-tun.org/main.php), which contains information on 273 plant antimicrobials. The database was queried for defensin and defensin-like molecules that had been shown previously to have antimicrobial activity. This query returned 53 potential candidate peptides.

Peptide sequences for the candidate peptides identified were retrieved from the UniProt database (http://www.uniprot.org/), and any containing the "knottin" peptide motif and scorpion toxin-like motifs were rejected. This reduced the candidate list to 34 peptides.

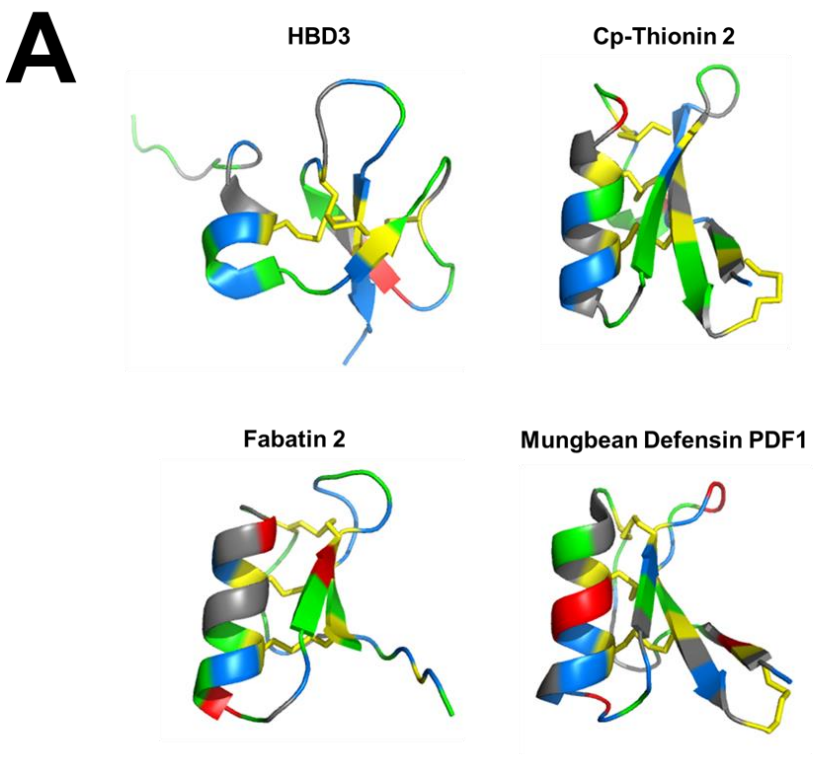
Where structures were available, they were retrieved from the Protein Data Bank server (http://www.rcsb.org/pdb/home/home.do). Candidate peptides with no experimentally determined structure were modelled using the protein structure prediction program Robetta (http://robetta.bakerlab.org/). All modelling was conducted using default protocols and parameters (Song *et al.*, 2013, Raman *et al.*, 2009). Template structures, against which modeling was conducted, were identified using the Ginzu PDB template identification and domain prediction protocol. Templates for modeling were chosen using an all-atom energy-based selection process. Structures were modeled using the Structure Prediction Tool (robetta.bakerlab.org; Domain Parsing & 3-D Modeling service), set to default parameters. The template and alignment most enriched in the lowest-energy population of all-atom refined models was then selected.

The candidate peptide structures were next quantitatively compared to the structure for the "Gold Standard" human beta defensin 3 (HBD3) structure. To allow for local structural differences between the molecules, a global alignment was performed between the structure of HBD3 and each of the candidate structures. This was carried out using the online TM-align tool (https://zhanglab.ccmb.med.umihttps://zhanglab.ccmb.med.umich.edu/TM-score/ch.edu/TM-score/). As described in the literature, a TM score above 0.3

was considered to sufficient to qualify two structures as having similar global folds (Xu and Zhang, 2010, Zhang and Skolnick, 2004). This analysis returned 3 candidate defensin-like peptides; Cp-Thionin-2 from *Vigna unguiculata*, Fabatin-2 from *Vicia faba* and the Mungbean defensin PDF-1 from *Vigna radiata* (Fig. 3.1(A)). An amino acid sequence alignment showed that there was no sequence homology between the candidate peptides and HBD3 (Fig. 3.1(B)). The primary amino acid sequences were used to synthesise the peptides which were used to carry out an *In vitro* analysis of the antimicrobial activity. Although these peptides had been previously reported, the novelty of this study was the fact that they were discovered based on tertiary structural comparisons to a benchmark peptide rather than sequence homology (Franco *et al.*, Zhang and Lewis, Lin *et al.*).

Name	Source Organism	Rejected after Motif Analysis	TM-Align Score vs. HBD3	Taken Forward for *In Vitro* Analysis
Cp-thionin-2	*Vigna unguiculata* (Cowpea)		0.38	
Fabatin-2	*Vicia faba* (Broad bean)		0.35	
PDF1	*Vigna radiata* (Mungbean)		0.31	
Cp-thionin-1	*Vigna unguiculata* (Cowpea)		0.27	
Defensin J1-1	*Capsicum annuum* (Bell pepper)		0.25	
Cysteine-rich antifungal protein 3	*Brassica napus* (Rape)		0.25	
LCR75	*Arabidopsis thaliana* (Mouse-ear cress)		0.22	
Antifungal protein AX2	*Beta vulgaris* (Sugar beet)		0.21	
Fabatin-1	*Vicia faba* (Broad bean)		0.21	
Defensin Tm-AMP-D1.2	*Triticum monococcum* (Einkorn wheat)		0.21	
Cysteine-rich antifungal protein 2B	*Sinapis alba* (White mustard)		0.20	
Defensin Tk-AMP-D6	*Triticum kiharae* (Wheat)		0.20	
Defensin Tk-AMP-D1.1	*Triticum kiharae* (Wheat)		0.19	
Defensin Tk-AMP-D1	*Triticum kiharae* (Wheat)		0.19	
Defensin Tk-AMP-D6.1	*Triticum kiharae* (Wheat)		0.19	
LCR68	*Arabidopsis thaliana* (Mouse-ear cress)		0.18	
MtDef2	*Medicago truncatula* (Barrel medic)		0.18	
Defensin Tk-AMP-D3	*Triticum kiharae* (Wheat)		0.18	
Gamma-zeathionin-2	*Zea mays* (Maize)		0.18	
Defensin Tk-AMP-D4	*Triticum kiharae* (Wheat)		0.18	
Antifungal protein AX1	*Beta vulgaris* (Sugar beet)		0.17	
Flower-specific gamma-thionin (Defensin SD2)	*Helianthus annuus* (Sunflower)		0.17	
LCR72	*Arabidopsis thaliana* (Mouse-ear cress)		0.16	
Defensin Tk-AMP-D5	*Triticum kiharae* (Wheat)		0.16	
At-AFP1	*Arabidopsis thaliana* (Mouse-ear cress)		0.15	
Defensin J1-2	*Capsicum annuum* (Bell pepper)		0.15	
Rs-AFP 4 (AFP4)	*Raphanus sativus* (Radish)		0.13	
LCR71	*Arabidopsis thaliana* (Mouse-ear cress)		0.13	
Rs-AFP At2g26010	*Arabidopsis thaliana* (Mouse-ear cress)		0.11	
MsDef1 (defensin 1.3)	*Medicago sativa* (Alfalfa)		0.11	
Gamma-thionin1	*Eutrema wasabi* (Wasabi plant)		0.11	
LCR73	*Arabidopsis thaliana* (Mouse-ear cress)		0.10	
Defensin Tk-AMP-D2	*Triticum kiharae* (Wheat)		0.10	
Gamma-zeathionin-1	*Zea mays* (Maize)		0.10	
Rs-AFP1	*Raphanus sativus* (Radish)		NA	
Ah-AMP1	*Aesculus hippocastanum* (Horse chestnut)		NA	
Defense-related peptide 1	*Pisum sativum* (Garden pea)		NA	
Defense-related peptide 2	*Pisum sativum* (Garden pea)		NA	
Floral defensin-like protein 1	*Petunia hybrida* (Petunia)		NA	
Floral defensin-like protein 2	*Petunia hybrida* (Petunia)		NA	
Flower-specific gamma-thionin (NaD1)	*Nicotiana tabacum* (Common tobacco)		NA	
Gamma-1-purothionin	*Triticum aestivum* (Wheat)		NA	
Gamma-2-purothionin	*Triticum aestivum* (Wheat)		NA	
Gamma-hordothionin	*Hordeum vulgare* (Barley)		NA	
Gamma-thionin homolog PPT	*Petunia integrifolia* (Violet petunia)		NA	
LCR66	*Arabidopsis thaliana* (Mouse-ear cress)		NA	
LCR69	*Arabidopsis thaliana* (Mouse-ear cress)		NA	
LCR70	*Arabidopsis thaliana* (Mouse-ear cress)		NA	
LCR74	*Arabidopsis thaliana* (Mouse-ear cress)		NA	
LCR76	*Arabidopsis thaliana* (Mouse-ear cress)		NA	
LCR77	*Arabidopsis thaliana* (Mouse-ear cress)		NA	
LCR78	*Arabidopsis thaliana* (Mouse-ear cress)		NA	
NP-THN1	*Nicotiana paniculata*		NA	

Table 3.1 Bioinformatic Workflow for Discovery of Defensin-like Plant Peptides Structurally Similar to HBD3



B

HBD3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK
Cp-Thionin-2	KTCMTKKEGWGRCLIDTTCAHSCRKYGYMGGKCQGITRRCYCLLNC
Fabatin-2	LLGRCKVKSNRFNGPCLTDTHCSTVCRGEGYKGGDCHGLRRRCMCLC
Mungbean Defensin PDF1	KTCENLANTYRGPCFTTGSCDDHCKNKEHLRSGRCRDDFRCWCTRNC

Fig. 3.1 Candidate defensin-like peptides. (A) Tertiary structures of the candidate defensin peptides. The structures for the candidate antimicrobial peptides were modelled using the homology modelling followed by structural refinement using the online molecular modelling tool Robetta. The structures were visualised using the PyMol molecular visualisation suite. The peptides were coloured as follows; Red represented negatively charged residues, positively charged residues were coloured blue and hydrophobic amino acids were coloured green. Disulfide bridges were displayed as yellow sticks. **(B)** Alignments of the amino acid sequences of the candidate defensin peptides.

3.2.2 *In-Vitro* Analysis of Antimicrobial Activity of Synthetic Peptide Candidates

3.2.2.1 Effect of salt on *In vitro* Antimicrobial Activity at pH7

To assess whether the structural based discovery process had succeeded, the synthesised defensin-like peptides were used to carry out an *In vitro* analysis of their antimicrobial activity. To achieve this, the synthetic peptides were used to challenge the beer spoiling bacterium *Lactobacillus brevis* and the data were compared to the efficiency of human beta defensin 3 (HBD3) at reducing bacterial survival. It had been shown on many occasions previously that the presence of ionic chemical species within the assay environment reduces the effectiveness of many AMP's by interfering with the initial interaction between the peptides and the bacterial cell surface (Maisetta *et al.*, 2008, Lee *et al.*, 1997, Wu *et al.*, 1999, Wang *et al.*, 2009). To determine if the presence of ions was having an effect on the antimicrobial activity of these peptides, the assay was carried out in a lower ionic strength.

Briefly, *L. brevis* at a final cell density of $2x10^3$ cfu/ml were challenged with varying concentrations of the synthetic peptides in either phosphate buffered saline, or the lower osmotic strength phosphate buffer which lacked the 137mM NaCl component, for 90 min at 30°C. Samples were plated onto De Man, Rogosa and Sharpe (MRS) agar containing 0.1% v/v Tween-80 (MRST) to allow enumeration of bacterial levels. Bacterial survival was represented as % survival in comparison to non-peptide treated control samples.

It was found that, under the higher osmotic strength assay conditions, HBD3 had a inhibitory concentration which killed 90% of bacteria (IC_{90}) of 0.16 µM (Fig. 3.2). Synthetic Cp-Thionin and Fabatin were found to have IC_{90} values of 0.92 µM and 1.2 µM respectively. It was also observed that the Mungbean defensin PDF-1 had no antimicrobial activity at any tested concentration under these conditions.

When the assay was conducted under the lower osmotic strength conditions the analysis showed that HBD3 had an IC_{90} value of 0.095 µM; a significant increase in antimicrobial activity in comparison to the higher ionic strength

buffer. The synthetic Cp-Thionin and Fabatin peptides displayed IC_{90} concentrations of 0.18 µM and 0.27 µM respectively; these were also significant increases in antimicrobial activity. Interestingly, the synthetic Mungbean defensin now displayed antimicrobial activity, albeit at a much lower level than the other peptides, with an IC_{90} concentration of 0.415 µM.

These data suggest that, due to the structural similarity between the candidate defensin-like peptides, structure was not the most important determinant of antimicrobial activity as only two of the candidate peptides produced an antimicrobial response under these assay conditions.

When the assays under high and low salt conditions were taken together, these data suggested the initial interaction between the AMP's and the bacterial cell surface was indeed sensitive to ions in the assay environment.

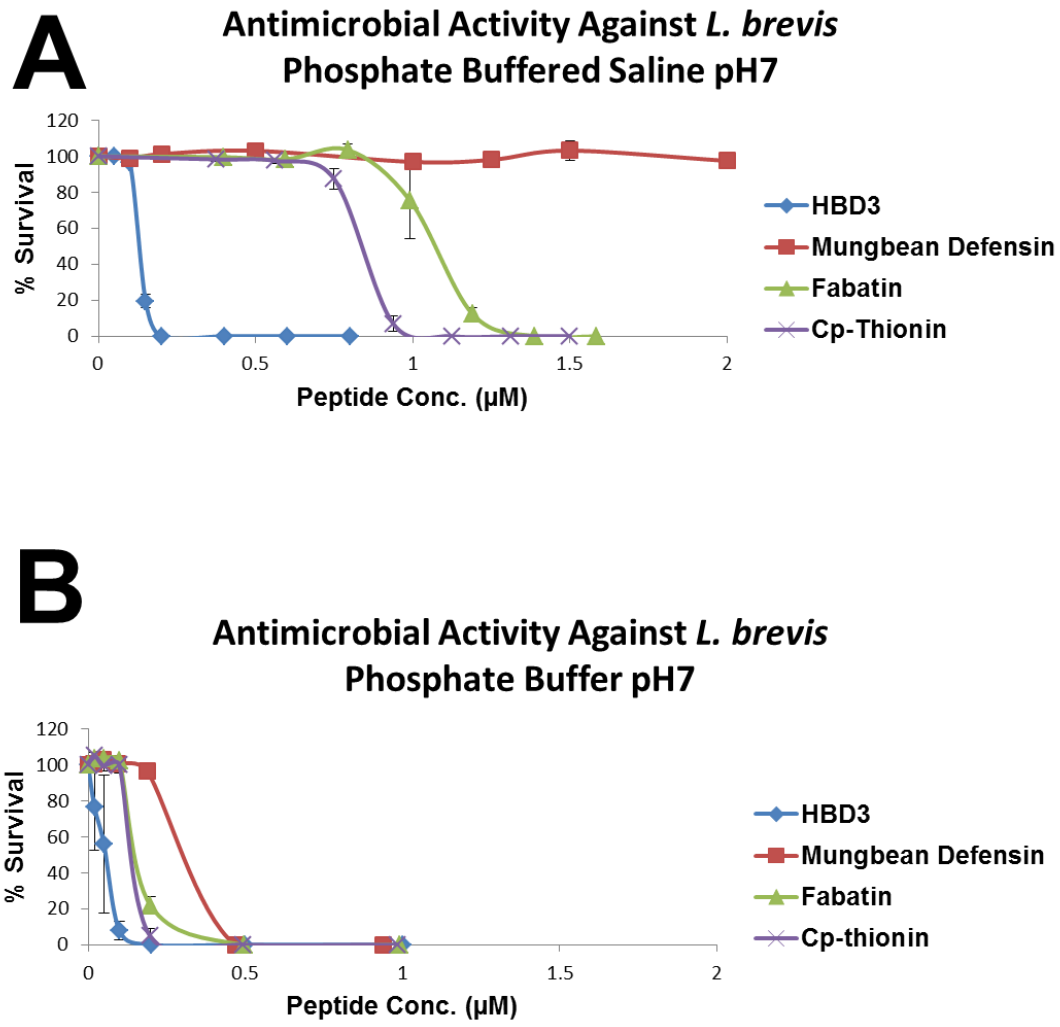


Fig. 3.2 *In vitro* Antimicrobial Activity of Candidate Defensin-like Peptides. (A) Antimicrobial activity in phosphate buffered saline at pH7 **(B)** Antimicrobial activity in phosphate buffer at pH7. *L. brevis* at a final cell density of $2x10^3$ cfu/ml were challenged for 90 min at 30°C with varying concentrations of the synthetic candidate defensin-like peptides in phosphate buffered saline and phosphate buffer. Samples were plated onto MRST agar to allow enumeration of bacteria. Data are represented as % survival in comparison to non-peptide treated controls. Assay was conducted on two independent occasions with 3 technical replicates each. Error bars are displayed as the standard deviation of the two biological replicates.

3.2.2.2 Effect of pH on the *In vitro* Antimicrobial Activity of the Candidate Peptides

The initial interaction between the AMP's and the bacterial cell surface is believed to be mediated by the net cationic charge of the peptide binding to the charged head groups of the cell membrane lipids of the bacterial cell. The charges of the peptides tested so far, at neutral pH, were predicted to be 11.6, 7.5, 6.7 and 3.7 for HBD3, Cp-Thionin, Fabatin and Mungbean defensin respectively. When the peptide antimicrobial activities described in the previous sections were ordered from most effective to least, a clear positive correlation could be seen between the net cationic charge and antimicrobial activity.

It was decided to further test this relationship between peptide cationic charge and antimicrobial activity. To achieve this it was decided to determine the effect of the pH of the assay environment on antimicrobial activity. This was decided as it has been demonstrated in the past that cationic proteins increase their positive character in low pH environments and decrease it in high pH environments. This was also predicted to be the case with these AMP's (Table 3.2).

Peptide	Peptide Net Charge		
	pH4	pH7	pH8
HBD3	13.4	11.6	9.9
Cp-Thionin 2	11	7.5	5.1
Fabatin 2	10.9	6.7	4.2
Mungbean Defensin	9.9	3.7	1.1

Table 3.2 Prediction of the Effect of pH on Net Charge

The *In vitro* antimicrobial assay was carried out as described in previous sections, in both high ionic strength phosphate buffered saline and low ionic strength phosphate buffer, at pH4 and pH8.

It was seen that at pH4, in the higher ionic strength buffer, HBD3 had an IC_{90} of 0.125 µM (Fig. 3.3(A)). Under the same assay conditions, synthetic Cp-Thionin and Fabatin were observed to have IC_{90} values of 0.48 µM and 0.78 µM respectively. Again it was shown that, in the higher ionic strength phosphate buffered saline, the Mungbean defensin showed no antimicrobial activity against *L. brevis* at the concentrations tested.

It was shown that at the same pH, in the lower ionic strength phosphate buffer, HBD3 was observed to have an IC_{90} of 0.047 µM (Fig. 3.3(B)). The Cp-Thionin and Fabatin peptides were observed to have IC_{90} values of 0.065 µM and 0.087 µM respectively while the Mungbean defensin peptide had an IC_{90} of 0.112 µM.

Taken together, these data suggested that decreasing the pH of the assay environment increased the antimicrobial activity of the peptides beyond that of their respective levels at neutral pH but the Mungbean data suggests that increasing the net charge of the peptide was not sufficient to overcome the competing ions in the assay environment under these experimental conditions.

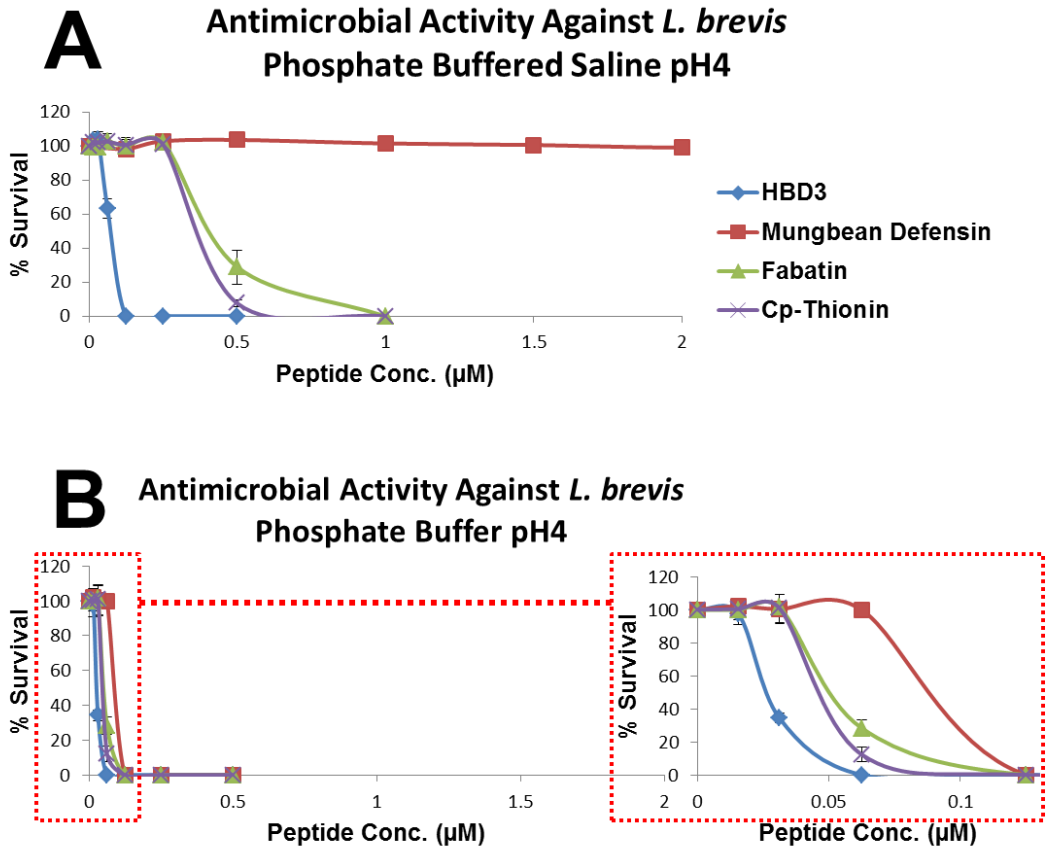


Fig. 3.3 *In vitro* Antimicrobial Activity of Candidate Defensin-like Peptides at pH4. *L. brevis* at a final cell density of $2x10^3$ cfu/ml were challenged for 90 min at 30°C with varying concentrations of the synthetic candidate defensin-like peptides in **(A) Phosphate buffered saline** or **(B) Phosphate Buffer** at pH4. An expanded view can be seen in the inset. Samples were plated onto MRST agar to allow enumeration of bacteria. Data are represented as % survival in comparison to non-peptide treated controls. Assay was conducted on two independent occasions with 3 technical replicates each. Error bars are displayed as the standard deviation of the two biological replicates.

When the same assay was conducted at pH8, using higher ionic strength phosphate buffered saline, HBD3 was found to have an IC_{90} concentration of 0.32 µM (Fig. 3.4(A)). It was found that Cp-Thionin and Fabatin had IC_{90} values of 1.815 µM and 1.832 µM respectively. Again, under the higher ionic strength condition, it was seen that the Mungbean defensin produced no antimicrobial activity.

When lower ionic strength phosphate buffer was used to conduct the assay at pH8, the IC_{90} of HBD3 was found to be 0.125 µM while Cp-Thionin and Fabatin were observed to have values of 0.4 µM and 0.448 µM respectively (Fig. 3.4(B)). The Mungbean defensin was seen to have an IC_{90} of 0.560 µM.

Taken together with the data from the lower pH assays it can be concluded that, by reducing the pH of the assay environment, the effectiveness of the AMP's at reducing the survival of *L. brevis* can be increased. Again this indicates that the increase in net cationic charge of the peptides in the lower pH environment greatly influences the antimicrobial activity but that the ionic strength of the medium has significant effect on this activity.

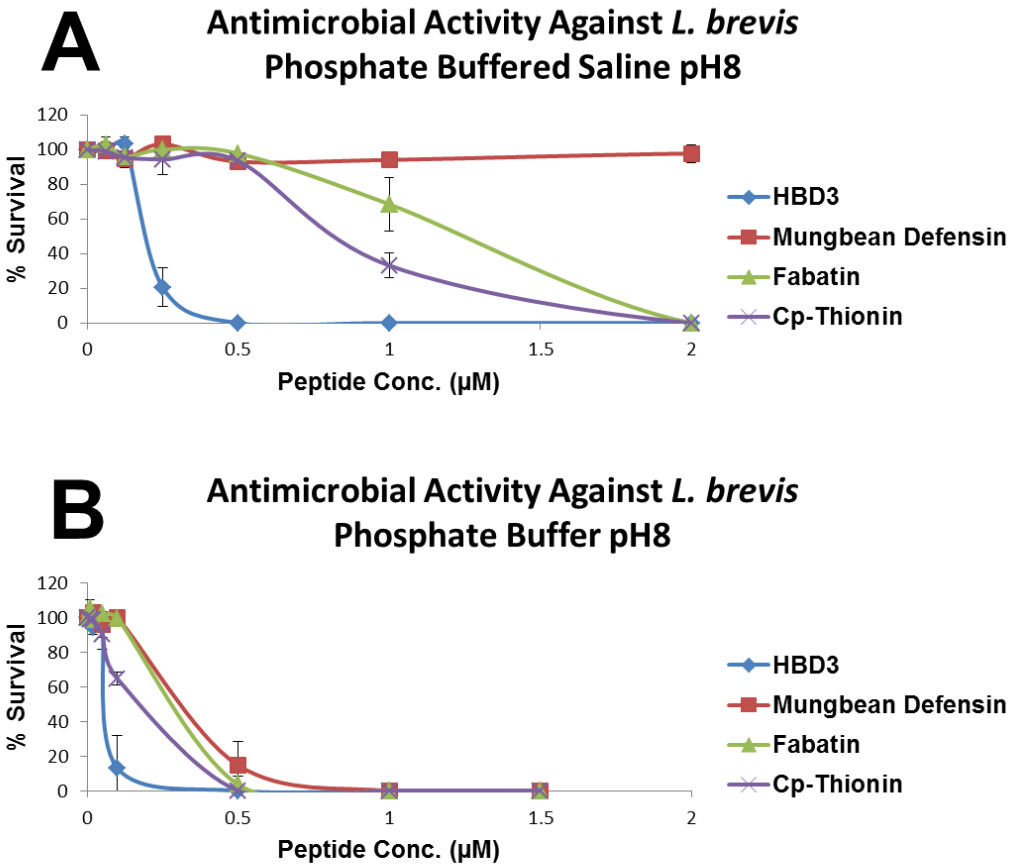


Fig. 3.4 *In vitro* Antimicrobial Activity of Candidate Defensin-like Peptides at pH8. *L. brevis* at a final cell density of $2x10^3$ cfu/ml were challenged for 90 min at 30°C with varying concentrations of the synthetic candidate defensin-like peptides in **(A) Phosphate buffered saline** or **(B) Phosphate Buffer** at pH8. Samples were plated onto MRST agar to allow enumeration of bacteria. Data are represented as % survival in comparison to non-peptide treated controls. Assay was conducted on two independent occasions with 3 technical replicates each. Error bars are displayed as the standard deviation of the two biological replicates.

3.2.2.3 Determination of the Importance of Disulfide Bonds for *In vitro* Antimicrobial Activity

Although the structures of Mungbean defensin, Cp-Thionin and Fabatin were all quite similar, the lack of antimicrobial activity for the Mungbean defensin suggested that the tertiary structure of the peptides was not the most important determinant of antimicrobial activity. It was decided to investigate if tertiary structure had any effect on antimicrobial activity. This decision was taken because of the evolutionary conserved nature of the tertiary structure despite the sequence diversity; this suggested that structure must play some role in determining antimicrobial activity. It was reasoned that the conserved disulfide bridge positions played an important role in holding the tertiary structure in place. Accordingly, it was decided that perturbations to the native structures of the peptides would most easily be achieved by breaking the disulfide bridges using the reducing agent dithiothreitol (DTT).

Briefly, *L. brevis* at a final cell density of $2x10^3$ cfu/ml were challenged with the concentration of each peptide which had previously been determined to be the IC_{50} in phosphate buffer at pH7. Two sets of peptides were used to conduct the assay. The first set included 5 mM DTT while the second set did not. The assays were carried out for 90 min at 30°C in phosphate buffer at pH7, after which samples were plated onto MRST agar. Results were calculated based on non-peptide treated control samples. Data were reported as % of retained activity based on non-DTT treated samples. It should be noted here that the inclusion of DTT in the assay did not affect the survival of the bacteria (Data not shown).

It was seen that the Mungbean defensin was the most sensitive to reduction of the disulfide bonds, retaining just 7.5% of the activity of the non-DTT treated sample (Fig. 3.5). The other peptides retained between 35.1% and 36.8% of their original antimicrobial activities. These data suggested that, although not the most important determinant of antimicrobial activity, tertiary structure was an important factor for effective antimicrobial activity against *L. brevis*. It can also be concluded that this effect is most likely not directly related to the disulfide bonds themselves as Cp-Thionin and the Mungbean both have 4 disulfide

bonds while the others have 3, yet for Cp-Thionin the retained activity was not significantly different from that of HBD3 or Fabatin.

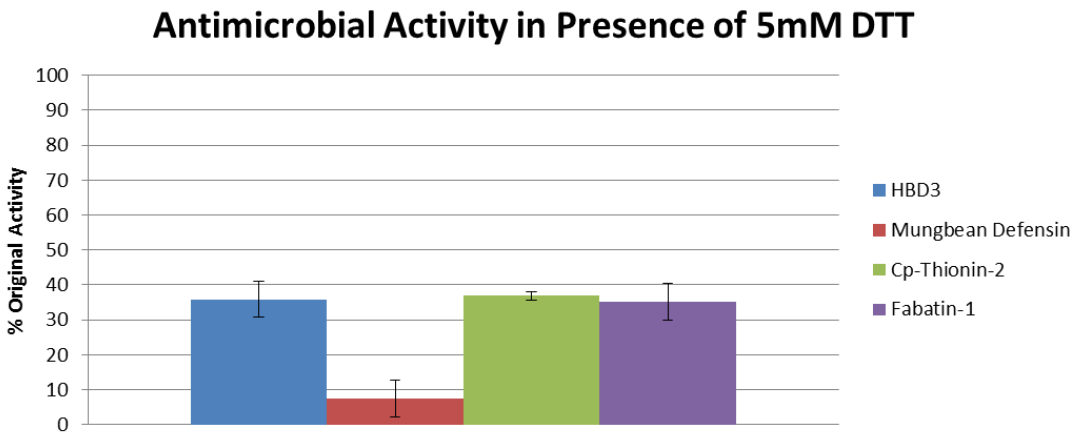


Fig. 3.5 *In vitro* Determination of the Importance of Disulfide Bridges for Antimicrobial Activity. *L. brevis* final cell density of $2x10^3$ cfu/ml were challenged with peptides at the previously established MIC_{50} concentration in phosphate buffer at pH7. This challenge was conducted for 90 min at 30°C with and without the reducing agent DTT. Following the challenge samples were plated onto MRST agar to allow enumeration of bacterial numbers. Results were calculated based on a non-peptide treated control. Data are represented as % of retained activity based on non-DTT treated control samples. Assays were conducted on two independent occasions with 3 technical replicates each. Error bars are reported as the standard deviation of the 2 biological replicates.

3.2.2.4 Determination of the Thermostability of *In vitro* Antimicrobial Activity

An important part of food production and preservation processes is the use of thermal treatments. Therefore, for use as a food preservative, the antimicrobial activity of the candidate peptides must be thermotolerant. To examine if this was the case, the antimicrobial activity of the peptides was tested after a thermal pre-treatment.

To accomplish this, *L. brevis* at a final cell density of $2x10^3$ cfu/ml were challenged with the AMP's at a final concentration which had previously been determined to be the IC_{50} concentration in phosphate buffer at pH7. Two sets of peptide were used to challenge the bacteria. The first set was composed of peptides which had been thermally pre-treated at 90°C for 10 min after which they were cooled to 20°C over 5 min. The other set contained peptides which were not thermally pre-treated. The challenge was carried out for 90 min at 30°C in phosphate buffer at pH7, after which, the bacteria survival were enumerated on MRST agar plates. Results were calculated based on non-peptide treated control samples and data were reported as the % of retained activity based on the non-thermally treated samples.

It could be seen that the antimicrobial activity of HBD3 was the most thermolabile, retaining only 25.4% of the activity of the non-thermally treated sample (Fig. 3.6). The Mungbean defensin was found to be the most thermostable peptide, retaining 63.8% of the original activity. Cp-Thionin and Fabatin were found to have retained 58.7% and 60.1% respectively of their original antimicrobial activities.

These data, taken together with the antimicrobial activity data observed with phosphate buffer at pH7, would suggest that the most effective defensin-like peptide for use in food which undergoes thermal treatment would be Cp-Thionin. Although the Mungbean defensin was found to be the most thermostable peptide, it was used at a significantly higher concentration in this assay than the other AMP's.

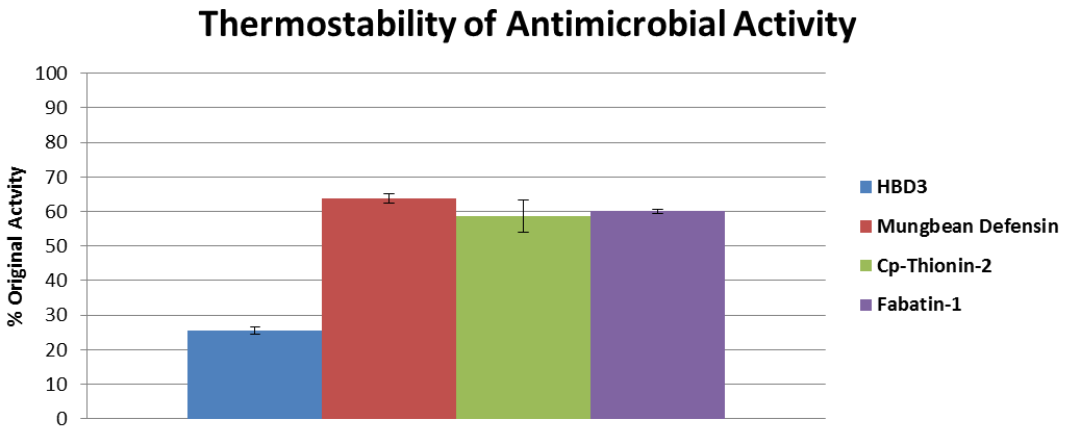


Fig. 3.6 Determination of the Thermostability of Antimicrobial Activity. *L. brevis* final cell density of $2x10^3$ cfu/ml were challenged with antimicrobial peptide at the previously established MIC_{50} concentration in phosphate buffer at pH7. For the duration of the challenge one set of samples contained peptide which had been thermally treated at 90°C for 10 min before being cooled to 20°C over 5 min. The other set of samples challenged the bacteria with non-thermally pre-treated peptides. Following the challenge , samples were plated onto MRST agar to allow enumeration of the bacteria. Results were calculated based on non-peptide treated samples. Data are reported as % of original activity based on the non-thermally treated samples. Assays were conducted on two independent occasions with 3 technical replicates each. Error bars are reported as the standard deviation of the two biological replicates.

3.3 Physio-chemical Characteristics of lead AMP's

A summary of the results of the antimicrobial assays against *L. brevis* (Table 3.3) yields some interesting correlations. The peptides were tested in either phosphate buffered saline (PBS) containing 137 mM NaCl or phosphate buffer (PB), which did not contain NaCl, at pH4, pH7 and pH8. All of the peptides were predicted to have increased net cationic charge at lower pH conditions compared to higher pH conditions.

Taking HBD3 as an example, when the peptide was tested for antimicrobial activity, the greatest activity was always observed at the lower pH compared to the higher pH. This correlation held true for both the high and low NaCl environments of PBS and PB assays respectively. The second correlation that could be clearly observed was the significant increase in IC_{90} in the higher NaCl PBS buffer compared to the low ionic strength PB buffer. Together these two correlations suggest that activity is indeed linked to ionic interactions between the antimicrobial peptide and the bacterial cell envelope, and that, this interaction may be perturbed by competing ions in the assay environment. This was particularly evident for the Mungbean defensin, which, under high ionic strength conditions, had no detectible antimicrobial activity. This peptide, when assayed in low ionic strength conditions, had antimicrobial activity which also showed an inverse relationship between pH and antimicrobial activity. When ranked from highest to lowest antimicrobial activity, it could be clearly seen that one of the most important determinants of antimicrobial activity was net cationic charge and that the presence of ions in the environment interfered with this activity. Further to this, the net positive charge of HBD3 at pH8 and Mungbean defensin at pH4 are both predicted to be +9.9. The IC_{90}, under these conditions, for HBD3 and Mungbean defensin were 0.125 µM and 0.112 µM respectively.

This again demonstrated that increasing positive charge could increase antimicrobial activity. As can also be seen from the summary of these data, there was no correlation observed between antimicrobial activity and either the number of cysteine residues within the molecules or their average hydropathy.

Taken together, these data suggest that a major determinant of antimicrobial activity, for these peptides, is net positive charge. These data also suggest that the ionic interactions necessary for effective antimicrobial activity can be negatively affected by the presence of free ions in the surrounding assay environments.

	pH	4.0	7.0	8.0	Number of Cysteines	Average Hydropathy
Human Beta Defensin 3	Net Charge	13.4	11.6	9.9	6	-0.7
	MIC_{90} PBS (μM)	0.125	0.160	0.320		
	MIC_{90} PB (μM)	0.047	0.095	0.125		
Cp-Thionin-2	Net Charge	11	7.5	5.1	8	-0.439
	MIC_{90} PBS (μM)	0.480	0.920	1.815		
	MIC_{90} PB (μM)	0.065	0.180	0.400		
Fabatin-1	Net Charge	10.9	6.7	4.2	8	-0.423
	MIC_{90} PBS (μM)	0.780	1.200	1.832		
	MIC_{90} PB (μM)	0.087	0.270	0.448		
Mungbean Defensin	Net Charge	9.9	3.7	1.1	8	-1.174
	MIC_{90} PBS (μM)	NA	NA	NA		
	MIC_{90} PB (μM)	0.112	0.415	0.560		

Table 3.3 Physiochemical Characteristics of Lead Antimicrobial Peptides

3.4 Discussion

The identification and characterization of new AMP's offers many novel avenues for the control of food borne pathogens and spoilage organisms. The development of novel therapeutics such as these will provide a *via*ble alternative to conventional antibiotics, the use of which has become cost prohibitive and unethical in the face of growing antimicrobial resistance (Casewell *et al.*, 2003, Anon, 2019). Previous work conducted by this lab had shown that human beta-defensin-3 (HBD3) to be an effective treatment measure for the control of food spoilage organisms such as the lactic acid bacterium *Lactobacillus brevis* (James *et al.*, 2014). It was felt however that the use of human derived food additives would be unpalatable to the consumer.

In an effort to alle*via*te some of these concerns, this study sought to identify plant based peptides which could provide a similar level of antimicrobial activity. It was felt that this approach would provide a more attractive alternative because the public have long accepted plant based additives in food products (Karunaratne and Pamunuwa, 2017). The proven track record of HBD3 meant that it was an excellent benchmark for use in the discovery and screening process.

Identification of novel defensin like peptides, with antimicrobial activity, is fraught with obstacles. Although much work had been done on plant based AMP's, the lack of standardized comparative analyses between different publications meant that identification could not be based on previously reported activity alone.

Defensin like peptides form part of the innate immune system of all higher eukaryotes (Lehrer and Lu, 2012). Due to the lengthy evolution of these molecules the primary amino acid sequence is quite divergent; a trait which hampers the discovery of active peptides using a sequence mining approach (Machado and Ottolini, 2015). Despite their divergent nature at the sequence level, defensin like peptides are highly conserved at the structural level (Antcheva *et al.*, 2013). This characteristic presented a unique method to identify active peptides by comparing their structures to that of a proven

benchmark such as HBD3. The use of this scheme for the identification of AMPs was novel.

The work discussed in this chapter sought to answer a number of important questions surrounding the discovery of plant based defensin-like peptides with effective antimicrobial activity against food spoiling bacteria. The first question which was asked was, “Can effective plant derived defensin-like peptides be discovered solely by comparing their tertiary structures to those of AMP’s of known efficacious activity?” The second question which was asked was “If the tertiary structure of a defensin-like peptide is not the most important determinant of antimicrobial activity, what is?” This work also sought to determine if any antimicrobial activity observed with any discovered peptides would be stable under industrially relevant conditions.

The initial stage of the bioinformatic discovery pipeline mined a plant specific antimicrobial database to create a list of defensin-like AMP’s. This process returned 53 potential defensin-like candidates (Table 3.1). A number of AMP families such as the “Knottins”, “Snakins” and “Scorpion-like toxins” were known to be cytotoxic to mammalian cells (Harrison *et al.*, 2014). Due to this, it was decided to remove any candidates which were structurally similar to these unwanted motifs. Although these molecules themselves were never proven to be cytotoxic it was felt, at the time, that the decision to remove them at the beginning of the process was a prudent one given that public perception was an important consideration throughout this study. This filtering process reduced the candidate peptide list to 34 molecules.

The final stage of the discovery process was to create a list of structural alignment scores between the candidate peptide tertiary structures and the structure of HBD3. Although the classical defensin fold is highly conserved, there remain subtle local differences with regard to the placement of secondary structural elements in the final tertiary structure which hamper direct structural alignments. To circumvent this problem, it was decided to use a global scoring method which could account for these subtle variations and identify structures which were overall similar to the structure of HBD3. This was achieved through the use of the online TM-align webtool with a threshold score of 0.3; this was

the minimum score with which two structures were considered to be, in some way, structurally similar (Xu and Zhang, 2010, Zhang and Skolnick, 2004).

This analysis identified 3 defensin like peptides which were taken forward for *In vitro* analysis; Cp-Thionin-2, Fabatin-2 and the Mungbean defensin PDF-1 (Fig. 3.1). Only the former two of these peptides displayed antimicrobial activity in physiologically relevant conditions (Fig. 3.2(A)). This suggested that tertiary structure was not the most critical determinant of antimicrobial activity. It should be noted however that this conclusion was based on structures which were modelled *in silico* and may not be representative of the true structures of the peptides. Based on the available models, analysis did however show that tertiary structure was an important factor for effective antimicrobial activity against *L. brevis* (Fig. 3.5). However it must be noted that there have previously been reports that reduction of disulfide bonds using DTT treatment can in some cases cause peptides to become susceptible to degradation(Marcos, 2019, pers. comm.). Further analysis would be required to determine if this was the case for these peptides.

The observation that, for all the peptides tested, antimicrobial activity increased when the assays were carried out under low osmotic conditions alluded to cationicity being the most important determinant of antimicrobial activity (Fig. 3.2(B)). To determine if this was the case, the antimicrobial assays were conducted in buffers which either increased or decreased the positive character of the peptides. It was found that in low pH conditions, which was predicted to increase the positive charge of the peptides, the antimicrobial activity was increased for all the tested peptides (Fig. 3.3(A,B)). The inverse was true in a higher pH assay environment (Fig. 3.4(A,B)). This suggested that net peptide charge was an important determinant for effective antimicrobial activity but that, based on the data from the Mungbean defensin, the increase in net charge was not sufficient to overcome competing ions in the assay environment. However under any given condition, when the peptides were ranked by antimicrobial activity, a clear positive correlation was observed between this activity and the net positive charge of the peptides.

Many industrial processes surrounding the production and preservation of food involve thermal treatments (Bressani, 2003). Thus it was necessary to

determine if the antimicrobial activity of the defensin-like peptides would be compatible with these processes. It was found that the most thermostable peptide was the Mungbean defensin but due to the significantly higher concentration of peptide required to produce an antimicrobial effect and the salt sensitivity of its activity it was deemed that this peptide would not be suitable for industrial use (Fig. 3.6).

Instead, being both relatively thermostable and active at physiological relevant ionic conditions, Cp-Thionin was found to be the most suitable for large scale use.

In conclusion, it has been demonstrated that a bioinformatic discovery strategy based solely on tertiary peptide structure could not by applied to uncover effective plant based AMP's. Based on the available data, it was however demonstrated that correct folding of the tertiary structure was critical for effective antimicrobial activity. The peptides were all shown to be salt sensitive and the most important determinant of antimicrobial activity was observed to be the net positive charge of the peptide. Finally, it was demonstrated that the newly uncovered peptides were more thermostable that the Gold Standard HBD3 molecule and that Cp-Thionin was the most appropriate peptide for industrial use.

Chapter 4:

Rational Design of Antimicrobial Peptides with Increased Activity

4.1 Introduction

The overall aim of the research outlined in this chapter was to design defensin-like AMP's with increased antibacterial activity against the beer spoiling microorganism *Lactobacillus brevis*. This research sought to answer three questions. First, given the physiochemical characteristics of defensin peptides of known efficacious antimicrobial activity, can a peptide be rationally designed to have increased activity based on a lead peptide which showed no antimicrobial activity to begin with? Second, can the antimicrobial activity of a defensin peptide be increased by reducing the structural rigidity of the molecule, thus allowing the peptide to act in a more fluid, "induced fit", manner? Finally, can a small peptide be designed which maintains all of the physiochemical characteristics of the larger variants and which also maintains effective antimicrobial activity?

To rationally design AMP's in this manner, one must first have a basic understanding of the mode of action of such peptides. It has been well established that many AMP's, such as defensins, attack and permeabilise the membranes of the bacterial cell envelope leading to leakage of cell contents, and ultimately, cell death (Sudheendra *et al.*, 2015). Although the exact mechanism by which this occurs has not been proven as of yet, it is generally accepted that the initial interaction between the antimicrobial peptide and the bacterial cell envelope is mediated by the attractive forces between the positively charged amino acids of the peptide and the negatively charged lipid head groups of the bacterial membrane. It is then believed that the hydrophobic regions of the peptides allow for their insertion into the hydrophobic region amongst the lipid tails of the membrane (Domadia *et al.*, 2010). It should be noted at this point that many AMP's, particularly when exerting antifungal activity, attack non-membrane targets (Scocchi *et al.,* 2016). This study focused however on the membrane-based antibacterial activity.

In chapter 3 it was shown previously that a major determinant of antimicrobial activity against *L. brevis* is net cationic charge of the antimicrobial peptide (Kraszewska *et al.*, 2016). A clear positive correlation could be seen between increasing net charge and increasing antimicrobial activity (Table 3.3). This work identified two plant derived peptides, Fabatin-2 and Cp-Thionin-2, which

showed good antimicrobial activity against *L. brevis* and one peptide, Mungbean defensin, which showed poor activity under test conditions. Although, it should be noted, that these peptides all showed significantly lower antimicrobial activity than the “Gold Standard” Human Beta Defensin-3 peptide used throughout these studies.

It was decided to use the Mungbean defensin as the lead chassis upon which to make changes to produce analogue peptides. This decision was based on the fact that this lead peptide displayed no antimicrobial activity under physiologically relevant conditions, and so, any observed increase in activity by altering the primary amino acid sequence would not be masked by existing activity. Given the correlation already observed between charge and antimicrobial activity, it was decided that the first analogues to be designed would seek to increase the positive charge of the lead Mungbean defensin. Indeed, this approach has previously been shown to increase the antimicrobial activity of a*via*n β-defensin-8 (Higgs *et al.*, 2007). Two analogue peptides were designed based on this approach. The first analogue was designed to remove 3 negatively charged residues and replace them with 3 positively charged arginine residues; 3-Arg analogue. The second peptide was designed to replace 5 negatively charged residues with arginine residues; 5-Arg analogue. To investigate if structural fluidity of the peptide had any effect on antimicrobial activity it was decided to create three further analogue peptides. These analogues were based on the 5-Arg analogue described above. Two approaches were taken to investigate this aspect. In the first instance it was decided to alter the number of cysteine residues in the 5-Arg analogue peptide. This peptide originally contained 8 cysteine residues predicted to form 4 disulfide bridges. Two analogue peptides were designed using this approach. With the first peptide, all the cysteine residues were replaced with alanine residues; All Cysteine Knockout Analogue. With the second peptide, all but the two cysteine residues which hold the C and N termini together were replaced with alanine residues; Internal Cysteine Knockout Analogue.

The second approach to increase the fluidity of the peptide took into account an observation about the structure of the “Gold Standard” HBD3 defensin. The N-terminus of HBD3 contains a short alpha helix leading into a largely

unstructured terminal tail. In contrast to this, the alpha helix of the 5-Arg analogue peptide is predicted to be longer and well formed. It is also located more centrally with regard to primary amino acid sequence. In an attempt to produce a peptide which more structurally resembled HBD3, it was decided to modify the 5-Arg analogue peptide such that the helix was located at the N-terminus, this is analogous to the helix position in the HBD3 molecule. This was achieved by designing an amino acid sequence which has the original C and N terminal residues joined by a glycine-glycine linker, and, now had the alpha helix located at the site of the new N-terminus.

To answer the final question, whether one could produce a small but effective antimicrobial peptide, it was decided to again base these analogues on the 5-Arg analogue. Two peptide analogues were designed. The first peptide was designed such that the N-terminal strand of the beta sheet was deleted. The second peptide was designed to have both the N-terminal strand and the most C-terminal proximal strands of the beta sheet deleted.

In an effort to ensure that the overall tertiary structure of the designed analogues did not drastically change, it was decided to model the structures *in silico* using the molecular dynamics simulation software suite “GroMacs”. This suite would also provide important data about other characteristics such as peptide structural stability, as measured by the radius of gyration during 1ns simulations.

The analogue peptides were tested, *In vitro*, for antimicrobial activity in a range of physiologically relevant conditions against *L. brevis*. Previous work during this research had shown the antimicrobial activity to be dependent on peptide net charge and was sensitive to increasing concentrations of NaCl. The proposed hypothesis that the initial interaction between the peptide molecule and the bacterial cell envelope was mediated by ionic activity was in line with these data. Accordingly, it was decided to test peptide antimicrobial activity not only in high and low NaCl conditions, but also, in both high and low pH conditions. It is well known that basic proteins and peptides, such as the peptides discussed here, show an increase in their positive character in acidic environments and a decrease in their positive charge under alkaline conditions. If the proposed hypothesis holds true then, under acidic conditions and low NaCl conditions, the peptide antimicrobial activity should be maximal. The inverse should hold true for high pH / high NaCl conditions.

4.2 Results

4.2.1 Rational Design of Peptides

4.2.1.1 Molecular Modelling of Peptide Structure

4.2.1.1.1 Peptide Design and Structural Characteristics

As per the conclusion reached at the end of the preceding section, the most important determinant of antimicrobial activity was the degree of net cationic charge. Accordingly, the first set of peptides to be rationally designed sought to increase antimicrobial activity of the relatively inactive Mungbean defensin lead by increasing it positive charge.

Two peptides were designed to achieve this. The first peptide replaced three negatively charged amino acids (Glu4, Asp21 and Asp22) with Arginine residues to create the 3-Arg analogue peptide. This increased the positive charge of the Mungbean peptide from +3.7 at pH7 to +9.7 at pH7 for the 3-Arg analogue. The second derivative peptide replaced 5 negatively charged amino acids (Glu4, Asp21, Asp22, Asp37 and Asp38) with 5 Arginine residues to create the 5-Arg analogue peptide. This derivative further increased the positive character of the peptide to +13.7 at pH7, which interestingly is greater than the positive charge of the “Gold Standard” HBD3 defensin which is +11.6 at pH7.

It was also decided to investigate if creating analogue peptides with reduced structural rigidity would increase the antimicrobial activity of the peptides. Two approaches were taken to design these analogues.

The first approach was to remove disulfide bridges. Two analogue peptides were designed in this manner. The first of these peptides, All Cysteine Knockout Analogue, substituted all cysteine residues with alanine residues. The second peptide designed using this approach replaced all but the two cysteine residues holding the C and N termini together with alanine residues to create the Internal Cysteine Knockout Analogue.

The second approach was based on an observation made about the structure of the HBD3 peptide. It was noted that the N-terminus of HBD3 is composed of a short helix leading into a largely unstructured terminal tail (Fig. 3.1). It was decided to design a peptide which more closely resembled the tertiary structural

layout of HBD3. To achieve this, a sequence was designed, based on the 5-Arg analogue peptide, with a Glycine-Glycine linker was joining the old N and C-termini, while the new N-termini was designed to be at the N-terminal end of the alpha helix. This formed the N-terminal fluid analogue peptide.

It was further decided to attempt to design the smallest possible antimicrobial peptide analogue, based on the 5-Arg analogue, 2 further analogues were designed. The first of these peptides was designed to have the N-terminal proximal strand of the 3 stranded beta sheet deleted; this formed the 1 strand deletion analogue. The second peptide was designed to have both the N and C-terminal strands of the beta sheet deleted; this formed the 2 strand deletion analogue.

A sequence alignment showing the amino acid changes to the rationally designed analogue peptides can be seen in Fig. 4.1(A) and (B).

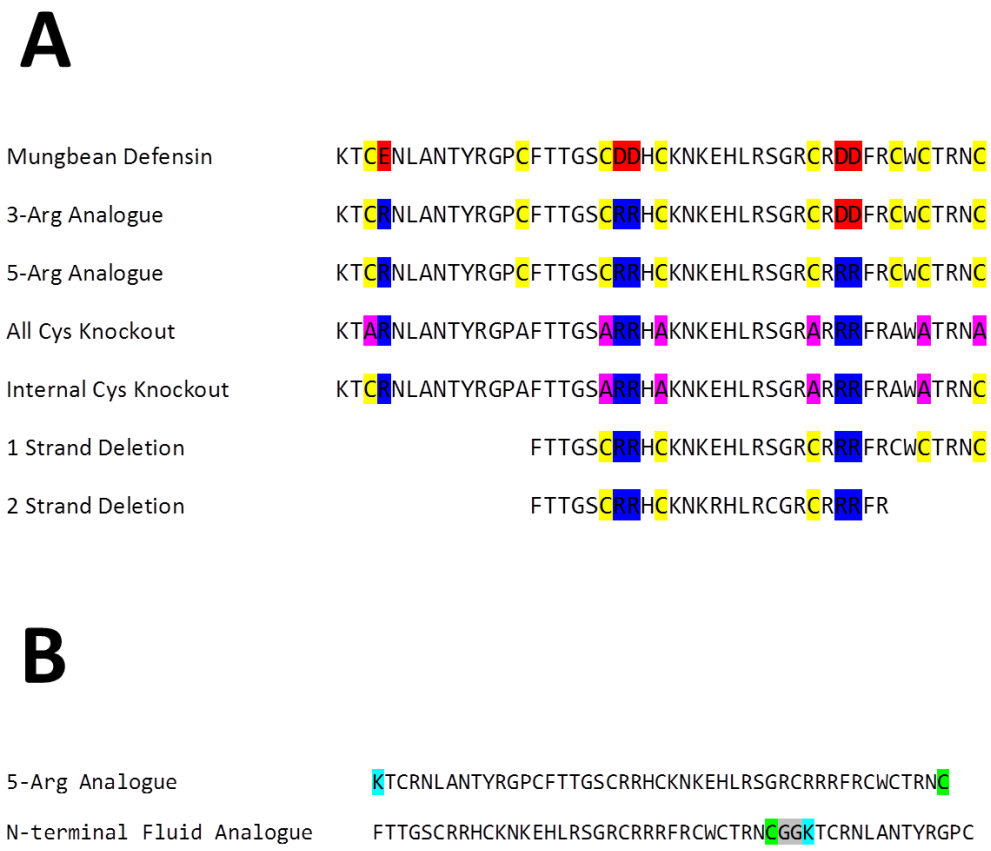


Fig. 4.1 Sequence Alignment of Rationally Designed Peptides. (A) Sequence Alignment of Rationally Designed Peptides Rationally designed peptides were based on the Mungbean Defensin lead sequence. Red indicates negatively charged residues. Blue indicates positively charged residues. Cysteine residues are highlighted in yellow. Alanine residues used to substitute for cysteines are highlighted in purple. **(B)** Sequence alignment of the N-terminal fluid analogue and the 5-Arg analogue peptide upon which its design was based. The original N- and C-termini are highlighted in light blue and green respectively. The glycine-glycine linker is highlighted in grey.

To ensure that any potential increase in antimicrobial activity observed was due to the increased cationicity of the peptide and not an alteration to the peptide tertiary structure it was decided to model the peptide structures to determine if there was any de*via*tion from the parental Mungbean defensin structure. To achieve this, the newly designed primary amino acid sequences were first modelled by homology modelling using the online tool “Phyre2”.

This analysis resulted in all analogue peptides being modelled on the Mungbean defensin peptide structure from the Protein Data Bank (PDB) database. These structures were next passed to the “GroMacs” molecular dynamics simulation suite for structural refinement and molecular modelling. After energy minimisation, the structures were modelled in a, charge balanced, water based environment over the course 1 ns with energy measurements and structural poses being recorded every 10 ps.

The results of these structural analyses indicate that the modifications made to create the analogue peptides did not alter the final tertiary structures of the 3-Arg and 5-Arg analogue peptides (Fig. 4.2) in comparison to the Mungbean defensin lead molecule. Indeed, aligning the structures of the lead Mungbean defensin each of the analogues using the “PyMol” molecular visualisation suite resulted in a RMSD between the structures of 0.074 and 0.091 for the 3-Arg and 5-Arg analogues respectively.

The results of the structural analyses for the cysteine knockout analogue peptides (Fig. 4.3) indicate that the modifications made to create the analogue peptides did not alter the final tertiary structures of the peptides in comparison to the 5-Arg analogue peptide based on the *in silico* model. Indeed, aligning the structures of the lead Mungbean defensin each of the analogues using the “PyMol” molecular visualisation suite resulted in a RMSD between the structures of 0.052 and 0.076 for the All Cysteine Knockout and Internal Cysteine Knockout analogues respectively.

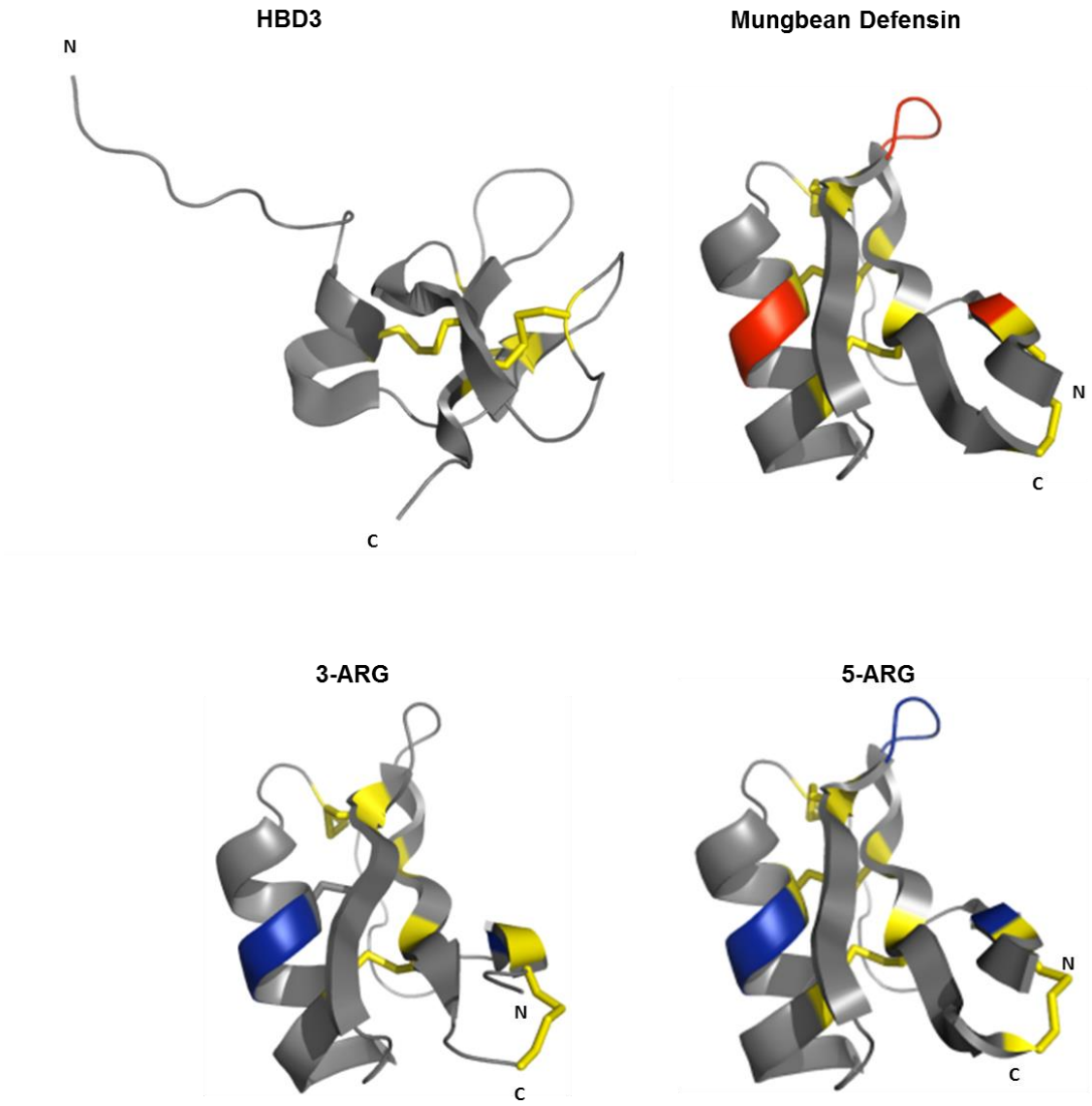


Fig. 4.2 Predicted Lead and Analogue Structures. Peptide structures were modelled using GroMacs package and visualised using PyMol. Positive and negative amino acids are coloured blue and red respectively. Disulfide bonds are displayed as yellow sticks. The N- and C-termini are also labelled.

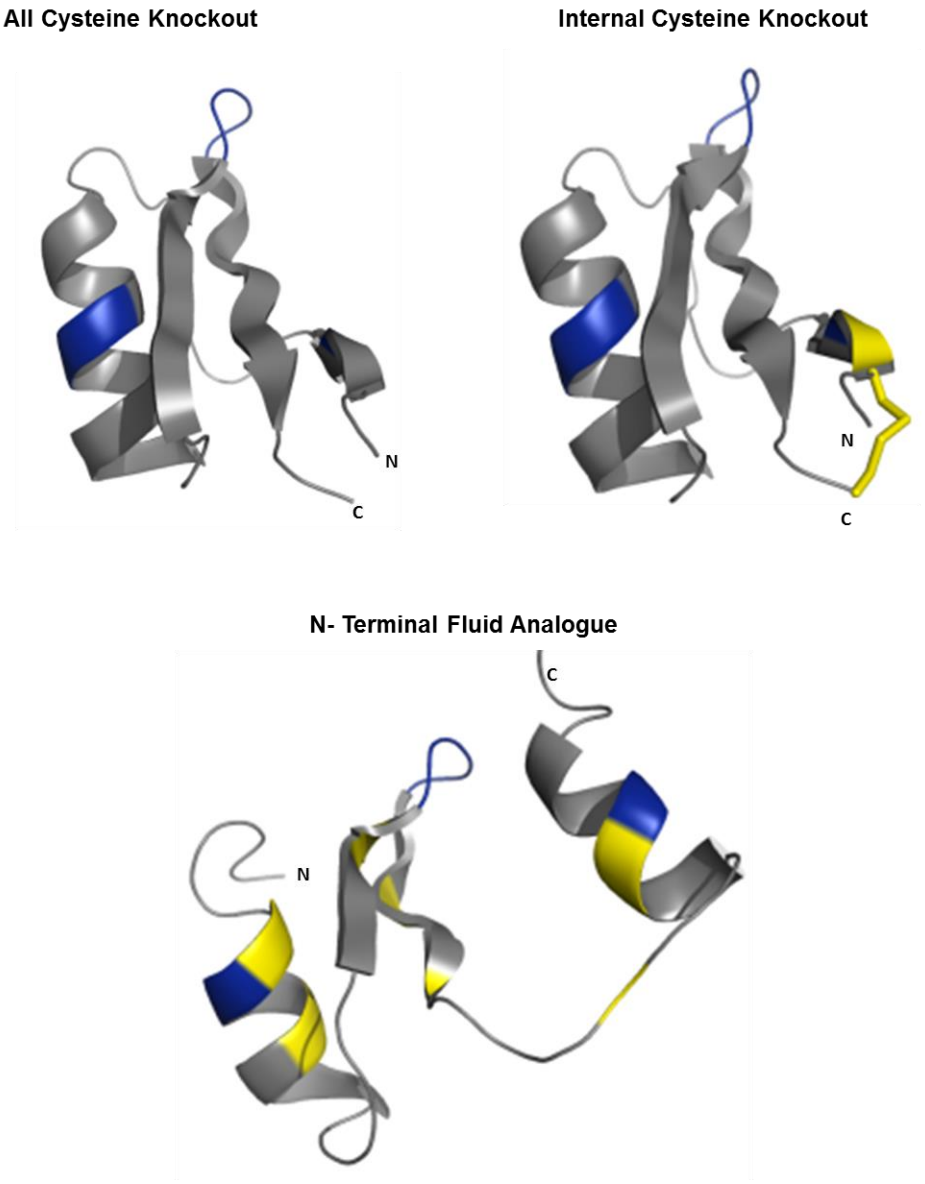


Fig. 4.3 Predicted Lead and Analogue Structures. Peptide structures were modelled using GroMacs package and visualised using PyMol. Positive amino acids are coloured blue. Disulfide bonds are displayed as yellow sticks. The N- and C-termini are also labelled.

The results of the structural analysis on the N-terminal fluid analogue showed significant de*via*tion from the tertiary structure of the 5-Arg analogue peptide (Fig. 4.3). Instead of having a single alpha helix at the N-terminus, the analogue peptide contained a helix at both termini with the C-terminal helix now being formed from what should have been the C-terminal proximal strand of the beta sheet. Furthermore, the model predicted that the disulfide bridges were no longer formed with this analogue peptide. A typical disulfide bridge has a bond length of 2 angstroms (Fig. 4.4). When the distances between the terminal atoms of the cysteine residues of the N-terminal fluid analogue were measured they were found to be between 3 angstroms and 13.8 angstroms, far longer than would be possible for a disulfide bridge to form (Fig. 4.5). These findings were unsurprising given the significant alterations made at the primary amino acid sequence level.

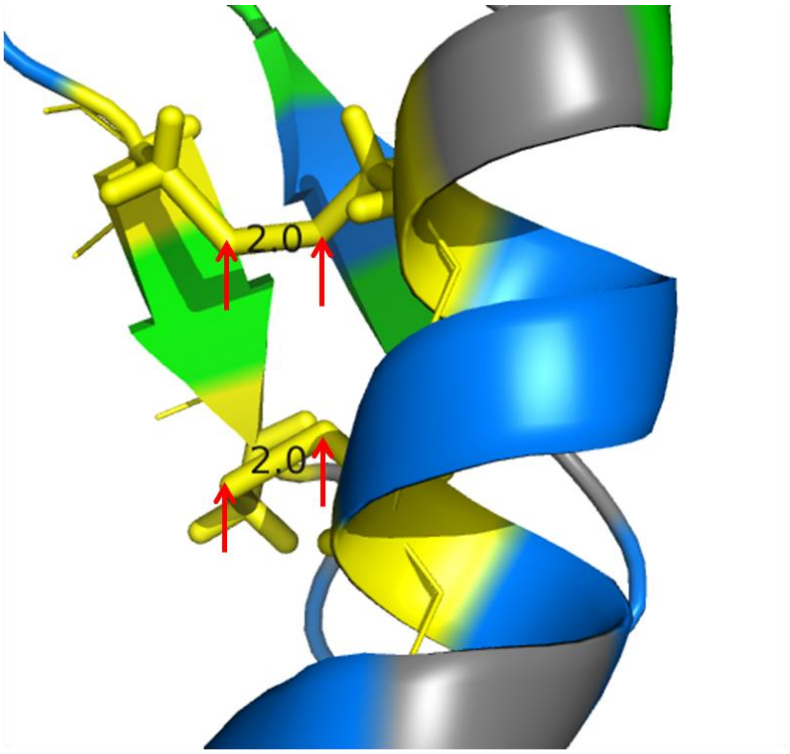


Fig. 4.4 Interatomic distances between cysteine residues in a typical disulfide bond. For a typical disulfide bond the spatial distances between thiol moieties of adjacent cysteine residues are known to be ~2 angstroms. Cysteine residues are coloured yellow. Cysteine side chains are displayed as sticks. The sulfur atoms in the S-S bond are indicated by red arrows. Measured distances are reported in angstroms. The distances were measured and the molecule was displayed using PyMol molecular visualisation suite.

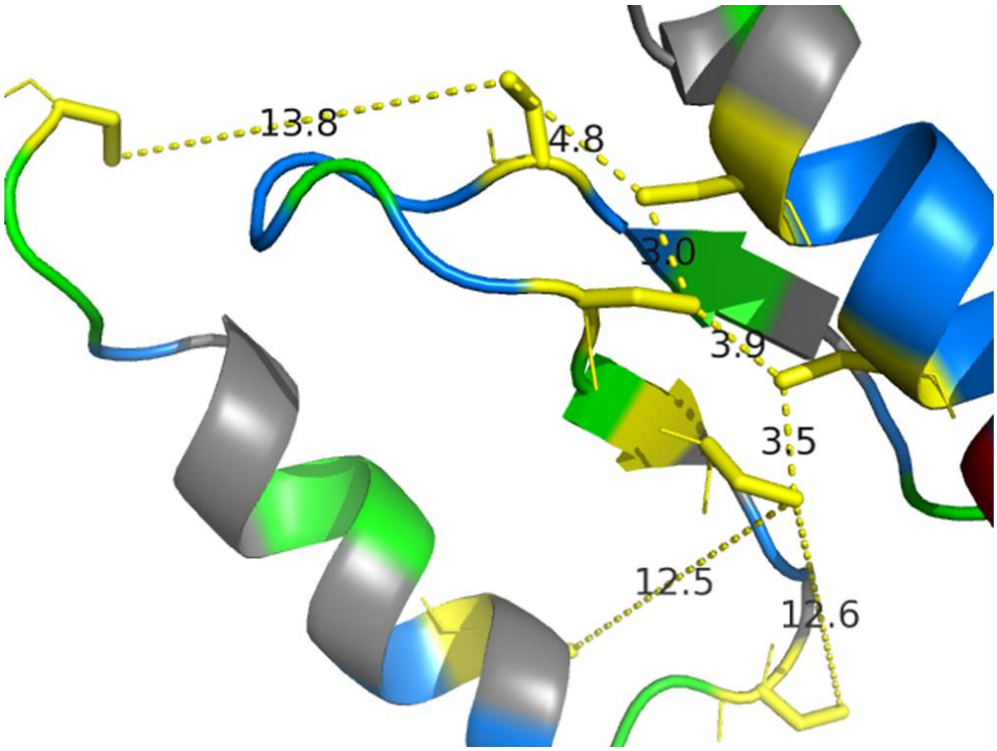
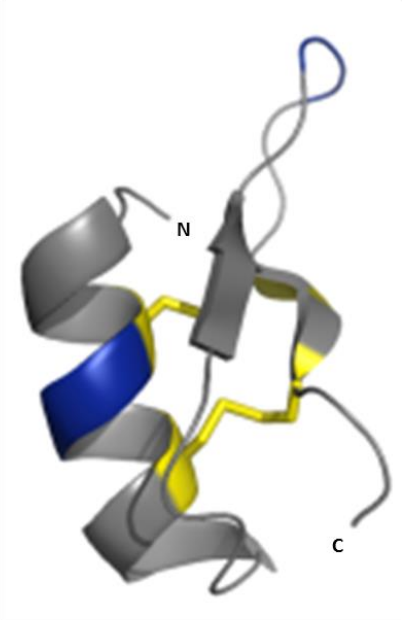


Fig. 4.5 Interatomic distances between cysteine residues in the N-terminal fluid analogue. To determine if cysteine residues were spatially close enough to theoretically form disulfide bridges the distances between thiol moieties of adjacent cysteine residues were measured. Cysteine residues are coloured yellow. Cysteine side chains are displayed as sticks. The yellow dashed lines indicate the S-S distances. Measured distances are reported in angstroms. The distances were measured and the molecule was displayed using PyMol molecular visualisation suite.

The results of the modelling process showed that the final structure of the 1-strand deletion analogue did not significantly de*via*te from the tertiary structure of the 5-Arg analogue peptide upon which it was based however the 2-strand deletion analogue did de*via*te to a larger extent (Fig. 4.6). The RMSD between the 5-Arg analogue and the 1 strand deletion analogue was found to be 0.089. The RMSD between the 5-Arg analogue and the 2 strand deletion analogue was found to be 0.148.

Although the only cysteine residues to be deleted were contained within the deleted strands, the remaining cysteine residues in the 2 strand deletion analogue were found to be 4.3 angstroms apart; this was outside the required maximum distance of 2 angstroms (Fig 4.7).

1 Strand Deletion Analogue



2 Strand Deletion Analogue

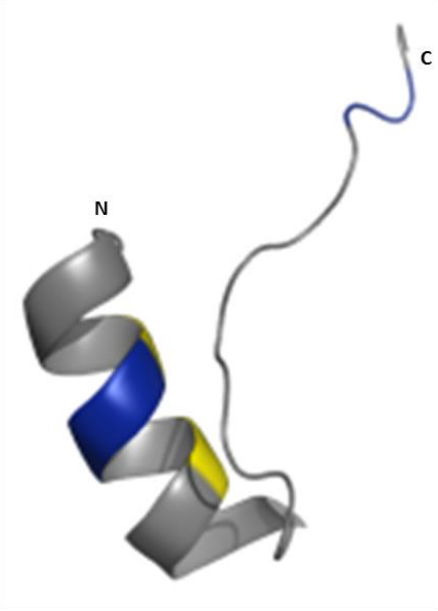


Fig. 4.6 Predicted Lead and Analogue Structures. Peptide structures were modelled using GroMacs package and visualised using PyMol. Positive amino acids are coloured blue. Disulfide bonds are displayed as yellow sticks. The N- and C-termini are also labelled.

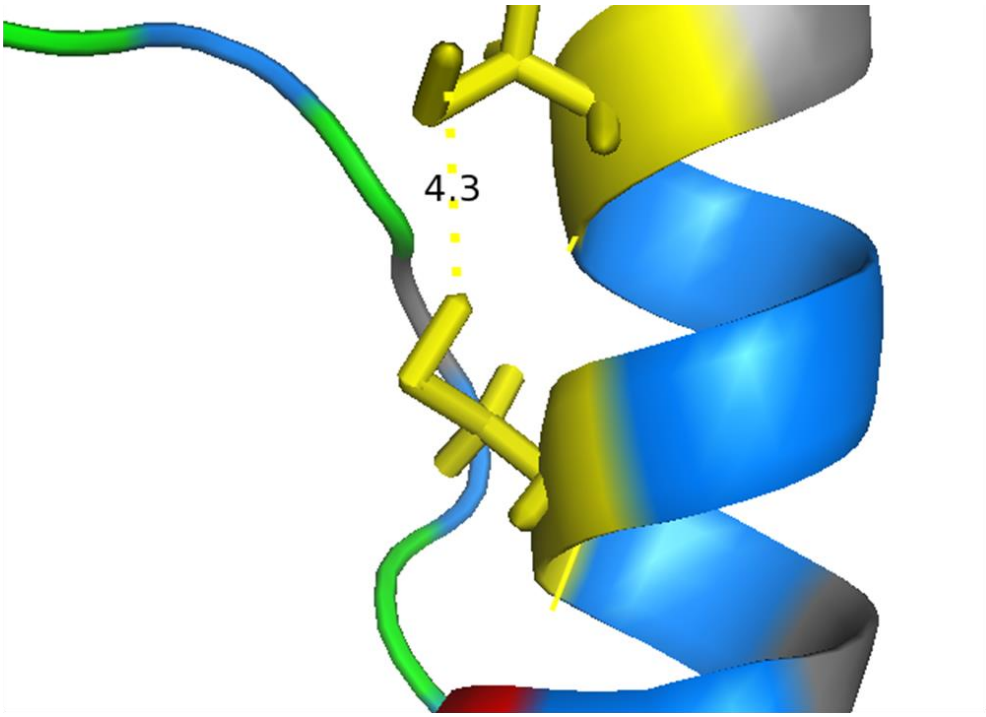


Fig. 4.7 Interatomic distances between cysteine residues in the 2-Strand deletion analogue. To determine if cysteine residues were spatially close enough to theoretically form a disulfide bridge the distance between thiol moieties of adjacent cysteine residues were measured. Cysteine residues are coloured yellow. Cysteine side chains are displayed as sticks. The yellow dashed lines indicate the S-S distance. Measured distances are reported in angstroms. The distance was measured and the molecule was displayed using PyMol molecular visualisation suite.

To gain an insight into the structural stability of these peptides, the radius of gyration, measured in nm, was recorded every 10 ps over the course of the 1ns molecular dynamic simulation. The recorded data (Fig. 4.8) showed that the radii of gyration of the peptides did not de*via*te significantly from their global averages over the course of the simulation. The data also indicates that there was no significant difference between the overall stability of the lead Mungbean defensin and the 3-Arg analogue which had average radii of gyration of 0.912 nm and 0.9 nm respectively. Interestingly the average radius of gyration of the 5-Arg analogue peptide was significantly lower at 0.825 nm indicating that this analogue was more stable of the course of the simulation.

The data also indicates that there was no significant difference between the overall stability of the 5-Arg analogue peptide and the Internal Cysteine Knockout analogue which had average radii of gyration of 0.825 nm and 0.911 nm respectively. Although the final structure of the All Cysteine Knockout Analogue was not dramatically different from that of the 5-Arg analogue, the radius of gyration was unsurprisingly significantly higher at 3 nm meaning that this molecule was much less structurally rigid. The data also indicated that the radius of gyration for the N-terminal fluid analogue was significantly higher, at 2.4 nm, than the 5-Arg analogue peptide upon which its design was based.

The radius of gyration of both the 1 strand and 2 strand deletion analogues does not significantly de*via*te over time from their respective global averages of 0.87 nm and 0.81 nm. Although at first glance, the 2 strand deletion analogue peptide may appear to be more stable due to the lower radius of gyration when compared to the 1 strand deletion analogue, one must take into account that these peptides are different sizes. The shorter a peptide is, the lower the degrees of freedom it has to gyrate. Due to this, no solid comparison can be made between these analogue peptides with regards to their stability. The only conclusion that can be made is that they remain relatively well folded over the course of the simulation.

When these available data were taken together they showed that the analogue peptides did not de*via*te structurally from the lead Mungbean defensin peptide to a significant degree, with the exceptions of the N-terminal fluid and 2-strand deletion analogues, and that all of the peptides remained very stable in their folded form over the course of the molecular dynamic simulation. This would suggest that any potential increase in antimicrobial activity with the analogue peptides would be due to the intended physiochemical or structural alterations rather than any unforeseen, and unintentional, structural change. However it must be noted that all these data were generated *in silico* by the molecular modelling process which may not represent their true structures. Circular dichroism spectroscopy would be necessary to determine the true structure.

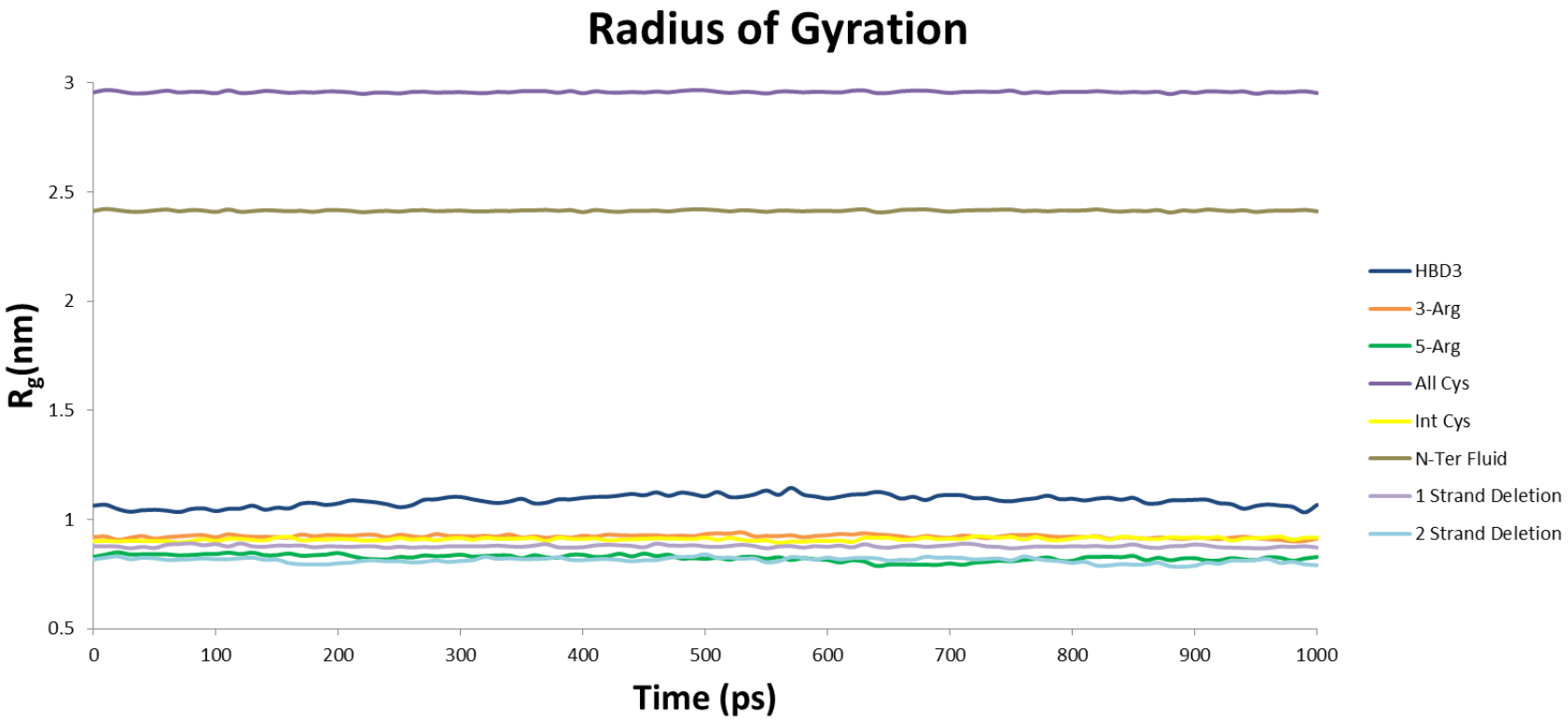


Fig. 4.8 Total Peptide Radius of Gyration. Molecular dynamic simulation was carried out over 1ns with radius of gyration measured every 10 ps. Radius of gyration was measured in nm and is displayed on the y-axis. Time is displayed on the x-axis in ps.

4.2.2 *In vitro* Antimicrobial Activity

4.2.2.1 Effect of Salt on Antimicrobial Activity

To determine if the antimicrobial activity of the analogue peptides was sensitive to the presence of ionic species in the assay environment, the peptides were tested for activity in high and low ionic strength buffers against *L. brevis*. The primary amino acid sequences were used to produce synthetic versions of the peptides which were used to carry out this *In vitro* analysis (Table 2.2). The high ionic buffer was composed of phosphate buffered saline which contained 137 mM NaCl. The low ionic strength phosphate buffer contained no NaCl. The bacteria, at a final concentration of $2x10^3$ cfu/ml, were challenged with each peptide for 90 min at 30°C before being plated onto MRST agar. The results were expressed as % survival in comparison to non-peptide treated controls.

Under the high ionic strength conditions the Mungbean defensin showed no antimicrobial activity at the concentrations tested (Fig. 4.9(A)). Both the 3-Arg and 5-Arg analogue peptides showed activity under these conditions with IC_{90} values of 0.35 µM and 0.17 µM respectively. Interestingly, the HBD3 IC_{90} value of 0.16 µM was not statistically significantly different from that of the 5-Arg analogue peptide. The All Cysteine Knockout analogue peptide had no observable antimicrobial activity. The Internal Cysteine Knockout analogue peptide had an IC_{90} value of 0.530 µM while the N-terminal fluid analogue peptide had an IC_{90} value of 1.410 µM. These values were significantly higher than the values observed for either HBD3 or the 5-Arg analogue peptide. In phosphate buffered saline at pH7 the 1 strand and 2 strand deletion analogue peptides had IC_{90} values of 1.3 µM and 2.05 µM respectively. These were significantly higher than the IC_{90} values for either HBD3 or the 5-Arg analogue which were 0.16 µM and 0.17 µM respectively.

Under the low ionic strength conditions the Mungbean defensin has an IC_{90} value of 0.415 µM (Fig. 4.9(B)). The 3-Arg and 5-Arg analogue peptides have IC_{90} values of 0.125 µM and 0.081 µM. Again, the HBD3 IC_{90} value of 0.095 µM is not statistically significantly different from that of the 5-Arg analogue peptide.

The All Cysteine Knockout analogue peptide had an IC_{90} value of 1.814 µM. The Internal Cysteine Knockout and the N-terminal fluid analogues had IC_{90} values of 0.115 µM and 0.250 µM respectively. Interestingly, although the Internal Cysteine Knockout analogue had a significantly higher IC_{90} than either HBD3 or the 5-Arg analogue peptide in high ionic strength conditions, under the low ionic strength conditions present in phosphate buffer, there was no significant difference in antimicrobial activity between these 3 peptides. The IC_{90} values for the 1 strand and 2 strand deletion analogues were found to be 0.17 µM and 0.34 µM respectively.

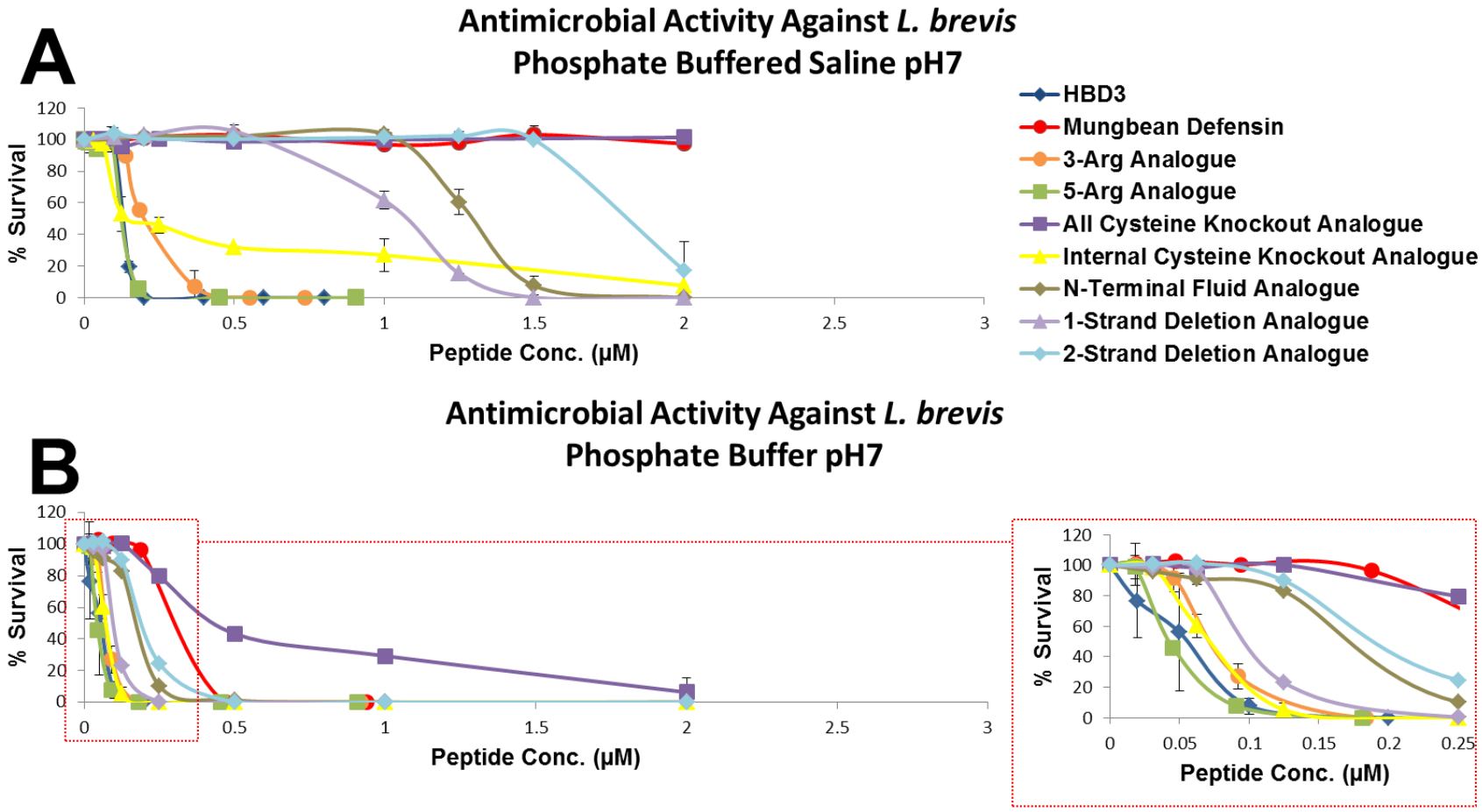


Fig. 4.9 *In vitro* antimicrobial activity of synthetic peptides to investigate the effect of NaCl on activity. *L. brevis* at a final concentration $2x10^3$ cfu/ml were challenged statically for 90min at 30°C with synthetic peptides suspended in either phosphate buffered saline (**A**) or phosphate buffer (**B**). An expansion of panel B is included in the inset. Samples were plated onto MRST agar and values were reported as % of untreated control samples. Assays were conducted on two independent occasions with three technical replicates per assay. Error bars are represented as the standard deviation of two biological replicates.

From these data, one can conclude that increasing the positive charge of the lead Mungbean peptide from +3.7 to +9.7 or +13.7 for the 3-Arg and 5-Arg analogues respectively increased the antimicrobial activity of the peptides in a charge dependant manner.

It can also be concluded that the antimicrobial activity of these peptides is sensitive to the presence of NaCl ions in the assay buffer. A comparison of the IC_{90} values for the analogue peptides showed that the 3-Arg analogue peptide was the most sensitive to the presence of NaCl ions with an IC_{90} value in phosphate buffer of just 35.71% that of the phosphate buffered. The 5-Arg analogue peptide had an IC_{90} value in phosphate buffer 47.64% that of the phosphate buffered saline value while HBD3 was shown to be the least sensitive with a phosphate buffer IC_{90} value 59.4% that of the phosphate buffered saline (PBS) value.

These data further suggest that altering the structural rigidity of the 5-Arg analogue peptide in this manner did not increase the antimicrobial activity of the molecule. These data also indicate that the analogue peptides are also sensitive to the concentration of ions in the assay environment.

Three conclusions can be made from the findings about the 1-Strand and 2-Strand deletion analogues. Firstly, the deletions made to produce these analogue peptides did not have a positive effect with regards to activity in comparison to the 5-Arg analogue upon which they were designed. Second, the most important determinant of antimicrobial activity, with these two analogue peptides was found not to be net positive charge. The net positive charge at pH7 for the 1 strand and 2 strand deletion analogues was predicted to be +10.9 and +12.0 respectively (Table 4.1). Although the 2 strand deletion analogue had the greatest net charge of the two peptides it had the lowest activity.

The third conclusion which could be made based on these findings was that the antimicrobial activity of these analogue peptides was extremely sensitive. A comparison of the results for the 1 strand deletion analogue showed that the absence of NaCl ions in the assay reduced the IC_{90} value was reduced to just 17.73% of that when NaCl was included in the assay. The same comparison with the results for the 2 strand deletion analogue showed the value reduced to 16.59% that of the phosphate buffered saline (PBS) value. This indicated that the 2 strand deletion analogue was the most NaCl sensitive peptide designed during this study.

		HBD3	Mungbean Defensin	3-Arg Analogue	5-Arg Analogue	All Cysteine Knockout	Internal Cysteine Knockout	N-Terminal Fluid Analogue	1 Strand Deletion Analogue	2 Strand Deletion Analogue
		Peptide Net Charge								
pH	**4.0**	13.4	9.9	13.9	16.4	16.1	16.2	16.3	13.4	13.8
	4.5	12.8	8.2	12.9	16.1	15.8	15.9	16.0	13.1	13.7
	5.0	12.3	6.7	12.1	15.7	15.5	15.6	15.6	12.7	13.6
	5.5	12.0	5.8	11.6	15.5	15.2	15.3	15.4	12.5	13.5
	6.0	11.9	5.1	11.2	15.1	14.8	14.9	15.0	12.1	13.2
	6.5	11.8	4.4	10.5	14.5	14.3	14.4	14.4	11.5	12.6
	7.0	11.6	3.7	9.7	13.7	13.7	13.7	13.6	10.9	12.0
	7.5	11.1	2.7	8.8	12.8	13.2	13.1	12.7	10.2	11.4
	8.0	9.9	1.1	7.2	11.2	12.8	12.4	11.1	9.2	10.5

Table 4.1 Predicted peptide net charge at varying pH. Peptide net charge predicted using the online protcalc webtool (http://protcalc.sourceforge.net/cgi-bin/protcalc)

4.2.2.2 Effect of pH on Antimicrobial Activity

To determine if the pH of the assay environment had an effect on the antimicrobial activity of the analogue peptides the antimicrobial assays were carried out at pH4 and pH8 in both high and low ionic strength buffers. The net charge of the peptides was predicted to increase as the pH of the environment decreased (Table 4.1). The assay was carried out by challenging *L. brevis* at a final concentration of $2x10^3$ cfu/ml for 90 min at 30°C with varying concentrations of the peptides, after which, the bacteria were plated onto MRST plates.

In PBS at pH4, the 3-Arg and 5-Arg analogue peptides had IC_{90} values of 0.111 µM and 0.105 µM respectively (Fig. 4.10(A)). Again, the IC_{90} value for HBD3, 0.125 µM, was not significantly different from that of the 5-Arg analogue peptide. The All Cysteine Knockout analogue peptide showed no antimicrobial activity at the concentrations tested. The Internal Cysteine Knockout analogue and the N-terminal fluid analogue had IC_{90} values of 0.79 µM and 0.945 µM respectively. The 1 strand and 2 strand deletion analogue peptides had IC_{90} values of 1.410 µM and 2.433 µM respectively.

In low ionic strength phosphate buffer at pH4, the 3-Arg and 5-Arg analogue peptides had IC_{90} values of 0.077 µM and 0.032 µM respectively (Fig. 4.10(B)). The lead Mungbean defensin and HBD3 had IC_{90} values of 0.112 µM and 0.047 µM respectively. The All Cysteine Knockout analogue had an IC_{90} of 0.525 µM. The Internal Cysteine knockout analogue and the N-terminal fluid analogue had IC_{90} values of 0.1 µM and 0.215 µM respectively. The 1 strand and 2 strand deletion analogues produced IC_{90} values of 0.160 µM and 0.200 µM respectively while HBD3 and the 5-Arg deletion analogues had values of 0.047 µM and 0.032 µM respectively.

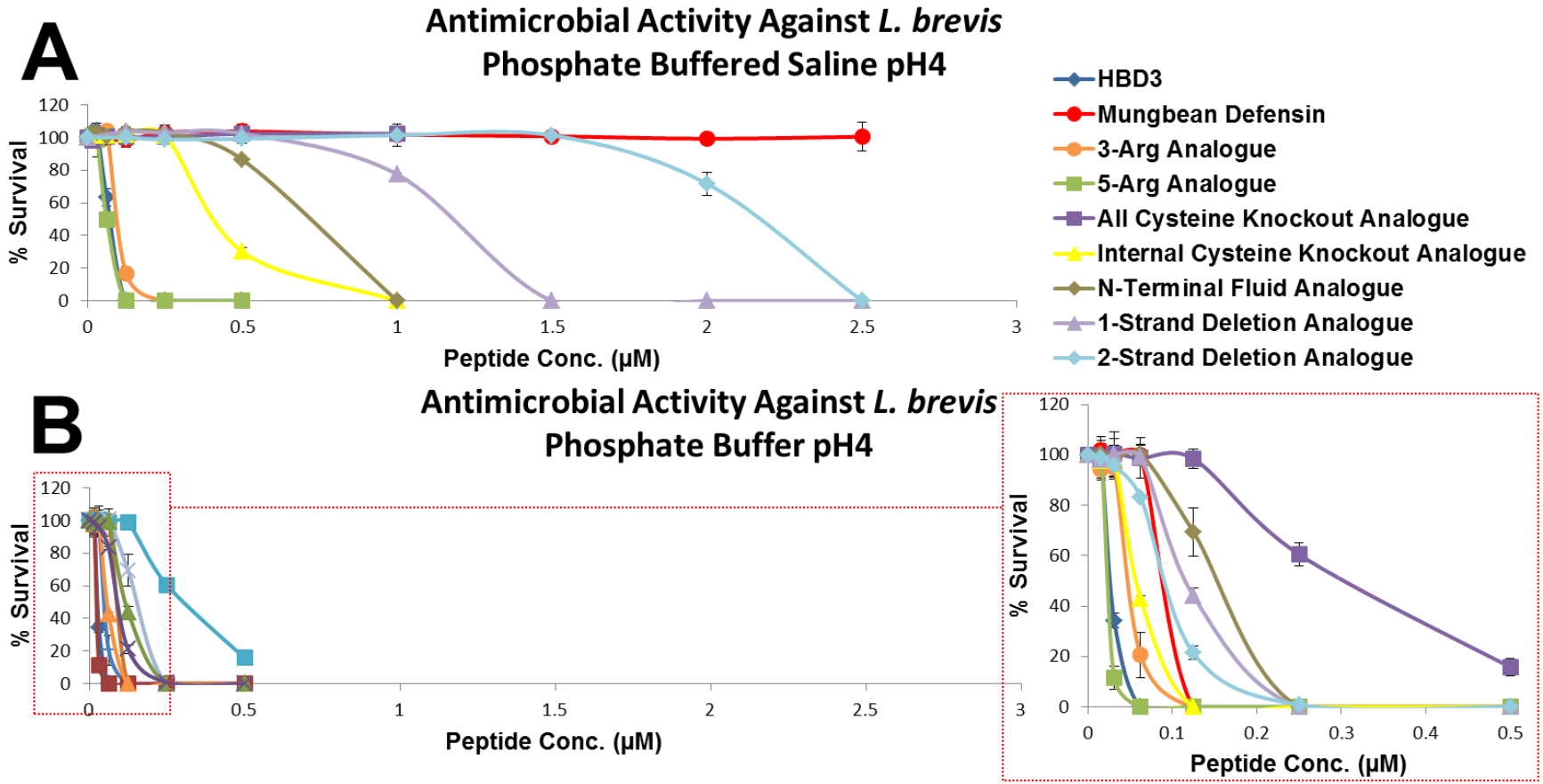


Fig. 4.10 *In vitro* antimicrobial activity of synthetic peptides to investigate the effect of pH on activity. *L. brevis* at a final concentration $2x10^3$ cfu/ml were challenged statically for 90min at 30°C with synthetic peptides suspended in either phosphate buffered saline pH4 (**A**) or phosphate buffer pH4 (**B**). An expansion of panel B is included in the inset. Samples were plated onto MRST agar and values were reported as % of untreated control samples. Assays were conducted on two independent occasions with three technical replicates per assay. Error bars are represented as the standard deviation of two biological replicates.

In PBS at pH8, the 3-Arg and 5-Arg analogue peptides showed IC_{90} values of 0.470 µM and 0.320 µM respectively (Fig. 4.11(A)). The Mungbean defensin peptide showed no antimicrobial activity under these test conditions while HBD3 produced an IC_{90} value of 0.320 µM. pH8 the All Cysteine Knockout analogue again showed no antimicrobial activity. The Internal Cysteine Knockout analogue and the N-terminal fluid analogue returned an IC_{90} of 1.82 µM and 1.84 µM respectively. The 1 and 2 stand deletion analogues returned IC_{90} values of 2.360 µM and 2.840 µM respectively. While both HBD3 and the 5-Arg analogue peptides were seen to an IC_{90} value of 0.320 µM.

Under low ionic strength, phosphate buffer, conditions at pH8 the 3-Arg and 5-Arg analogue peptides had IC_{90} values of 0.410 µM and 0.095 µM respectively (Fig. 4.11(B)). The Mungbean defensin had an IC_{90} value of 0.560 µM with HBD3 returning an IC_{90} value of 0.125 µM. The All Cysteine Knockout analogue displayed an IC_{90} value of 2.14 µM. The Internal Cysteine Knockout analogue and the N-terminal fluid analogue were found to have IC_{90} values of 0.27 µM and 0.5 µM. The 1 strand and 2 strand deletion analogue peptides were seen to have IC_{90} values of 0.448 µM and 0.492 µM respectively (Fig. 4.24 (B)). In comparison to this HBD3 and the 5-Arg analogue had values of 0.125 µM and 0.095 µM respectively.

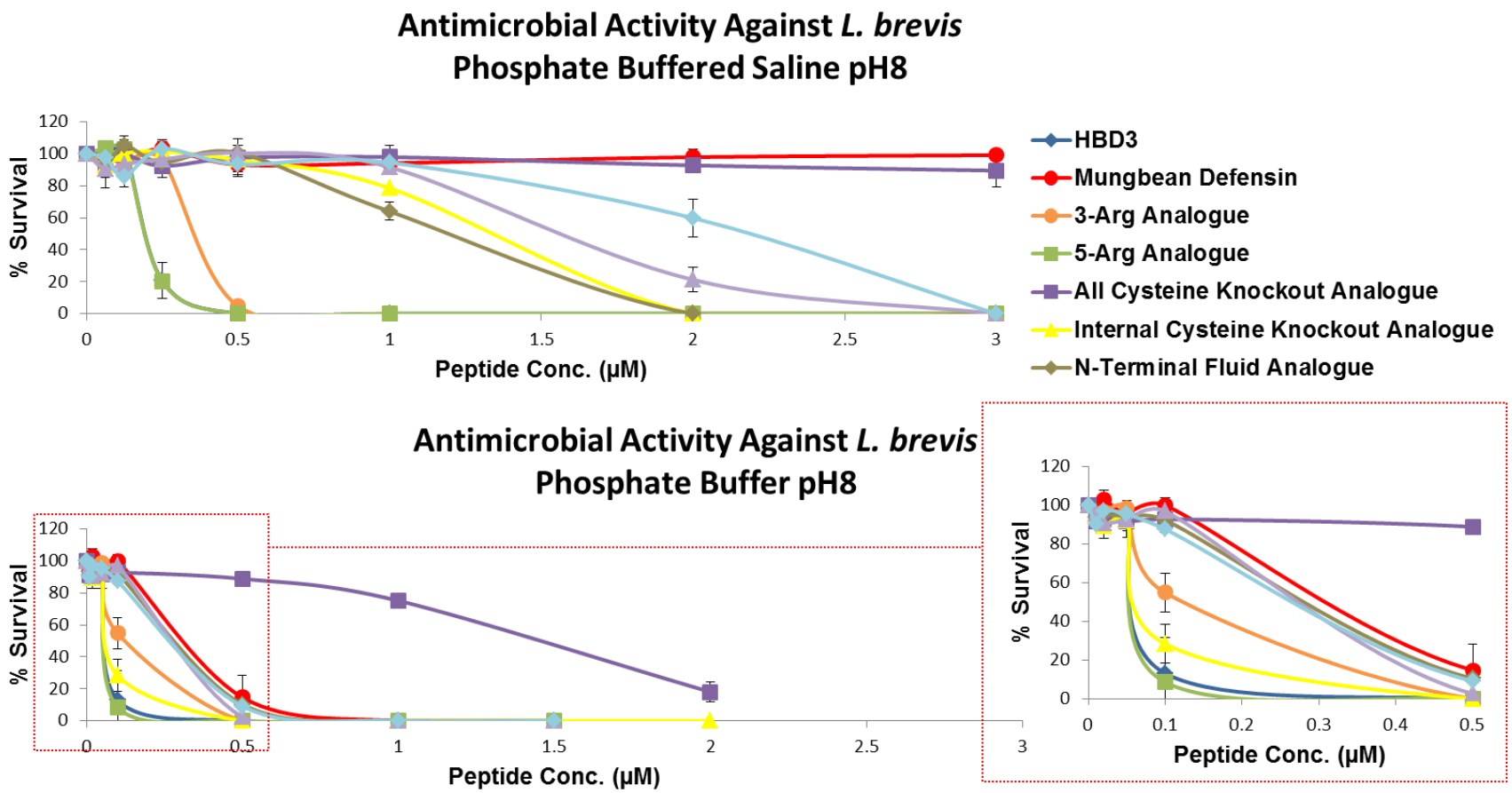


Fig. 4.11 *In vitro* antimicrobial activity of synthetic peptides to investigate the effect of pH on activity. *L. brevis* at a final concentration $2x10^3$ cfu/ml were challenged statically for 90min at 30°C with synthetic peptides suspended in either phosphate buffered saline pH8 (**A**) or phosphate buffer pH8 (**B**). An expansion of panel B is included in the inset. Samples were plated onto MRST agar and values were reported as % of untreated control samples. Assays were conducted on two independent occasions with three technical replicates per assay. Error bars are represented as the standard deviation of two biological replicates.

These data show an inverse relationship between the pH of the assay medium and the antimicrobial activity of the peptides. This finding would suggest that the peptide net charge is an important determinant of antimicrobial activity. These data also show that, under the pH conditions tested here, the peptides still have an inverse relationship between the ionic strength of the assay environment and their antimicrobial activity. It should also be noted that, for the 3-Arg analogue peptide, the ionic strength also negatively affected the dose response of its antimicrobial activity.

A closer examination of the data generated by the cysteine knockout peptides and the N-terminal fluid analogue peptide reveals an interesting phenomenon. In low ionic strength conditions, an inverse relationship can observed between the assay pH and antimicrobial activity for all the peptides. However, under the higher ionic strength conditions of phosphate buffered saline, the same relationship does not hold true for the Internal Cysteine Knockout analogue peptide. In the presence of NaCl the antimicrobial activity is most potent at neutral pH with an IC_{90} of 0.530 µM. In comparison, at pH4 and pH8 the IC_{90} climbs to 0.790 µM and 1.820 µM respectively. One could hypothesise that, at the higher pH, the predicted decrease in net positive charge could account for the decrease in antimicrobial activity.

The reason for the decrease in antimicrobial activity at the lower pH is unknown at this time but one possible explanation could be that the reduced structural rigidity due to the removal of cysteine residues could make the peptide activity sensitive to any additional ions in the system; be they NaCl or H^+ ions. The additional positive charge conferred by the low pH eventually becomes counteracted by the presence of H^+ ions which interfere with ionic interactions between the peptides and the bacterial surface.

A number of conclusions can be derived from the data generated by the strand deletion analogue peptides. First, in the lower ionic strength phosphate buffer, the antimicrobial activity of both analogue peptides increases in an inverse relationship with the pH of the assay buffer. Again, as we already observed with the cysteine knockout analogues, this does not hold true in the presence of NaCl ions in the assay environment. This again suggested that any alterations

made to the primary sequence which may affect the rigidity of the tertiary structure would make the antimicrobial activity of that peptide sensitive to the presence of competing ions in the assay environment. Another explanation may be that the two stages to membrane insertion of the peptides, initial ionic interaction and subsequent hydrophobic insertion into the lipid bilayer are affected independently by net charge and peptide structural changes respectively.

4.2.2.3 Importance of Disulfide Bonds

To examine the relationship between disulfide bond formation and antimicrobial activity of the analogue peptides it was decided to carry out the antimicrobial assay under reducing conditions. To achieve this, *L. brevis* at a final concentration of $2x10^3$ cfu/ml, was challenged for 90 min at 30°C with peptides at concentrations equal to their IC_{50} value in phosphate buffer at pH7. One set of peptides contained the reducing agent dithiothreitol (DTT) at a final concentration of 5mM. The results of the assay were represented as a % of a set of samples not containing DTT.

The results of this assay showed that the 3-Arg and 5-Arg analogue peptides retained 37.5% and 38.25% of their original antimicrobial activity respectively (Fig. 4.12). These results were not statistically significantly different from the retained activity of HBD3 which was seen to have retained 35.79% of original activity. These results would suggest that correct disulfide bond formation is important for antimicrobial activity. This finding would indicate that, although a major determinant of antimicrobial activity is net positive charge, maintaining the correct tertiary structure is also important. The difference in sensitivity to DTT treatment between the lead Mungbean defensin and the two analogue peptides which are structurally very similar cannot be explained at this time. It would seem that the increase in net charge allows the anaolgue peptides to overcome any structural perturbation caused by the DTT treatment.

the Internal Cysteine Knockout analogue retained 51.03% of its original antimicrobial activity. Unsurprisingly, the All Cysteine Knockout analogue was found to have retained all of its original antimicrobial activity. In all likelyhood this was due to the fact that the peptide had no amino acids to the reducing agent to act upon. The N-terminal fluid analogue was found to have retained only 30.82% of original activity despite the modelled prediction that the disulfide bonds had not formed for this peptide.

It can be concluded from this analysis, when compared to the 5-Arg analogue peptide with four disulfide bonds, that a greater amount of original antimicrobial activity could be retained for peptides containing few or no cysteine residues.

It was observed that the 1 strand deletion analogue retained only 36.29% of its original antimicrobial activity. This was comparable to, and not statistically significantly different from, HBD3 and the 5-Arg deletion analogue which retained 35.8% and 38.25% of their respective antimicrobial activities. In contrast to this, the 2 strand deletion analogue peptide retained 93.58% of its original antimicrobial activity.

The latter observation was unsurprising due to the fact that no disulfide bridges were predicted to form in this peptide so the reducing agent would have no substrate to act upon, ergo with this peptide the antimicrobial activity is not related to disulfide bonds. The reduction in antimicrobial activity of the 1 strand deletion analogue again suggested that tertiary structure was also important for efficient antimicrobial activity. However, as with section 3.2.2.3, it had previously been observed that peptides can in some cases become susceptible to degradation when their disulfide bonds are reduced by treatment with DTT ((Marcos, 2019), pers. comm.).

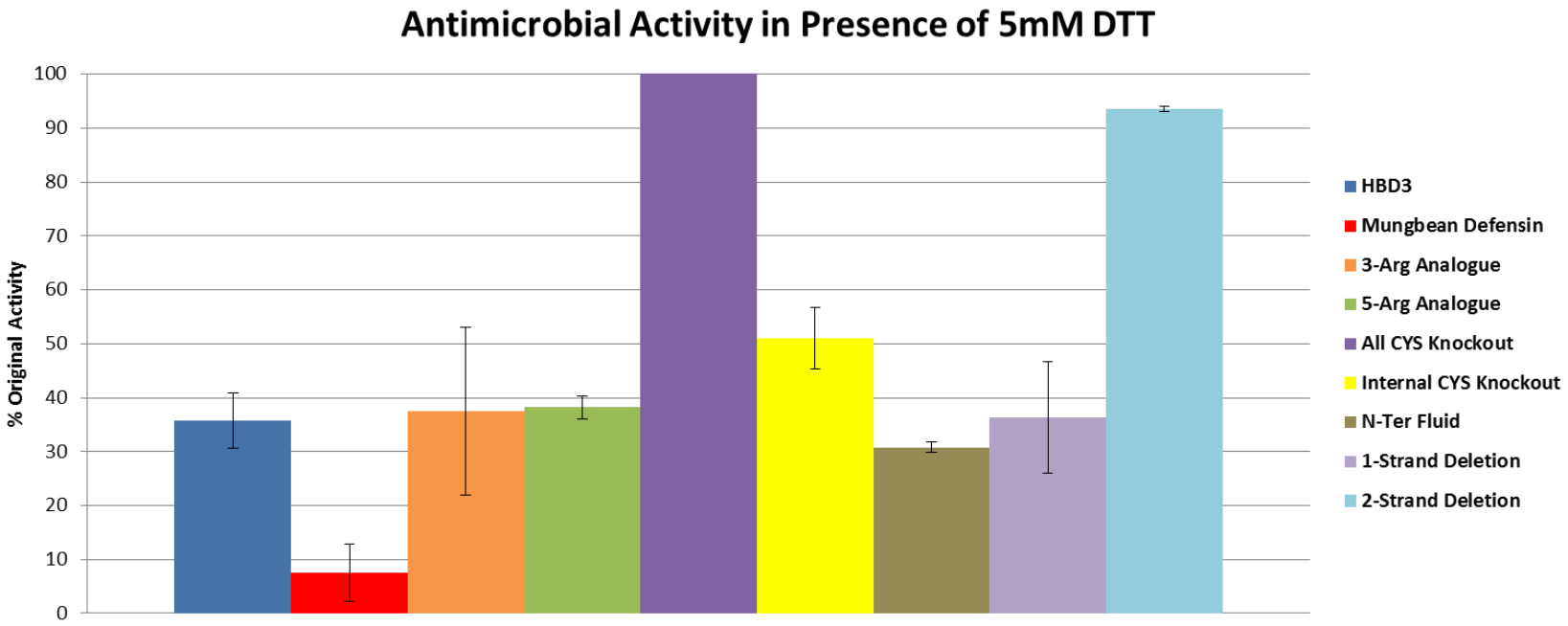


Fig. 4.12 *In vitro* antimicrobial assay investigating the importance of disulfide bridges for activity. *L. brevis* at a final concentration of $2x10^3$ cfu/ml were challenged with peptides at the previously established MIC_{50} concentration in phosphate buffer for 90min at 30°C. For the duration of the challenge one set of samples contained the reducing agent DTT at 5mM and one set did not. Following the challenge, samples were plated onto MRST agar. Results were calculated based on non-peptide but DTT treated samples. Values are reported as % of non-DTT treated samples. Assays were conducted on two independent occasions with three technical replicates per assay. Error bars are reported as the standard deviation of the two biological replicates.

4.2.2.4 Thermal Stability of Antimicrobial Activity

To determine if the analogue peptides antimicrobial activities were thermostable, it was decided to thermally pre-treat the peptides before carrying out the antimicrobial assay. The peptides, at their phosphate buffer pH7 IC_{50} concentrations, were heated to 90°C for 10min before being cooled to 20°C over 5 min. After thermal treatment, the peptides were used to challenge *L. brevis* at a final concentration of $2x10^3$ cfu/ml for 90 min at 30°C, after which, samples were plated on MRST plates. The results were expressed as a % of the activity of a set of peptides which did not undergo the thermal pre-treatment.

It was found that the 3-Arg and 5-Arg analogue peptides retained 57.65% and 62.11% respectively of their original antimicrobial activity after thermal pre-treatment (Fig. 4.13). This showed that these analogue peptides were significantly more thermostable than HBD3 which only retained 25.38% of its antimicrobial activity after thermal treatment. The hypothesis for loss of antimicrobial activity after thermal pre-treatment would be that the peptides unfolds while heated and cannot refold correctly upon cooling. This may be a result of structural rigidity within the molecule caused by disulfide bond formation. Again this would suggest that maintaining the correct tertiary structure is important for efficient antimicrobial activity, particularly for HBD3. HBD3 was seen to be the least thermotolerant and has three disulfide bonds while the other three peptides each have four disulfide bonds and have increased thermotolerance. A plausible explanation for this may be that the reduced number of disulfide bonds allows a greater degree of unfolding during thermal treatment increases the likelihood of misfolding when the thermal treatment is stopped.

It could be seen that the All Cysteine Knockout analogue peptide retained 99.4% of the originally reported activity after thermal pre-treatment (Fig. 4.18). It was found that the Internal Cysteine Knockout analogue and the N-terminal fluid analogue peptides retained 88.81% and 58.36% respectively of their original antimicrobial activities.

It can be concluded from these data that, for these analogue peptides, an inverse correlation exists between the number of cysteine residues present in

the molecule and the antimicrobial activity of that peptide. The hypothesis for why this might occur could be that the more fluid a peptide is, the easier it is for that peptide to correctly refold after thermal treatment.

It was seen that after thermal pre-treatment the 1 strand and 2 strand deletion analogue peptides retained 74.67% and 84.90% of their respective original antimicrobial activities (Fig. 4.26). This was in comparison to HBD3 and the 5-Arg deletion analogue which were observed to have retained 25.38% and 62.11% respectively of their original antimicrobial activities.

Although the differences in retained activity between the 5-Arg, 1 strand deletion and 2 strand deletion analogues is not statistically significant, a subtle trend could be observed. Again an inverse correlation between the number of intra-molecular disulfide bonds predicted to form within the molecule and the retained activity post thermal treatment could be observed.

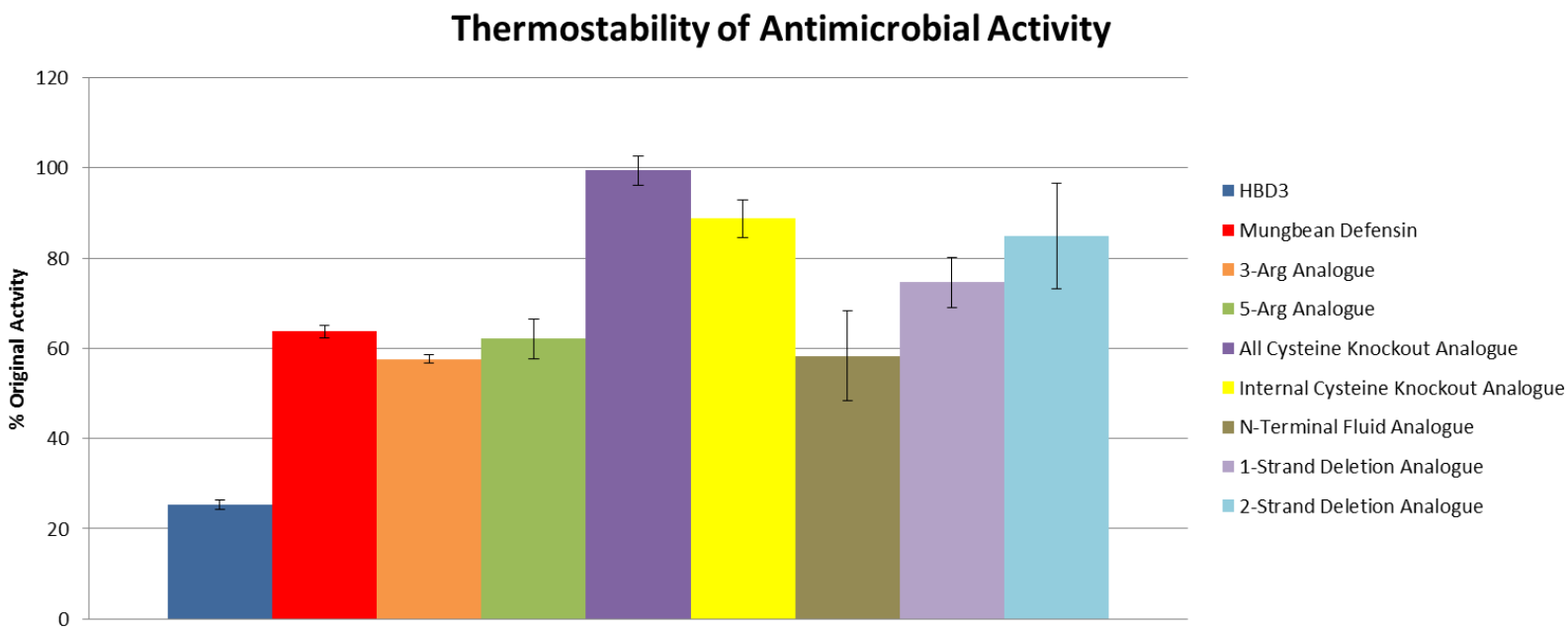


Fig. 4.13 *In vitro* antimicrobial assay investigating the thermostability of peptide activity. *L. brevis* at a final concentration of $2x10^3$ cfu/ml were challenged with peptides at the previously established MIC_{50} concentration in phosphate buffer for 90min at 30°C. For the duration of the challenge one set of samples contained peptides which were thermally treated at 90°C for 10min before being cooled to 20°C over 5min. The other set of samples contained peptides which were not thermally treated. Following the challenge, samples were plated onto MRST agar. Results were calculated based on non-peptide treated samples. Values are reported as % of non-thermally treated samples. Assays were conducted on two independent occasions with three technical replicates per assay. Error bars are reported as the standard deviation of the two biological replicates.

4.3 Discussion

The work discussed within this chapter sought to answer 3 important questions surrounding the rational design of effective AMP's based on the physiochemical characteristics of peptides which had previously been observed to have good antimicrobial activity. These characteristics were engineered into an antimicrobial peptide backbone which had previously been observed to have little antimicrobial activity.

Three approaches were taken for this rational design, each of which sought to answer a different question surrounding the optimum physiochemical characteristics of highly active AMP's.

The first approach was based on correlations observed in chapter 3 of this work. A clear positive correlation was observed between antimicrobial activity and the net positive charge of the AMP's (Kraszewska *et al.*, 2016)(Table 3.3). The Mungbean defensin PDF-1 was seen to only have antimicrobial activity in low ionic strength phosphate buffer.

This peptide was chosen as the lead peptide backbone upon which to engineer physiochemical and structural changes. The rationale behind this decision was based on the fact that no activity, antimicrobial or otherwise, had previously been observed for the Mungbean defensin (Lin *et al.*, 2007). Using this peptide as a parental scaffold sequence would allow the elucidation of the peptide characteristics required for antimicrobial activity without any interference from other residues which may possess antimicrobial activity; the antimicrobial activity caused by these residues on peptides of known activity may mask any changes in activity caused by the creation of analogue peptides.

It was hypothesised that increasing the net positive charge of the Mungbean defensin would increase the antimicrobial activity. Two analogue peptides were designed using this approach. The first replaced 3 negatively charged residues (Glu4, Asp21 and Asp22) with arginine residues to create the 3-Arg analogue peptide. The second replaced 5 negatively charged residues (Glu4, Asp21, Asp22, Asp37 and Asp38) to create the 5-Arg analogue peptide.

The second approach to the rational design methodology asked the question; can one increase the antimicrobial activity of a peptide by reducing the

structural rigidity of the molecule? The rationale behind this approach was based on allowing the peptides to interact with the bacterial surface in an “Induced Fit” manner; the more flexible the molecule the easier it would be to bind to its target.

To answer this question, 3 further peptides were designed, based on the 5-Arg analogue peptide. The first two peptides attempted to reduce the overall structural rigidity of the molecule by removing cysteine residues. The first such peptide had all cysteine residues replaced; this formed the All Cysteine Knockout analogue. The second such peptide analogue to be designed replaced all of the internal cysteine residues, leaving only the two cysteine residues which formed the disulfide bridge between the N and C-termini; this formed the Internal Cysteine Knockout analogue.

The final peptide to be designed based on this approach took into account an observation made about the tertiary structure of the “Gold Standard” defensin HBD3. It was noted that the N-terminus of HBD3 was composed of a short alpha helix leading into a largely unstructured terminal tail. A peptide was designed which more closely resembled the tertiary structure of HBD3. To achieve this, an amino acid sequence was designed where the N and C-termini of the original 5-Arg analogue were now connected by a glycine – glycine linker. The new N-terminus was now located at the N-terminal end of the alpha helix; this produced the N-terminal fluid analogue.

The third and final approach to the rational design methodology sought to answer the question; can an antimicrobial peptide of minimal size be designed which maintains all of the activity of the parental molecular backbone upon which its design was based?

To investigate this question, two further peptides were designed. The first peptide was designed to have the N-terminal proximal strand deleted. This was the 1 Strand Deletion analogue. The second peptide had both the N and C-terminal proximal strands deleted to form the 2 Strand Deletion analogue peptide.

To ensure that any change in antimicrobial activity observed with the analogue peptides was due to the newly engineered characteristics, and not drastic

alterations to the tertiary structures of the peptides, their structures were modelled by molecular dynamic simulation. It was found that for the 3-Arg and 5-Arg analogue peptides that the alterations to the primary amino acid sequences did not affect the overall tertiary structures of the peptides (Fig. 4.2). This was also observed for the cysteine deletion analogue peptides (Fig. 4.3). The structural similarity between the parental 5-Arg analogue peptide and both the All Cysteine Deletion analogue and the Internal Cysteine Deletion analogue peptides was unexpected considering the important role disulfide bridges have on structure formation. The predicted structure of the N-terminal fluid analogue was very different from that of the parental 5-Arg analogue structure (Fig. 4.3). The formation of the C-terminal alpha helix was completely unexpected although given the drastic nature of the changes to the primary amino acid sequence, some structural alterations were expected. Furthermore, although no alterations were made to the cysteine residues of this analogue, the results of the structural modelling process predicted that no disulfide bridges could be formed. Measurements of the distances between the cysteine residues of this peptide showed that no two cysteine residues were within the 2 angstrom distance necessary for disulfide bond formation (Fig. 4.5). The structures of the 1 Strand and 2 Strand deletion analogues were also found to be structurally similar to the 5-Arg analogue structure upon which their designs were based (Fig. 4.6).

The structural stability of the peptides were analysed by recording the radii of gyration over the course of the molecular dynamic simulations. It was seen that the radius of gyration for all of the peptides did not de*via*te significantly from their respective global averages (Fig. 4.8). This suggested that the peptides achieved their folded form early in the molecular dynamic simulation and maintained this fold throughout the simulation. These data must be interpreted carefully however. Only the radii of gyration from peptides of roughly the same size and amino acid composition can be directly compared. There is generally a direct correlation between a proteins size and its radius of gyration (Smilgies and Folta-Stogniew, 2015, Fitzkee and Rose, 2004). With this in mind, unsurprisingly, the All Cysteine knockout analogue peptide had the greatest radius of gyration followed by the N-terminal fluid analogue and HBD3. This is

most likely due to the lack of disulfide bond formation. Of the peptides that could be directly compared, the 5-Arg analogue peptide was found to be the most stable followed by the 3-Arg analogue, both of which were found to be more stable than the lead Mungbean defensin upon which their design was based. Surprisingly, the Internal Cysteine knockout analogue peptide was found to be more stable than HBD3 even though it had fewer disulfide bonds and was comparable in size to HBD3. The presence of the largely unstructured N-terminal tail of the HBD3 molecule could account for this result. In contrast to this, the Internal Cysteine knockout analogue had both termini connected *via* a disulfide bond which would be expected to reduce the degrees of freedom for the molecule. A direct comparison could not be made between the aforementioned analogue peptides and either the 1 strand or 2 strand deletion analogues due to the significant difference in overall peptide size and sequence length. The only comment that could be made about the radii of gyration for these peptides was that they achieved their folded form early in the simulations and maintained that form throughout the simulations. These data taken together would suggest that the stability of the analogue peptides was directly correlated with the number of disulfide bonds within the molecule.

When the antimicrobial activity against the food spoilage bacteria *L. brevis* was tested at pH7 it was found that there was a negative correlation between the ionic strength of the assay buffer and the antimicrobial activity of the peptides (Fig. 4.9 (A) and (B)). At pH7 the most salt sensitive peptide was found to be the 1 strand deletion analogue, followed by the 2 strand deletion analogue. The N-terminal fluid and Internal Cysteine analogues followed next. The least salt sensitive peptide was found to the 5-Arg deletion analogue followed by the 3-Arg analogue, although, both of these analogues were more salt sensitive than HBD3. These data would suggest that alterations to the peptides which perturb their structures increase their sensitivity to competing ions in the assay environment. The comparisons between HBD3, the 3-Arg and 5-Arg analogues would also suggest that this sensitivity can be counteracted by increasing the net positive charge of the peptide. It is likely that the presence of NaCl ions in the assay interfere with ionic interactions between the bacterial surface and the AMP’s. This phenomenon has been previously modelled for the interaction

between Magainin and POPC lipid bilayers (Kandasamy and Larson, 2006). The salt sensitivity of AMP's such as these analogue peptides has been previously well documented also (Maisetta *et al.*, 2008, Lee *et al.*, 1997, Wu *et al.*, 1999, Wang *et al.*, 2009).

The net cationic charge of all of the analogue peptides was predicted to increase as the pH of the assay environment decreased (Table 4.1). When the peptides were tested for antimicrobial activity at pH4 and pH8, it was seen that under low ionic strength conditions the peptides had an inverse relationship between antimicrobial activity and pH (Fig 4.10 (A) and (B) and Fig. 4.11 (A) and (B)). When the ionic strength of the assay environment was increased to physiologically relevant conditions an unusual phenomenon was observed. For the majority of the peptides tested, the same inverse relationship between pH and antimicrobial activity was observed. When the Internal Cysteine knockout, 1 strand deletion and 2 strand deletion were tested, it was seen that either increasing or decreasing the assay pH had a negative effect on the antimicrobial activity in comparison to the activity at pH7. The reason for this effect is unknown at this time but the data would suggest that any increase in ions in the assay environment, be they NaCl, H+ or OH- ions, decreases the antimicrobial activity of this subset of peptide analogues. The inverse relationship between pH and antimicrobial activity for membrane active cationic peptides has been documented previously (Maisetta *et al.*, 2010, Yeaman *et al.*, 1997). The conclusions reached by these researchers are in agreement with the hypothesis proposed by this work; decreasing the pH of the assay environment increases the antimicrobial activity of the peptides in a charge dependant manner. Another explanation may be that the two stages to membrane insertion of the peptides, initial ionic interaction and subsequent hydrophobic insertion into the lipid bilayer are affected independently by net charge and peptide structural changes, respectively.

When the importance of disulfide bond formation was investigated, it was found that in all cases, disulfide bonds were vital for effective antimicrobial activity (Fig. 4.12). Unsurprisingly, a negative correlation was observed between the number of cysteine residues present in the peptide sequence and the antimicrobial activity in the presence of the reducing agent DTT. These data

also suggested that analogue peptides had correctly formed their disulfide bridges. This was an important observation as no experimental evidence of this had been previously been seen throughout this study. The data, reported throughout the primary literature to date, were not in agreement on the importance of disulfide bonds for effective antimicrobial activity (Ma *et al.*, 2016, Haag *et al.*, 2012). In direct contrast to what has been observed throughout this work it had been previously reported that HBD3 did not require disulfide bonds for activity against *Streptococcus pneumonia* and *E. coli* (Kluver *et al.*, 2005). It should be noted however that the concentration of HBD3 used in that study was significantly higher than was used in this study. The fact that this study challenged a different bacterium than the ones used in the reported study could also account for the discrepancy in results. Overall the results of this study are very clear about the importance of disulfide bonds for antimicrobial activity.

The results from the analysis of peptide thermostability uncovered some interesting correlations (Fig 4.13). It was seen that the 3-Arg analogue and the 5-Arg analogue peptides, which each have 4 disulfide bonds, retained equal amounts of antimicrobial activity after thermal pre-treatment which was also the lowest amount of retained for any of the analogue peptides. The 1 strand deletion analogue peptide which has the next best retained post thermal treatment antimicrobial activity has 2 disulfide bonds. This inverse relationship between the number of disulfide bonds and retained antimicrobial activity was seen to continue with the other analogue peptides. The generally accepted hypothesis surrounding the thermostability of larger proteins states that disulfide bridges increases the thermostability of the protein activity (Bulaj, 2005, Matsumura *et al.*, 1989, Wetzel *et al.*, 1988). The results presented here would seem to be in contradiction to this hypothesis. One possible explanation for this observation could be that there is a fine balancing act between the number of disulfide bonds which a peptide has, its ability to resist thermally induced unfolding and the propensity for misfolding when it cools down. For example, it was shown here that peptides with no or few disulfide bonds are the most thermotolerant meaning they easily unfold during thermal treatment but are not structurally restricted while refolding. The peptides with four disulfide bonds were found to be the next most thermotolerant. They may resist thermally

induced unfolding to a greater extent than the other peptides, and so, do not require a high degree of refolding. While peptides, such as HBD3, with three peptide bonds, unfold to a high degree during thermal treatment but become trapped in a local energy minimum upon refolding due to the presence of disulfide bonds.

In conclusion the work contained within this chapter has answered each of the 3 research questions surrounding rational design of active AMP's. One can indeed increase the activity of an antimicrobial peptide by increasing the net positive charge of the peptide. Secondly, decreasing the structural rigidity of the peptide decreases the antimicrobial activity of the peptide. It does however confer thermo-tolerance upon the peptide. Finally, one can design a small peptide which has antimicrobial activity but not one which retains all of the antimicrobial activity of the parental molecule.

Chapter 5:

In Vivo Expression of Antimicrobial Peptides in *Saccharomyces* sp.

5.1 Introduction

The research outlined in this chapter sought to answer a number of important questions surrounding the use of *Saccharomyces* sp. as microbial cell factories for the *In Vivo* production of AMP's. This was done with the aim of using these systems to combat bacterial spoilage of yeast based foods in an industrial setting without the need antibiotic or other selective pressure for the intracellular maintenance of the expression systems; a characteristic which would be extremely economically and ethically desirable (Casewell *et al.*, 2003).

Yeasts such as *Saccharomyces* have long been microbial cell factory workhorses for the production of pharmaceuticals, industrial enzymes and other metabolite based products (Ferrer-Miralles *et al.*, 2009, Colao *et al.*, 2006, Resina *et al.*, 2007, Corchero *et al.*, 2013, Singh *et al.*, 2008, Gurramkonda *et al.*, 2009). It should be noted however that other yeasts, such as *Pichia pastoris*, have been used to produce AMP's on an industrial scale as they display higher levels of protein expression than *Saccharomyces* sp. (Garrigues *et al.,* 2017). However, given that the intended use of these expression systems was within a brewery setting, the choice to use *S. pastorianus* was justified.

The use of *Saccharomyces* sp. as microbial cell factories has traditionally been done either by integrating the expression cassettes for the desired products, or pathways, into the chromosome or by expressing the genes from vectors which require selective pressure for their maintenance within the cell. Several problems present themselves with these systems. Firstly, integrating protein expression cassettes into the genome and subsequent removal of any antibiotic selection cassette is a cumbersome and time consuming process. Moreover, after successful integration of the expression cassette, further genetic manipulation of the system is again a time consuming process. The use of plasmid based systems may expedite the process and alle*via*te some of the problems surrounding integration; however this presents its own problems. Plasmid based systems require constant selective pressure, either antibiotic or auxotrophic based, to be maintained within the cell. The use of antibiotics on an industrial scale is neither cost effective nor ethically *via*ble considering the

growing problem of antibiotic resistant microorganisms (Larsson *et al.*, 2007, Collignon, 2015, Hensing *et al.*, 1995, Casewell *et al.*, 2003).

The use of auxotrophic selection allays the problems associated with antibiotic use but most industrial *Saccharomyces* strains do not have auxotrophic selective markers and, due to the fact that most are not haploid, creation of auxotrophic mutants based on these strains is difficult (Akada, 2002). A further problem with the use of auxotrophic selection in industrial settings is the complex nature of the medium upon which the yeasts feed; Auxotrophic selection only works if the auxotrophic substrate is missing from the medium.

To overcome these problems, it was decided to attempt to design and construct a modular expression vector which remains stable over multiple generations without the need for selective pressure. This system would then be used to express multiple copies of antimicrobial expression cassettes. This vector was based upon the yeast artificial chromosome pYAC4 which has been shown in the past to be stably maintained within the cell due to the presence of yeast centromeric and telomeric sequences. This system was also designed in a manner which would allow easy and rapid insertion of multiple copies of antimicrobial peptide expression cassettes in a modular, “drag and drop”, fashion thus reducing the time cost associated with classical vector construction methods.

This system was used to answer a number of important questions surrounding expression of AMP’s *in situ* from *Saccharomyces*. First, can an expression platform by constructed which is stable without the need for selective pressure? Second, can a system be created which allows easy modular insertion of multiple copies of an antimicrobial expression cassette? If multiple copies of an antimicrobial expression cassette are present on the expression platform, will peptide production increase linearly in a gene dosage dependent manner or is there an upper limit to peptide production? Lastly, can such an expression system effectively control bacterial growth in an industrially relevant environment?

The second part of this chapter sought to answer questions surrounding the *in situ* expression of plant derived defensin-like AMP’s from *Saccharomyces* to

control bacterial growth. Previous work conducted in this lab used human beta-defensin 3, integrated into the genome of *Saccharomyces*, to control the growth of the beer spoiling bacteria *Lactobacillus brevis* in an industrially relevant setting (James *et al.*, 2014). It was felt that the public perception surrounding the use of a human derived antimicrobial peptide would not be well received. People have long been accustomed to the use of plant based additives in their food however. Based on this assumption, it was decided to construct a set of genes for the expression of the plant based AMP's Cp-Thionin, Fabatin and the 3-Arg analogue of the Mungbean defensin discussed in previous chapters.

Here, the questions which were asked were, can these peptides be expressed *in situ* from *Saccharomyces*? And, can these peptides, expressed from *Saccharomyces* be used to control the growth of the beer spoiling microorganism *L. brevis*?

5.2 Results

5.2.1 Construction of a Stable Modular Expression System Based on the Yeast Artificial Chromosome pYAC4 Vector

5.2.1.1 Construction of pYAC-Kan Expression System

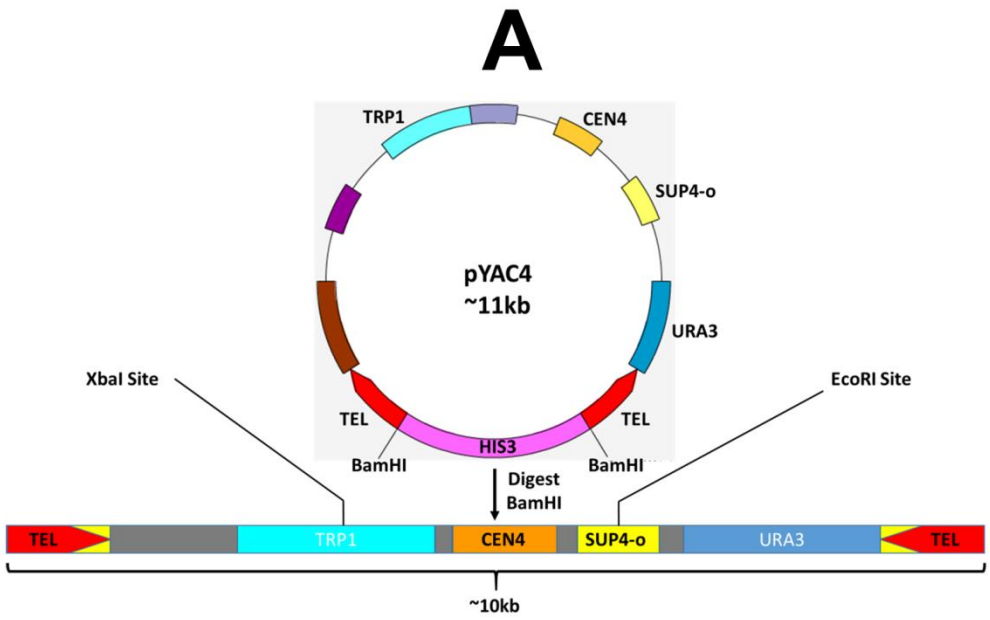
The yeast artificial chromosome pYAC4 is an 11kb shuttle vector which can be transferred between *Saccharomyces* and *Escherichia coli* (5.1 (A)). It is a single copy vector containing the centromeric sequence CEN4 and two *Tetrahymena* derived telomeric sequences. This vector has the auxotrophic selectable markers TRP1, URA3 and, while in its circular form, HIS3. Upon digestion of the vector using the restriction enzyme BamHI the HIS3 gene is excised which linearises the vector and exposes the telomeres. The vector also contains a multiple cloning site within the SUP4-o gene.

The lager yeast *Saccharomyces pastorianus* has previously been shown to have a significantly higher level of recombinant protein production than the more commonly used baker's yeast *Saccharomyces cerevisiae (*Kricka *et al.,* 2015*)*. It is also the primary fermentation yeast for most beer production. Due to this, it was decided to use the lager yeast *Saccharomyces pastorianus* CMBS33 for the *in situ* production of recombinant AMP's from the artificial chromosome expression platform. The pYAC4 vector contains auxotrophic selection marker genes but does not contain any antibiotic selection markers for use with yeast. *Saccharomyces pastorianus* CMBS33 does not have any auxotrophic selection markers. Due to this, it was necessary to construct the vector pYAC-Kan which would have KanMX gene cassette inserted into the TRP1 gene of the parental pYAC4 vector which would allow for antibiotic selection using the aminoglycoside G418. Once it was verified that the vector had been successfully transformed into *S. pastorianus,* and was stably maintained without selective pressure, the use of the antibiotic would in theory no longer be necessary.

To construct this vector, the KanMX cassette was amplified by PCR from the donor vector pRS42K using the primer pair YAC-Kan F and YAC-Kan R which had flanking tails which were homologous to sites within the TRP1 gene of pYAC4 (Table 2.3). The parental pYAC4 vector was digested using the

restriction enzyme XbaI, a unique recognition site of which was contained within the TRP1 gene of pYAC4. The PCR product and linearised vector were subsequently co-transformed into *S. cerevisiae* and successful transformants which had integrated the KanMX cassette into the TRP1 gene, using *Saccharomyces* native homologous recombination ability, were selected on agar plates containing G418. It was necessary to transform the vector into *S. cerevisiae* first as its transformation and recombination efficiency is higher than that of *S. pastorianus.*

Successful construction of the vector was initially confirmed by colony PCR screening (5.1 (B)). Subsequent DNA sequencing of the vector from the candidate colony confirmed that the pYAC-Kan vector had been faithfully constructed without mutations. The newly constructed vector was then transformed into *S. pastorianus*.



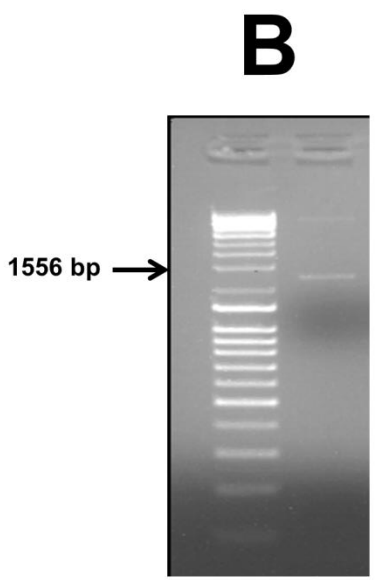


Fig. 5.1 (A) Schematic representation of the yeast artificial chromosome pYAC4 expression vector. The low copy number expression vector pYAC4 contains the *Saccharomyces* centromeric sequence CEN4 (orange), the SUP4-o gene (yellow) and a pair of *Tetrahymena* telomere sequences (red) flanking a HIS3 auxotrophic selection marker (purple). The vector also contains the auxotrophic selection marker genes TRP1 (light blue) and URA3 (dark blue). The HIS3 selection marker is flanked by two BamHI restriction enzyme recognition sites which, upon cleavage, excise the HIS3 marker from the 11kb circular vector to expose the telomere sites to yield the linearised 10kb pYAC4 vector. Also indicated are the restriction enzyme recognition sites EcoRI in the SUP4-o gene and XbaI in the TRP1 gene. **(B) Colony PCR verification of the expression vector pYAC-Kan.** Colony PCR was carried out using the primer pair YAC-KanMX(TRP)-F and YAC-KanMX(TRP)-R. The PCR product was separated by gel electrophoresis on a 1% agarose gel. Lane 1 contains 5µl of Q-Step 4 DNA ladder. The expected product size was 1556 bp.

5.2.1.2 Sequential Cloning to Construct a Multi-copy rHBD3 Expression Platform based on the pYAC-Kan Vector

Increasing peptide production can be achieved in a number of ways including through the use of high expression level constitutive promoters. It can also be increased through the use of high copy vectors which increase the, per cell, gene copy number. The latter would require the use of antibiotics or other selective pressures for stable intracellular maintenance system. This project sought to find a stable expression system which could act as a platform from which multiple copies of a given recombinant gene could be expressed.

This system could also be used to answer the questions surrounding the relationship between gene dosage and the levels of recombinant antimicrobial peptide production, it was necessary to construct a set of vectors with varying copy numbers of a recombinant antimicrobial expression cassette. This donor expression cassette was previously constructed in this lab and was composed of a rHBD3 gene translationally fused to an alpha-factor secretion signal which mediated secretion into the extracellular supernatant (James *et al.*, 2014). Transcription from the expression cassette was driven from a phosphoglycerate kinase (PGK) promoter and terminated by an alcohol dehydrogenase (ADH) terminator.

To construct the multi-copy system, the donor cassettes were amplified using a set of primers designed to create unique regions of homology between the multiple cassettes (Fig. 5.2(A)). These unique sequences formed “barcodes” which could be used to confirm integration of the cassettes into the pYAC-Kan vector by PCR using sets of barcode specific primers (Fig. 5.2(B)).

This system was designed to insert each copy of the recombinant expression cassette into the pYAC-Kan vector in a sequential manner. The cassettes were first amplified by PCR using a set of primers which added a tail on the 3’ end which was composed of the unique barcode sequence, a NotI restriction enzyme recognition site and a sequence homologous to the pYAC-Kan vector (Fig. 5.3(A)). The workflow to create the multi-copy vectors using this system was as follows. The first donor cassette was amplified using the primer pair YAC F1, which added a 5’ tail homologous to the pYAC-Kan vector upstream of

an EcoRI restriction enzyme recognition site in the SUP4-o gene, and Seq YAC NotI R1 which added the 3' tail described previously (Table 2.3). This PCR product was co-transformed into *S. cerevisiae* along with the pYAC-Kan vector which had been digested using an EcoRI restriction enzyme (Fig. 5.3(B1)). After the integration of the first cassette had been confirmed, the newly created pYAC-Kan 1xHBD3 vector was transformed into *S. pastorianus.*

The newly constructed vector was then digested using the restriction enzyme NotI which linearised the vector downstream of the first integrated cassette. The linearised vector was co-transformed into *S. cerevisiae* along with the second donor cassette which was amplified by PCR using the primer pair YAC F2, which added a 5' tail which was homologous to the unique barcode sequence of the first integrated cassette and Seq YAC NotI R2 which added a 3' tail homologous to the pYAC-Kan vector downstream of the first cassette (Fig. 5.3(B2)). This reverse primer also contained a second unique barcode sequence and a NotI restriction enzyme recognition site. When the second cassette had been successfully integrated to form the pYAC-Kan 2xHBD3 vector, the NotI restriction enzyme recognition site was reformed downstream of the second expression cassette while the first NotI site was no longer present (Fig. 5.3(B3)). Rebuilding the restriction site like this allowed recycling of the process continuously. Each time a new vector was confirmed by DNA sequencing it was subsequently used to transform *S.pastorianus.* This process was repeated using the primer pair YAC F3 and Seq YAC NotI R3 to create the vector pYAC-Kan 3xHBD3.

At each stage of construction, colony PCR using barcode specific Chk primer pairs, designed to generate a 1.3 kb product, were used to confirm the successful integration of the expression cassettes into the vector (Table 2.3) (Fig. 5.4 (A,B,C)). Subsequent DNA sequencing confirmed the faithful construction of the multi-copy vectors.

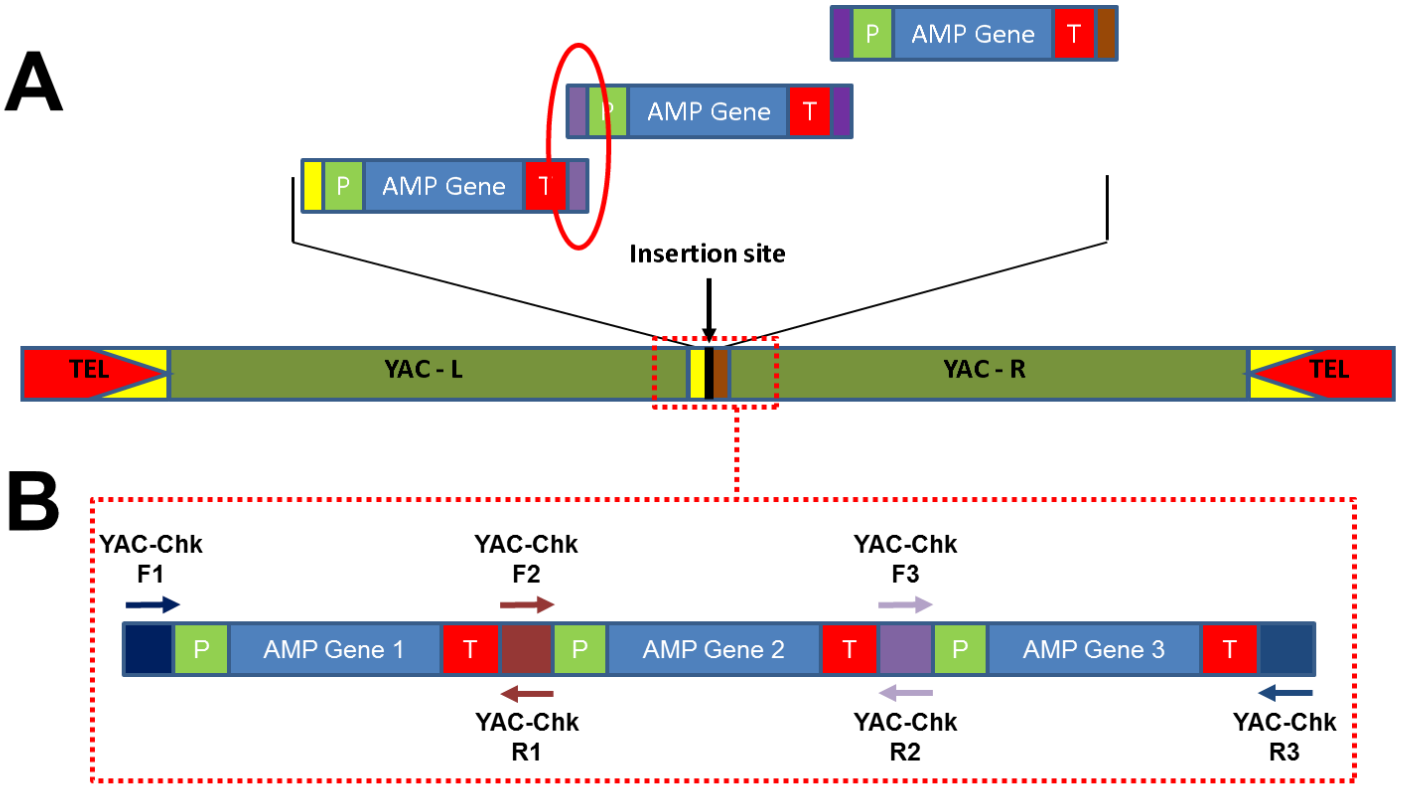


Fig. 5.2 (A) Schematic representation of recombination strategy used to construct the multi-rAMP cassette pYAC-Kan vectors. Each copy of the rAMP gene cassettes is first amplified by PCR using primers designed to add a tail which is homologous to the pYAC-Kan vector. As subsequent cassette integrations are performed, a unique "barcode" sequence forms between each cassette which allows PCR based confirmation of the cassette integration using a set of barcode specific primers. The overlapping barcode regions are circled in red. **(B) Schematic representation of the positions of the barcode specific "Chk" primer sets.** The primers were designed to amplify the rAMP gene cassettes by priming within the unique DNA sequences of the barcode regions. Panel B is also an exploded view of the inserted sequence of panel A.

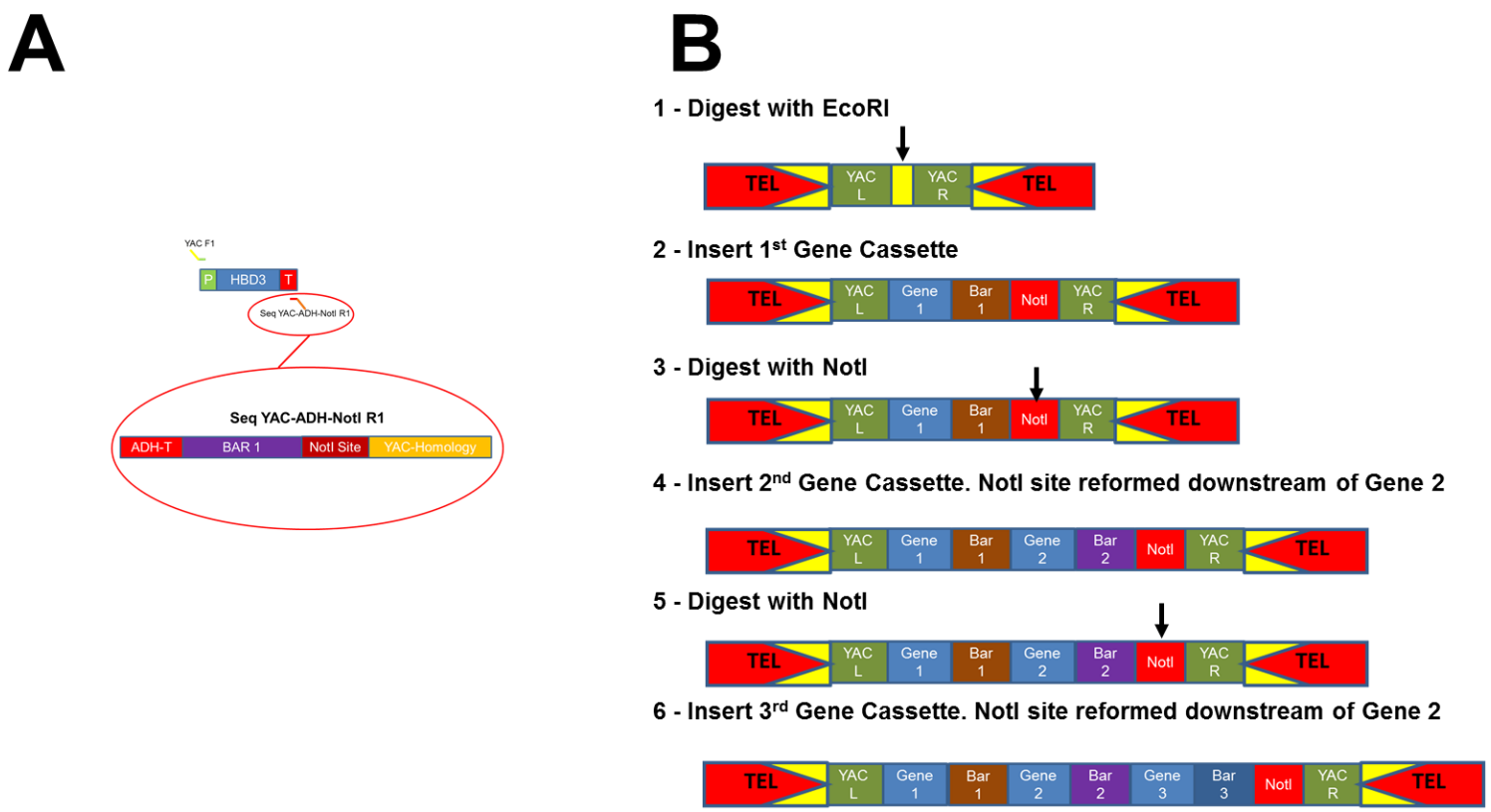


Fig. 5.3 (A) Schematic representation of the sequence formed by the barcoded NotI reverse primers. The primers were designed to prime the ADH terminator (red) of the rAMP gene cassettes adding a tail which is composed of the unique barcode sequence (purple), the NotI restriction enzyme recognition site (wine) and a sequence homologous to the site of integration within the pYAC-Kan vector. **(B) Schematic representation of the workflow to construct the multi-rAMP cassette copy vectors using the NotI system.** The rAMP expression cassette is first amplified using primers with homology to the pYAC-Kan vector. This product is transformed into *Saccharomyces* which integrates the cassette into the vector via homologous recombination (1). This forms the NotI recognition site between the gene cassette and the vector backbone. To insert the next expression cassette, the vector is first digested with the NotI restriction enzyme. The second cassette recombines with the barcode sequence from the first cassette and the vector backbone (2). The NotI restriction is now reformed downstream of the second integrated cassette. This workflow is repeated until the desired number of cassette integrations has been completed.

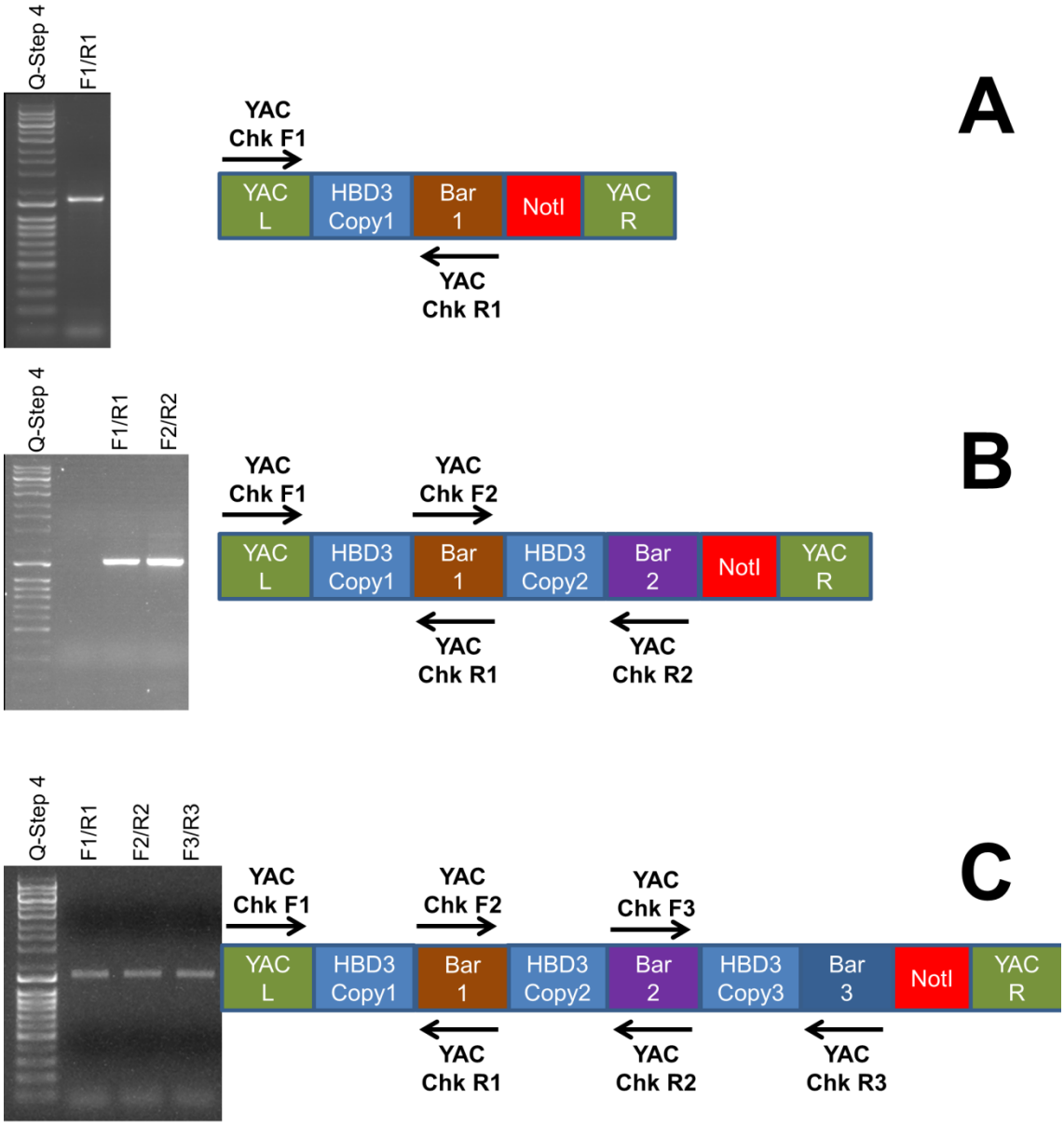


Fig. 5.4 Colony PCR verification of multi-rHBD3 pYAC-Kan expression vectors. Colony PCR was carried out using the barcode specific "Chk" primer sets. The products were separated by electrophoresis on a 1% agarose gel. The expected product size for all products was 1.3 kb. The schematic diagram to the right of each gel image represents the resultant construct. Indicated are the barcode sequences (Bar), the HBD3 expression cassettes (light blue), the NotI recognition sites (red) and the pYAC-Kan backbone (green). Also indicated are the priming positions of the Chk primers. **(A) pYAC-Kan 1xHBD3. (B) pYAC-Kan 2xHBD3. (C) pYAC-Kan 3xHBD3**

5.2.2 ELISA Based Analysis of *In-vivo* Antimicrobial Peptide Production from Multi-Copy HBD3 pYAC-Kan Expression System.

To analyse the level of recombinantly produced HBD3 from the multi-copy pYAC-Kan expression platforms, an anti-HBD3 enzyme linked immunosorbent assay (ELISA) was used. This analysis also used the high copy-number rHBD expression plasmid pRS42H-HBD3 which had previously been constructed in this lab and contained the same rHBD3 expression cassette used throughout this study. This study also sought to uncover the relationship between gene copy number and the relative levels of *in situ* peptide production.

Briefly, *S. pastorianus* carrying the expression platforms were inoculated into minimal medium, containing the appropriate selective antibiotic, at a cell density of $2x10^6$ cfu/ml and grown for 48h at 30°C. $2x10^{10}$cfu/ml equivalent samples were harvested for analysis. Samples were taken from intracellular lysates, cell surface washes and concentrated extracellular supernatants.

It was found that within the intracellular lysate samples, the single, double, and triple rHBD3 cassette pYAC-Kan vectors produced 2.425 μg/L, 3.617 μg/L and 6.553 μg/L respectively while the high copy pRS42H-HBD3 vector produced 9.532 μg/L (Fig. 5.5 (A)). Relative to the single rHBD3 cassette pYAC-Kan vector, the double gene copy and triple gene copy pYAC-Kan vectors produced 1.49 and 2.7 times the amount of the recombinant peptide. The high copy vector produced 3.9 times the amount of peptide.

Within the cell surface wash samples it was observed that single, double and triple cassette vectors produced 0.206 μg/L, 0.345 μg/L and 0.597 μg/L respectively while the high copy expression plasmid produced 0.96 μg/L (Fig. 5.5(B)). Relative to the single rHBD3 expression cassette vector the double and triple cassette copy vectors produced 1.7 and 2.9 times the amount of rHBD3 while the high copy vector produced 4.7 times the amount of recombinant peptide.

The levels of recombinant peptide found within the extracellular supernatant for the single gene copy, double gene copy and triple gene copy pYAC-Kan was found to be 0.021 μg/L, 0.042 μg/L and 0.059 μg/L respectively (Fig. 5.5(C)). The high copy expression plasmid was found to have secreted 0.083 μg/L into

the supernatant. Again, relative to the single gene copy pYAC-Kan vector the double and triple gene copy vectors produced 2 times and 2.8 times the levels of recombinant peptide while the high copy expression plasmid produced 3.97 times the amount of peptide.

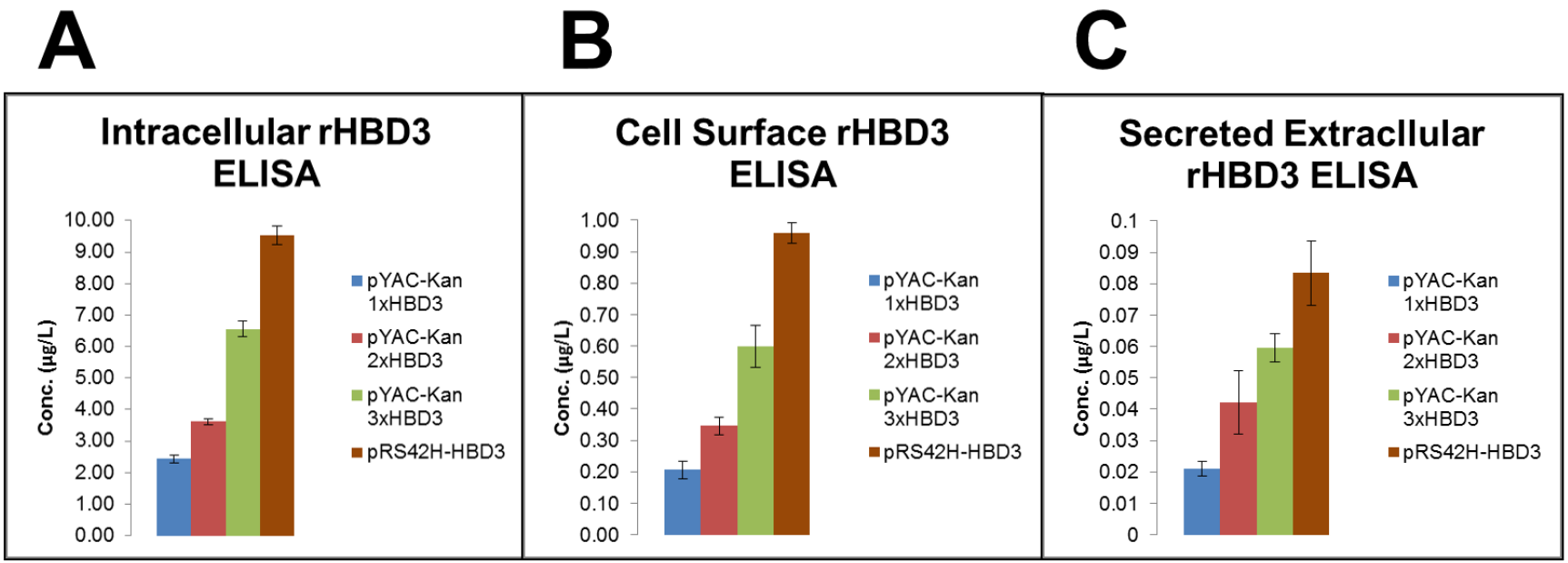


Fig. 5.5 Analysis of *in vivo* expression of rHBD3 peptide from multi-copy pYAC-Kan expression vectors and high copy pRS42H-HBD3 vector. *Saccharomyces pastorianus* carrying the pYAC-Kan expression vectors were seeded into CSM complete minimal media containing G418 at a cell density of $2x10^6$ cfu/ml. Cells were grown for 48h at 30°C. Samples corresponding to $2x10^{10}$ cells were harvested. The concentration of rHBD3 was determined by αHBD3 sandwich ELISA. Samples were analysed from **(A)** intracellular lysates, **(B)** cell surface and **(C)** extracellular supernatant. The recombinant peptide concentration is represented as µg/L. The assay was performed on 3 independent occasions each with 3 technical replicates. The error bars represent the standard deviation of the 3 biological replicates.

For a complete analysis of the relationship between gene copy number and the level of recombinant peptide production, it was necessary to determine the copy number of the high copy pRS42H-HBD3 expression plasmid relative to the single copy pYAC-Kan expression platforms. To achieve this, whole genome DNA, including the expression vector DNA, was isolated from *S. pastorianus* carrying the vectors and used to perform quantitative PCR (qPCR) analysis. Both the high copy pRS42H plasmid and the pYAC-Kan expression platform contain the ampicillin resistance gene AmpR which was used to determine the relative vector copy number. The *Saccharomyces* chromosomal Glyceraldehyde-3-phosphate dehydrogenase 3 (TDH3) gene was used to normalise all samples. All vector copy number data was expressed relative to the copy number of pYAC-Kan without a rHBD3 expression cassette.

It was found that the vector copy number of the single, double and triple rHBD3 cassette pYAC-Kan expression vectors did not vary significantly from that of the empty vector control with vector copy numbers of 0.99, 1.15 and 1.08 copies respectively (Fig. 5.6). The high copy number pRS42H-HBD3 vector was observed to have 26.01 copies in comparison to the vector control. Interestingly it was observed that the pRS42H vector control was present in the cell at a significantly higher copy number, 39.82 copies, than the same vector carrying the rHBD3 expression cassette.

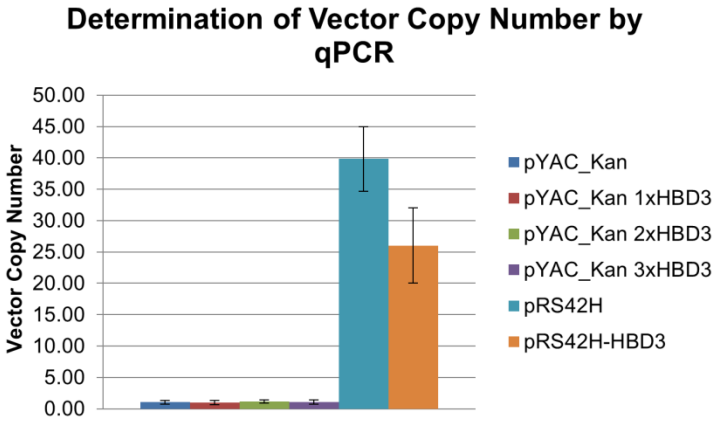


Fig. 5.6 Determination of the intracellular copy number of expression vectors. Whole genomic DNA was extracted from *Saccharomyces pastorianus* carrying the expression vectors. Extracted DNA was used to carry out qPCR analysis of the intracellular vector copy number. Primers specific to the AmpR gene carried by all vectors were used to determine the copy number. Threshold cycle values were normalised to the genomic housekeeping gene TDH3. Vector copy number values are expressed relative to the copy number values for pYAC-Kan. Analysis was carried out on two independent occasions with 3 technical replicates each. The error bars represent the standard deviation of two biological replicates.

These data taken together suggest a number of important conclusions. First, increasing the rHBD3 expression cassette copy number within the cell does indeed increase the total amount of *in situ* produced peptide. This increase was however not found to have a linear relationship with gene copy number. Further to this, when taken together with the recombinant peptide production data for the high copy number pRS42H-HBD3 vector, it would seem that there is an upper limit to the increase in amount of peptide that can be produced by increasing gene copy number; although 26.01 copies, on average, of the high copy number vector were found to be present within the cell, the level of recombinantly produced peptide was found only to increase by around 4 times that of the single expression cassette copy pYAC-Kan vector.

It could also be concluded that the majority of *in situ* produced recombinant peptide, ~90%, remains within the cell and is not secreted into the extracellular space. It was also seen that, of the total amount of peptide that was secreted by the cell, only around 10% of this was found in the extracellular supernatant with the remainder being found associated with the cell surface.

5.2.3 Analysis of Stability of circular *vs.* linear pYAC4 Expression Systems under Non-Selective Conditions

One of the major goals of the study described in this chapter was to create an expression platform which was maintained stably within the cell without the need for selective pressure. As described previously, the pYAC-Kan expression platform can exist in two forms; a circular plasmid form and, when digested with the restriction enzyme BamHI, a linearised form (Fig. 5.1(A)). When linearised in this manner, the telomere sequences which flank the BamHI restriction enzyme recognition sites are exposed and become the terminal ends of the linear vector.

To assess the intracellular stability of both of these forms of the vector, *S. pastorianus* carrying the pYAC-Kan circular and linear expression platforms were grown overnight in the presence of G418. The overnight cultures were subculture into YEPD medium without selective pressure at a cell density of $1x10^6$ cfu/ml and grown for 48h at 30°C. Samples were harvested, diluted appropriately, and plated onto both YEPD and YEPD + Geneticin agar. The data were represented as % plasmid in comparison to the total colony counts for the non-selective YEPD agar plates.

It was found that, for the circular forms of the expression platform, the plasmid loss for the empty pYAC-Kan, pYAC-Kan 1xHBD3, pYAC-Kan 2xHBD3 and pYAC-Kan 3xHBD3 was 39.9%, 56.9%, 63.1% and 73.2% respectively (Fig. 5.7(A)).

With regard to the linearised vectors, the plasmid loss for empty pYAC-Kan, pYAC-Kan 1xHBD3, pYAC-Kan 2xHBD3 and pYAC-Kan 3xHBD3 was found to be 12.5%, 28.1%, 42.2% and 51.2% respectively (Fig. 5.7(B)).

These data suggested a number of things. First, the circular form of the expression platform was not found to be very stable. When the vectors were linearised, thus exposing the telomere sequences, the intracellular stability of the vectors was found to have increased.

The third important conclusion suggested by these data was that the presence of a rHBD3 expression cassette on the expression platform created some

burden to the cell and reduced the intracellular stability of the vector both in the circular and linear forms. This burden was seen to increase with increasing cassette copy number.

Overall these conclusions would suggest that, due to the intracellular instability of a yeast artificial chromosome based expression platform, their use in an industrial setting would provide further challenges.

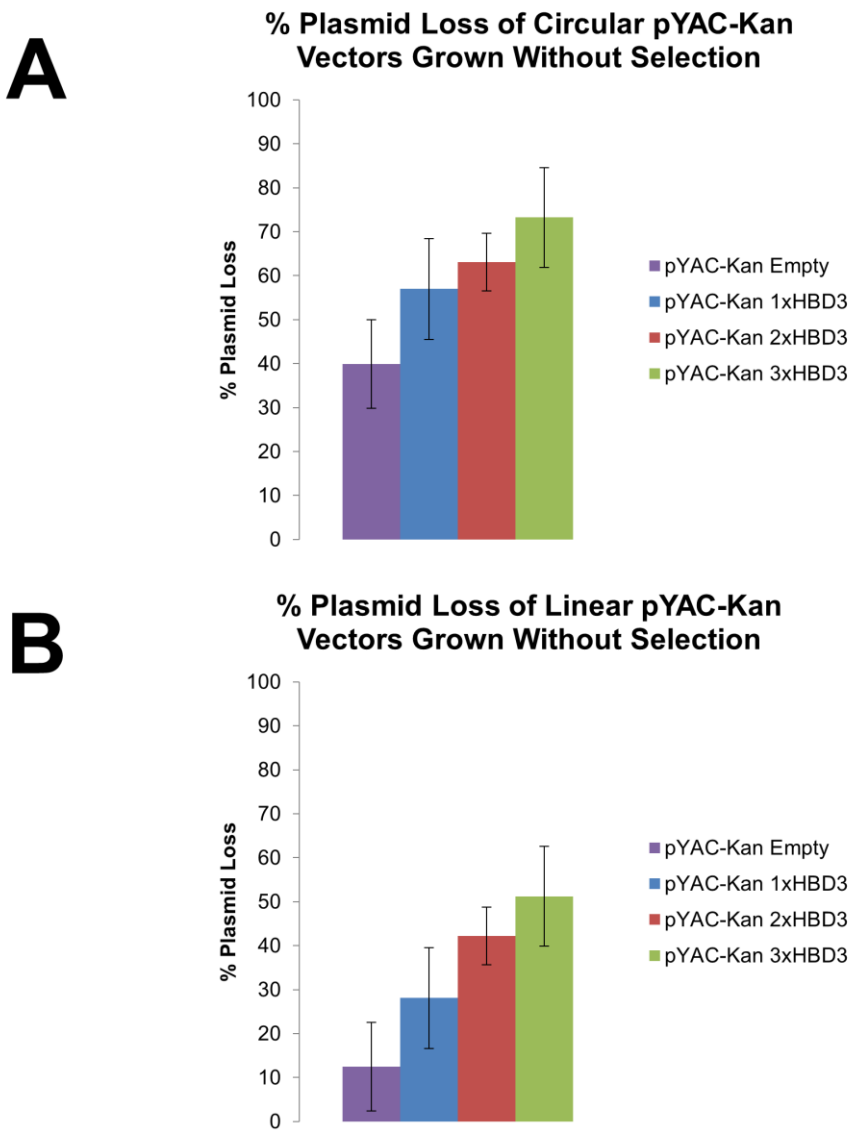


Fig. 5.7 Analysis of the *in vivo* stability of the multi-rHBD3 copy pYAC-Kan expression vectors without selective pressure. *Saccharomyces* strains containing the respective pYAC-Kan vectors were inoculated into YEPD medium without selective pressure at a cell density of $1x10^6$ cfu/ml and incubated for 48h. Cultures were diluted and samples were plated onto YEPD agar plates. One set of samples were plated onto media without selective pressure while the other set were plated onto media containing the antibiotic G418. Results are reported as % plasmid loss based on the non-selective plate counts. Error bars are reported as standard deviation of two independent biological replicates each comprising 3 technical replicates.

5.2.4 Construction of New Antimicrobial Peptide Expression Systems

5.2.4.1 Construction of *Saccharomyces*-optimised Antimicrobial Peptide Genes

As described in the introduction to this chapter, the second major part of the work described in this part of the study was to express plant derived AMP's, discussed in previous chapters of this work, *in situ* from *S. pastorianus*.

The genes for the AMP's Cp-thionin, Fabatin and the 3-Arg analogue of the Mungbean defensin PDF-1 do not exist in nature in a compatible codon bias to be efficiently expressed *in situ* for *Saccharomyces*. Due to this, it was necessary to create the open reading frames for these genes *In vitro*. To achieve this, the primary amino acid sequences of these peptides were reverse translated into nucleic acid sequences which were optimised to the *Saccharomyces* codon bias. A set of overlapping oligonucleotides for each of these optimised open reading frames were designed and synthesised (Table 2.4). These oligonucleotides were used to synthesise the full length gene open reading frames using polymerase chain assembly (PCA). The success of this assembly was analysed by resolving the PCA products by agarose gel electrophoresis. The expected product sizes of the Cp-Thionin, Fabatin and 3-Arg analogue peptide open reading frames were 218 nt, 216 nt and 216 nt. Gel electrophoresis analysis confirmed that the assembly protocol had been successful.

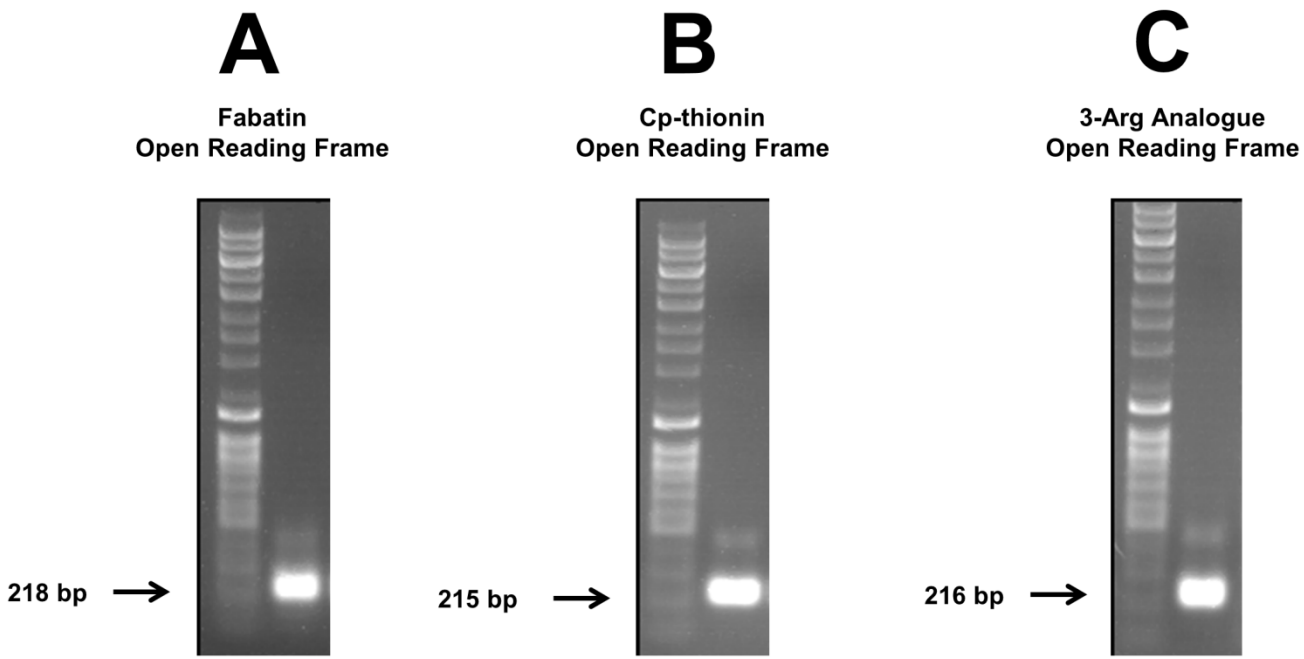


Fig. 5.8 PCR verification of polymerase chain assembly products. Polymerase chain assembly products were amplified by PCR using primers which added a tail homologous to the alpha factor secretion signal and the ADH terminator of pRS42H-HBD3. Products were separated on a 1% agarose gel. Lane 1 of each gel contains 5µl of Q-Step 4 DNA Ladder. **(A)** Fabatin open reading frame amplified using PGK-αFactorSS-LL44C-F and LL44-ADH R primers. Expected product was 218bp. **(B)** Cp-thionin open reading frame amplified using PGK-αFactorSS-KT43C-F and KT43-ADH R primers. Expected product was 215bp. **(C)** 3-Arg analogue peptide open reading frame amplified using PGK-αFactorSS-KC47-F and KC47-ADH R primers. Expected product was 216bp.

5.2.4.2 Construction of High Copy pRS42H based plasmid expression systems

The assembled PCA products were next used to create a set of high copy expression vectors based on the pRS42H plasmid system. The donor vector used to achieve this was pRS42H-HBD3 previously used in this study. This vector contained the selective marker for resistance to Hygromycin B; hphNT1. This vector also contained the rHBD3 expression cassette previously described in this study (Fig. 5.9). This cassette was composed of the rHBD3 open reading frame translationally fused to an alpha-factor secretion signal. Transcription from the expression cassette was driven by a *Saccharomyces bayanus* phosphoglycerate kinase (sbPGK) promoter and was terminated by an alcohol dehydrogenase (ADH) terminator (Fig. 5.9).

To construct the expression vectors for the newly constructed genes, the PCA products were first amplified using primer pairs which added a 5' tail which was homologous to the alpha-factor secretion signal nucleotide sequence and a 3' tail which was homologous to the ADH terminator nucleotide sequence (Table 2.3). A unique AscI restriction enzyme recognition site was contained within the rHBD3 open reading frame of the donor vector. After digestion of the donor vector using this enzyme it was co-transformed into *Saccharomyces pastorianus* along with the tailed PCR products. After selection of transformants on Hygromycin B plates, candidate colonies which had correctly integrated the open reading frames for the newly synthesised genes were screened by colony PCR using the same primer pairs YAC-F1 and Seq-YAC NotI R1 (Table 2.3), which amplified from the start of the PGK promoter and the end of the ADH terminator respectively (Fig. 5.9(A,B,C)). Subsequent DNA sequencing confirmed that the expression vectors had been faithfully constructed.

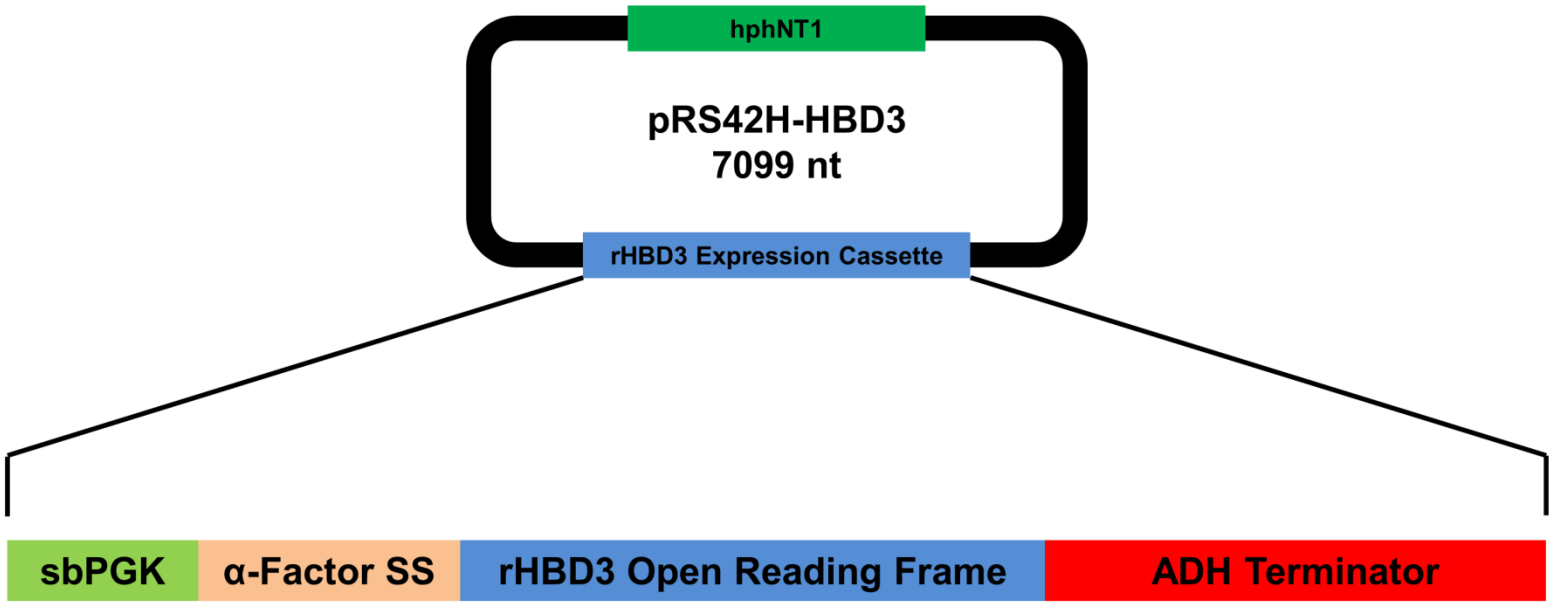


Fig. 5.9 Schematic representation of the pRS42H-HBD3 rHBD3 expression vector. The high copy number rHBD3 expression vector contains the Hygromycin B resistance cassette hphNT1 (dark green). The rHBD3 expression cassette is composed of the *Saccharomyces bayanus* PGK promoter (light green) driving transcription of the *Saccharomyces* alpha factor secretion signal (light brown) and the recombinant HBD3 open reading frame (blue). Transcription is terminated by the ADH terminator (red).

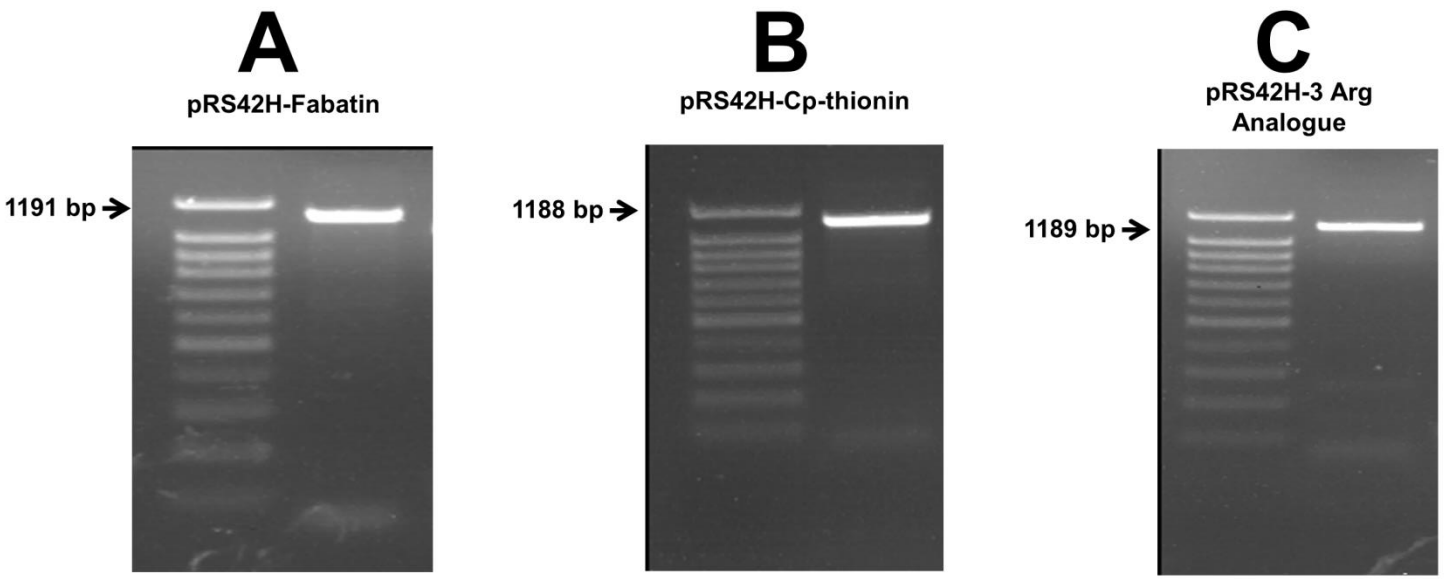


Fig. 5.10 Colony PCR verification of high copy number plasmid expression systems. Colony PCR was performed using primers located 50 bp upstream and downstream of the insert site (pRS42H Check F and pRS42H Check R). Products were separated on a 1% agarose gel. Lane 1 of each gel contains 5µl of Q-Step 1 DNA Ladder. **(A)** pRS42H-Fabatin. Expected product was 1191bp. **(B)** Prs42h-Cp-thionin. Expected product was 1188bp. **(C)** pRS42H-3Arg Analogue. Expected product was 1189bp.

5.2.5 Analysis of *In-vivo* Peptide Production from High Copy pRS42H Expression Systems

Before any further analysis could be conducted using the newly created recombinant expression vectors discussed in the previous section, it was necessary to confirm that the peptides were being produced *in situ* from *S. pastorianus*. Recombinant expression from the HBD3 expression cassettes was confirmed and quantified using ELISA analysis. This was facilitated using an antibody raised against the HBD3 peptide directly. At the time of this study no antibodies directed against the primary amino acid sequence of Cp-Thionin, Fabatin or the 3-Arg analogue peptide (rationally designed in chapter 4) were available. To circumvent this problem it was decided to determine if the recombinant peptides were being produced by mass spectrometry analysis.

To achieve this, *S. pastorianus* strains carrying the high copy expression vectors for Cp-Thionin, Fabatin and the 3-Arg analogue peptide were grown for 48h at 30°C and samples of intracellular lysates, cell surface washes and extracellular supernatant were harvested. The harvested samples were further processed by reverse phase high precision liquid chromatography (RP-HPLC). Candidate RP-HPLC fractions were collected based upon the elution profiles of synthetic versions of the AMP's previously discussed in this study. The isolated candidate fractions were fragmented by trypsin digestion and analysed using a Q-Exactive hybrid quadrupole-orbitrap mass spectrometer. The raw data were analysed with the Proteome Discoverer software suite using a custom library composed of the primary amino acid sequences of Cp-Thionin, Fabatin and the 3-Arg analogue peptide (Table 2.2).

In silico analysis of the primary amino acid sequence of Cp-Thionin predicted that trypsin digestion would yield 8 peptide fragments (Fig. 5.11(A)). The analysed data discovered 16 Cp-Thionin peptide fragments from the intracellular lysate fraction, 16 from the cell surface wash fraction and 12 fragments from the extracellular supernatant fraction (Fig. 5.11(B)). The largest of these fragments was 17 amino acids long which equated to 37.8% coverage of the 45 amino acid sequence.

The digest profile of the primary amino acid sequence of the Fabatin peptide predicted that 9 fragments should be present after trypsin digestion (Fig. 5.12(A)). The data indicated that 8 peptide fragments were found in the intracellular lysate fraction, 7 were found in the cell surface wash fraction and 6 in the extracellular supernatant fraction (Fig. 5.12(B)). The largest discovered fragment was 21 amino acids long, covering 44.7% of the 47 amino acid sequence.

The primary amino acid sequence of the 3-Arg analogue peptide was predicted to yield 11 peptide fragments after trypsin digestion (Fig. 5.13(A)). The results of the mass spectrometry analysis showed that 14 peptide fragments were found in the intracellular lysate fraction, 12 in the cell surface was fraction and 9 in the extracellular supernatant fraction (Fig. 5.13(B)). The longest of these was found to be 18 amino acids in length and covered 38.3% of the total 47 amino acids.

A

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KTCMTKKEGWGRCLIDTTCAHSCRKYGYMGGKCQGITRRCYCLLN

B

Confidence	Sequence	# PSMs	ΔCn	XCorr	Charge	MH+ [Da]	ΔM [ppm]	RT [min]	# Missed Cleavages
Intracellular Lysate									
High	KYGYMGGKcQGITR	3	0	4.27	3	1618.78324	0.21	19.88	2
High	EGWGRcLIDTTcAHScR	1	0	3.66	4	2078.89443	-0.47	25.03	1
High	cLIDTTcAHScR	5	0	3.21	3	1493.63202	1.73	21.04	0
High	YGYmGGKcQGITR	3	0	2.62	2	1506.68169	-0.77	20.02	1
Medium	RcYcLLNc	3	0	1.79	2	1158.48491	-0.35	26.06	1
Medium	cYcLLNc	3	0	1.63	2	1002.38329	-0.9	32.27	0
Medium	YYcRVRGGR	3	0	1.29	2	1186.58818	-1.41	24.04	2
Low	KYGYmGGK	1	0	0.77	2	919.43352	-0.79	17.23	1
Low	KYGYmGGKcQGITR	4	0	0.66	3	1634.78702	5.63	22	2
Low	TcmTKKEGWGR	2	0	0.66	2	1369.63249	-1.97	22.56	2
Low	YGYmGGKcQGITRR	1	0	0.64	2	1662.79021	3.75	30.88	2
Low	EHLRSGR	1	0	0.6	2	854.45195	-8.44	31.5	1
Low	YGYMGGKcQGITRR	2	0	0.54	2	1646.80046	6.93	35.8	2
Low	KTcMTKK	2	0	0.26	2	896.47185	2.88	21.23	2
Low	cQGITRR	1	0	0.22	2	890.46031	-2.49	42.07	1
Low	cLIDTTcAHScRK	1	0	0.19	2	1621.71575	-5.34	33.13	1
Cell Surface									
High	EGWGRcLIDTTcAHScR	1	0	4.14	4	2078.8976	1.05	25.03	1
High	YGYMGGKcQGITR	1	0	3.81	2	1490.6873	-0.42	21.36	1
High	cLIDTTcAHScR	6	0	3.61	2	1493.62712	-1.55	21.4	0
High	YGYmGGKcQGITR	2	0	3.36	2	1506.67998	-1.91	20.03	1
High	RcYcLLNc	1	0	2.33	2	1158.481	-3.72	26.07	1
High	KYGYMGGK	1	0	2.12	2	903.43907	-0.29	18.83	1
Medium	KYGYMGGKcQGITR	2	0	1.98	3	1618.78141	-0.93	19.92	2
Medium	KYGYmGGK	1	0	1.17	2	919.43389	-0.39	17.23	1
Medium	KTcMTKK	2	0	0.98	2	896.46044	-9.85	28.28	2
Low	cLIDTTcAHScRK	3	0	0.71	3	1621.72263	-1.09	19.66	1
Low	YGYMGGKcQGITRR	1	0	0.66	2	1646.79546	3.89	48.16	2
Low	TcmTKKEGWGR	3	0	0.59	2	1369.6436	6.14	29.73	2
Low	TcMTKKEGWGR	1	0	0.58	3	1353.63864	-1.2	20.53	2
Low	cQGITRR	1	0	0.3	2	890.46147	-1.19	42.03	1
Low	YGYmGGKcQGITRR	1	0	0.25	2	1662.7907	4.05	30.03	2
Low	KYGYmGGKcQGITR	1	0	0.15	2	1634.78557	4.74	20.82	2
Extracellular Supernatant									
High	cLIDTTcAHScR	3	0	3.55	2	1493.62712	-1.55	21.39	0
High	KYGYMGGK	1	0	2.58	2	903.4392	-0.15	18.8	1
Medium	KYGYmGGKcQGITR	1	0	1.25	3	1634.7808	1.82	25.7	2
Low	TcmTKKEGWGR	2	0	0.7	2	1369.62517	-7.32	35.47	2
Low	YGYmGGKcQGITR	1	0	0.65	2	1506.66924	-9.04	44.03	1
Low	HcKNKEHLR	3	0	0.52	2	1221.61846	-6.97	22.17	2
Low	YGYmGGKcQGITRR	1	0	0.42	2	1662.78765	2.21	29.98	2
Low	cLIDTTcAHScRK	1	0	0.24	2	1621.73467	6.33	25.06	1
Low	KTcMTKK	1	0	0.24	2	896.47215	3.22	21.25	2
Low	TcMTKKEGWGR	1	0	0.07	2	1353.63689	-2.5	23.32	2
Low	cQGITRR	1	0	0.06	2	890.46251	-0.03	42.13	1
Low	KTcmTKK	1	0	0.05	2	912.46269	-1.63	42.97	2

Fig. 5.11 Confirmation of *in vivo* expression of recombinant Cp-thionin from high copy number pRS42H vector by mass spectrometry analysis. Samples of intracellular lysates, cell surface washes and extracellular supernatant were digested with trypsin and analysed using a Q-Exactive LC/MS system. **(A) Amino acid sequence of Cp-Thionin.** Arrows indicate predicted trypsin digest sites. **(B) Positively identified peptide fragments.** Q-Exactive raw files were queried using a custom peptide sequence library with the proteome discoverer software suite.

A

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LLGRCKVKSNRFNGPCLTDTHCSTVCRGEGYKGGDCHGLRRRCMCLC

B

Confidence	Sequence	# PSMs	ΔCn	XCorr	Charge	MH+ [Da]	ΔM [ppm]	RT [min]	# Missed Cleavages
Intracellular Lysate									
High	FNGPcLTDTHcSTVcRGEGYK	2	0	6.61	4	2459.05263	-0.46	23.17	1
High	FNGPcLTDTHcSTVcR	13	0	5.32	3	1924.80777	-1.12	23.9	0
High	SNRFNGPcLTDTHcSTVcR	2	0	4.71	4	2281.9894	1.48	21.94	1
High	GEGYKGGDcHGLR	2	0	2.57	3	1405.62796	0.13	17.7	1
Medium	GGDcHGLR	1	0	1.2	2	871.38432	0.42	42.59	0
Low	GEGYKGGDcHGLRR	1	0	0.64	2	1561.73357	2.99	29.67	2
Low	GGDcHGLRRR	1	0	0.35	2	1183.59477	7.25	28.75	2
Low	GGDcHGLRR	1	0	0.21	2	1027.47832	-6.58	40.97	1
Cell Surface									
High	FNGPcLTDTHcSTVcRGEGYK	2	0	5.62	3	2459.05057	-1.3	23.15	1
High	SNRFNGPcLTDTHcSTVcR	2	0	4.67	4	2281.98305	-1.3	21.87	1
High	FNGPcLTDTHcSTVcR	8	0	4.6	3	1924.81034	0.21	22.21	0
High	GEGYKGGDcHGLR	4	0	2.58	3	1405.62769	-0.07	17.77	1
Medium	GGDcHGLR	4	0	1.4	2	871.38414	0.21	41.94	0
Low	GEGYKGGDcHGLRR	3	0	0.69	3	1561.72849	-0.27	26.48	2
Low	GGDcHGLRR	1	0	0.24	2	1027.47661	-8.24	40.86	1
Extracellular Supernatant									
High	FNGPcLTDTHcSTVcR	2	0	5.29	3	1924.80777	-1.12	23.97	0
High	GEGYKGGDcHGLR	3	0	2.4	3	1405.6276	-0.13	17.73	1
Medium	GEGYKGGDcHGLRR	5	0	1.35	3	1561.72702	-1.2	29.65	2
Low	FNGPcLTDTHcSTVcRGEGYK	1	0	0.8	3	2459.04874	-2.04	23.48	1
Low	LLGRcKVK	2	0	0.67	2	973.60362	6.2	35.53	2
Low	GGDcHGLRR	1	0	0.28	2	1027.47795	-6.93	40.86	1

Fig. 5.12 Confirmation of *in vivo* expression of recombinant Fabatin from high copy number pRS42H vector by mass spectrometry analysis. Samples of intracellular lysates, cell surface washes and extracellular supernatant were digested with trypsin and analysed using a Q-Exactive LC/MS system. **(A) Amino acid sequence of Fabatin.** Arrows indicate predicted trypsin digest sites. **(B) Positively identified peptide fragments.** Q-Exactive raw files were queried using a custom peptide sequence library with the proteome discoverer software suite.

A

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KTCRNLANTYRGPCFTTGSCRRHCKNKEHLRSGRCRDDFRCWCTRNC

B

Confidence	Sequence	# PSMs	ΔCn	XCorr	Charge	MH+ [Da]	ΔM [ppm]	RT [min]	# Missed Cleavages
Intracellular Lysate									
High	cRDDFRcWcTR	3	0	4.33	4	1631.66298	0.31	21.36	2
High	TcRNLANTYR	5	0	3.92	3	1268.61783	1.08	18.97	1
High	GPcFTTGScR	6	0	3.07	2	1142.47258	0.69	21.77	0
High	DDFRcWcTR	3	0	2.98	3	1315.53046	-0.19	25.13	1
Medium	NLANTYRGPcFTTGScR	2	0	2.09	3	1974.89129	0.17	24.34	1
Medium	KTcRNLANTYR	5	0	2.03	3	1396.71155	0.08	17.76	2
High	NLANTYR	3	0	2.02	2	851.43614	-1.02	20.74	0
Medium	cRDDFR	1	0	1.73	2	868.37334	0.33	47.62	1
Medium	SGRcRDDFR	1	0	1.21	2	1168.5258	-1.6	21.97	2
Medium	HcKNKEHLR	4	0	1.06	2	1221.63371	5.52	20.97	2
Low	NKEHLRSGR	3	0	0.65	2	1096.60539	7.6	47.17	2
Low	EHLRSGR	1	0	0.59	2	854.45195	-8.44	31.53	1
Low	GPcFTTGScRR	1	0	0.41	2	1298.57048	-1.87	18.02	1
Low	EHLRSGRcR	2	0	0.33	2	1170.57939	-9.86	29.29	2
Cell Surface									
High	cRDDFRcWcTR	2	0	4.27	4	1631.6625	0.01	21.36	2
High	TcRNLANTYR	4	0	4	2	1268.61601	-0.36	18.98	1
High	GPcFTTGScR	3	0	3.11	2	1142.47466	2.51	22.9	0
High	KTcRNLANTYR	8	0	3.11	2	1396.71099	-0.32	17.74	2
High	DDFRcWcTR	4	0	2.97	3	1315.53019	-0.4	25.16	1
High	NLANTYR	6	0	1.97	2	851.43535	-1.95	20.69	0
Medium	cRDDFR	1	0	1.7	2	868.37334	0.33	47.54	1
Medium	SGRcRDDFR	1	0	1.43	2	1168.52226	-4.63	22.01	2
Low	GPcFTTGScRR	1	0	0.7	2	1298.57329	0.29	17.8	1
Low	EHLRSGRcR	1	0	0.43	2	1170.59966	7.45	22.85	2
Low	NLANTYRGPcFTTGScRR	2	0	0.41	3	2130.99643	2.04	44.46	2
Low	HcKNKEHLR	2	0	0.24	2	1221.63591	7.32	47.78	2
Extracellular Supernatant									
High	GPcFTTGScR	1	0	3.02	2	1142.47173	-0.05	21.04	0
High	NLANTYR	10	0	1.95	2	851.43706	0.06	20.2	0
Medium	KTcRNLANTYR	4	0	1.37	3	1396.71164	0.15	17.84	2
Low	HcKNKEHLR	3	0	0.76	2	1221.6314	3.62	20.92	2
Low	TcRNLANTYR	2	0	0.67	2	1268.6176	0.9	19.01	1
Low	NKEHLRSGR	2	0	0.56	2	1096.58806	-8.21	40.44	2
Low	SGRcRDDFR	1	0	0.5	2	1168.52287	-4.11	21.89	2
Low	EHLRSGRcR	4	0	0.41	2	1170.60088	8.49	25.68	2
Low	NLANTYRGPcFTTGScRR	1	0	0.02	2	2130.99785	2.71	52.55	2

Fig. 5.13 Confirmation of *in vivo* expression of recombinant 3-Arg analogue peptide from high copy number pRS42H vector by mass spectrometry analysis. Samples of intracellular lysates, cell surface washes and extracellular supernatant were digested with trypsin and analysed using a Q-Exactive LC/MS system. **(A) Amino acid sequence of 3-Arg analogue peptide.** Arrows indicate predicted trypsin digest sites. **(B) Positively identified peptide fragments.** Q-Exactive raw files were queried using a custom peptide sequence library with the proteome discoverer software suite.

Taken together, these data show that the peptides are being produced *in situ* from the *S. pastorianus* strains harbouring the newly constructed expression plasmids. The discovery of a greater number of peptide fingerprint fragments than was predicted by the *in silico* trypsin digest analysis was due to missed cleavages. Although a number of these fragments were returned with a low confidence score, they were still above the analysis threshold.

These data also indicated that the recombinant peptides were being secreted from the cell and were found in the same intra- and extra-cellular locations that recombinant HBD3 was found by ELISA analysis. Although no quantitative analysis with regard to peptide concentration was conducted during this analysis, qualitative conclusions can be drawn from these data. The fact that fewer peptide fragments in total, and fewer high confidence fragments, were found in the extracellular supernatant fractions would indicate that the peptide concentrations in these fragments were lower than that of the other analysed sample fractions.

5.2.6 Analysis of *In-vivo* Antimicrobial Activity of High Copy pRS42H Expression Systems

The overall goal of this work was to construct a set of expression vectors for the *in situ* production plant based AMP's in *S. pastorianus* in order to control the growth of food spoiling microorganisms. To access the efficacy of these systems, it was decided to test their *In Vivo* antimicrobial activity against the beer spoiling bacterium *Lactobacillus brevis*.

To achieve this *S. pastorianus* strains expressing the AMP's were co-cultured with *L. brevis* in YEPD or brewers' wort at a specific gravity of 1.04. The yeast were seeded at a cell density of $1x10^{10}$ cfu/ml while the bacteria were seeded at a cell density of $2x10^{3}$ cfu/ml. The challenges were conducted for 24h after which samples were plated onto agar plated containing the eukaryotic specific antibiotic cyclohexamide which allowed enumeration of the bacteria. These assays were conducted at both 30°C and the more industrially relevant temperature of 10°C which is the average temperature used for beer fermentation. Control of bacterial growth was assessed in comparison to an empty vector control.

It was found that in YEPD at 30°C, after 24h, the rHBD3 expressing yeast strain had limited bacterial levels to $2.1x10^{5}$ cfu/ml which was just 5.03% of the bacterial levels found in the control samples (Fig. 5.14(A)). Neither the recombinant Cp-Thionin nor Fabatin expressing strains controlled bacterial growth to any significant degree. The recombinant 3-Arg analogue peptide expressing strain limited bacterial growth to $1.6x10^{6}$ cfu/ml. This was 39.3% of the bacterial levels observed in the control samples.

At the more industrially relevant temperature of 10°C, rHBD expressing strain had suppressed bacterial growth to $1.1x10^{4}$ cfu/ml which was 2.8% that of the control samples (Fig. 5.14(B)). At this temperature rCp-Thionin expressing yeast strain suppressed bacterial growth to $2.9x10^{5}$ cfu/ml which equated to 74.1% of the control samples. The rFabatin expressing strain limited bacterial growth to $3.4x10^{5}$ cfu/ml, this was 84.8% of the bacterial levels observed in the control samples. The 3-Arg analogue peptide expressing yeast strain reduced bacterial growth to $1.3x10^{5}$ cfu/ml which was 31.2% of the control samples.

When the assay was conducted in brewers' wort it was found that at 30°C, the rHBD3 expressing *S. pastorianus* strain reduced bacterial growth to $6.1x10^3$ cfu/ml which was 2.1% of the levels of bacteria found in the control samples (Fig. 5.14(C)). The recombinant Cp-Thionin expressing yeast limited bacterial levels to $2.3x10^5$ cfu/ml or 78.7% that of the control samples. The Fabatin expressing strain constrained bacterial growth to $2.6x10^5$ cfu/ml, this was 89.9% of the control samples. The 3-Arg analogue peptide expressing strain reduced bacterial growth to $1.2x10^5$ cfu/ml which was 41.7% of control bacterial levels.

At 10°C it was observed that the rHBD3 expressing strain decreased growth of *L. brevis* to $1.5x10^3$ cfu/ml which was 0.9% of the levels of growth observed in the control samples (Fig. 5.14(D)). The Cp-Thionin producing yeast strain reduced bacterial growth to $9.7x10^4$ cfu/ml or 63.2% that of the control. The Fabatin expressing strain limited bacterial growth to $1.3x10^5$ cfu/ml, this was 83.7% of the bacterial levels in the control samples. The yeast strain producing the recombinant 3-Arg analogue peptide reduced bacterial growth to $3.9x10^4$ cfu/ml which was 25.4% that of the control samples.

A number of conclusions could be drawn from these data. First, the recombinant HBD3 expressing yeast strain was the most effective at controlling bacterial growth. Second, at the lower temperature, all expressing strains were more effective at controlling bacterial spoilage. Finally, when the levels of bacterial control were compared between the various antimicrobial expressing yeast strains a clear correlation could be observed between the levels of control produced *In Vivo* and the *In vitro* IC_{90} values observed in previous sections of this study.

Overall it was observed that a higher level of bacterial control could be produced when the assay was conducted in brewers' wort in comparison with the assays conducted in YEPD. A number of factors could explain this observation. First, the lower pH of brewers' wort, ~pH5.5, in comparison to that of YEPD which has a pH of ~6.5 could partially explain the increase in *In Vivo* antimicrobial activity. As discussed in previous sections of this study, these AMP's were all observed to have increased activity in lower pH in *In vitro* antimicrobial assays. A second factor which could have an effect is the

concentration of sugars within the growth medium. Brewers' wort with a specific gravity of 1.04 is composed of approximately 12% w/w of a complex mixture of sugars. In comparison to this, YEPD contains just 2% sugar.

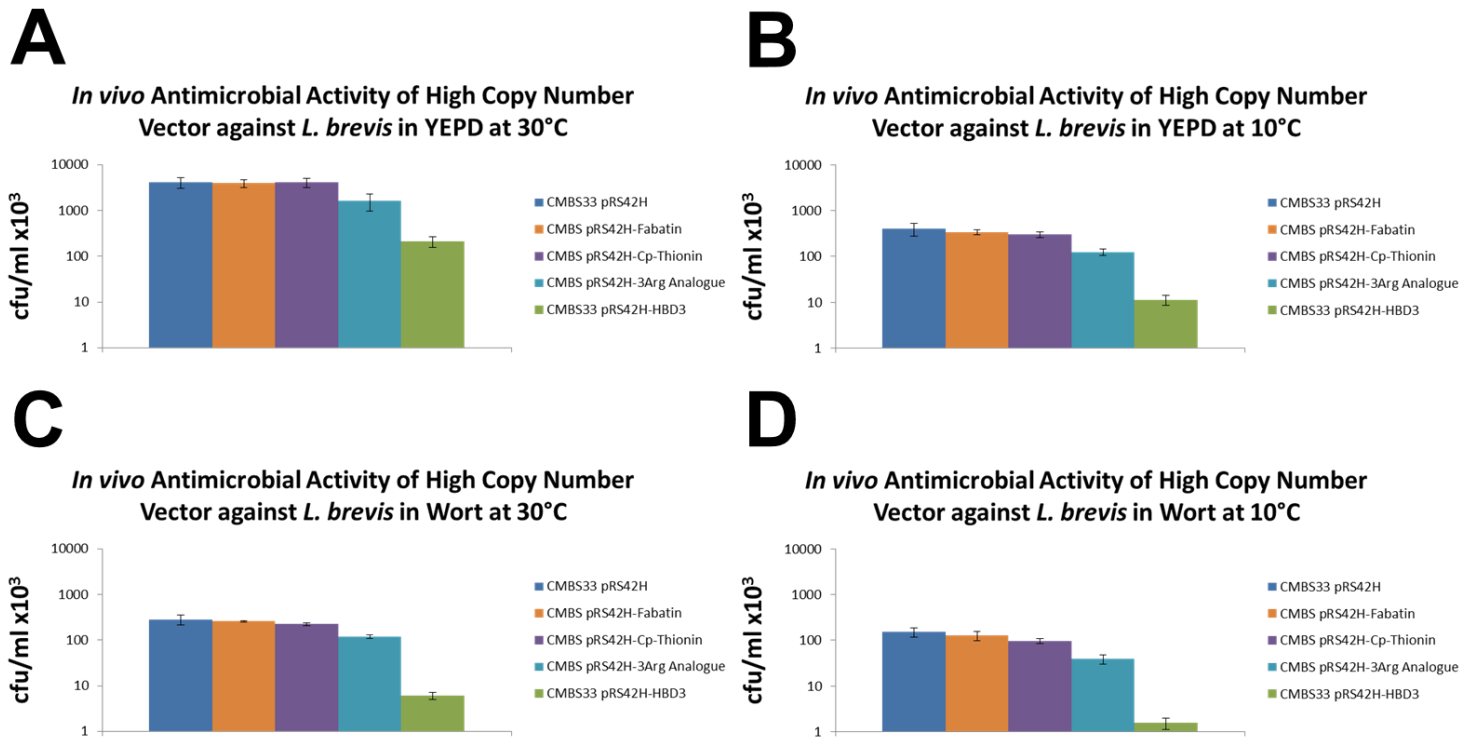

Fig. 5.14 Determination of the *in vivo* antimicrobial activity against *L. brevis* of recombinant antimicrobial peptides expressed from *Saccharomyces pastorianus*. *Saccharomyces pastorianus* strains expressing the antimicrobial peptides from the high copy number pRS42H vector were co-cultured with *L. brevis* in (A,B)YEPD media or (C,D) Brewers wort. The *Saccharomyces* strains were seed at a density of $1x10^{10}$ cfu/ml while *L. brevis* were seeded at a density of $2x10^{3}$ cfu/ml. After being challenged for 24h at **(A,C)** 30°C or **(B,D)** 10°C, bacteria were diluted and plated onto YEPD agar containing the eukaryotic specific antibiotic cyclohexamide which allowed the enumeration of the bacteria. The assay was conducted on 3 independent occasions with 3 technical replicates each. The error bars represent standard deviation of the 3 biological replicates.

5.3 Discussion

The work discussed within this chapter sought to answer a number of important questions surrounding the *In Vivo* expression of recombinant AMP's from *Saccharomyces pastorianus*. As described in the introduction, classic recombinant expression systems are time consuming to construct. Also, when not integrated into the genome, require selective pressure for the expression vectors to be stably maintained within the cell. This selective pressure comes in the form of either antibiotic or auxotrophic selection. The former is neither cost effective nor ethical for large scale industrial use while the latter is not possible with most industrial yeasts and processes in use today (Casewell *et al.*, 2003).

To circumvent these limitations, it was attempted to design a synthetic expression platform which was modular in nature, allowing easy and rapid integration of multiple recombinant expression. This platform was also designed to be stably maintained within the cell without the need for selective pressure.

This expression system was based on the, single copy, yeast artificial chromosome pYAC4 and was intended to be used within the larger yeast *Saccharomyces pastorianus* (Fig. 5.1(A)). Unfortunately the only selectable markers, compatible with yeast, on this vector, were auxotrophic in nature and the *S. pastorianus* CMBS33 used in this study has no auxotrophies. This required that an antibiotic resistance cassette be integrated into the TRP1 gene of the pYAC4 parental vector, to create the vector pYAC-Kan, to allow for initial selection of successful transformants (Fig. 5.1(B)). The initial hypothesis was that once successful transformants were confirmed, antibiotic selection would not be necessary for downstream applications.

To make this synthetic expression platform modular in nature, a system was designed which would make use of the natural ability of *Saccharomyces* to carry out homologous recombination of DNA (Fig. 5.2(A)). To achieve this, a set of primers were designed, which, when used amplify donor recombinant peptide expression cassettes, would add 5' and 3' tails to the cassettes. These tails would create unique regions of homology between the expression cassettes which would allow their integration by homologous recombination and would create unique, cassette specific, barcodes which could be used to confirm the

correct integration of the cassettes (Fig. 5.3(A)). This system was also designed to insert a recyclable NotI restriction enzyme recognition site which would increase the cassette integration success rate by allowing the vector to be linearised at the intended site of integration (Fig. 5.3(B)).

Using this system 3 vectors were constructed which carried 1, 2 or 3 copies of a recombinant human beta defensin (rHBD3) expression cassette; these vectors were designated pYAC-Kan 1xHBD3, pYAC-Kan 2xHBD3 and pYAC-Kan 3xHBD3 (Fig. 5.4(A,B,C)). This part of the study showed that indeed a vector could be constructed which was modular in nature, allowing the rapid insertion of multiple expression cassettes.

Although this part of the study was successful, when the question of the intracellular stability of the vector without selection was queried, it was shown that the vector was, overall, unstable (Fig. 5.7(A,B)). This disproved the initial hypothesis that antibiotic selection would not be necessary for the stable intracellular maintenance of the vector. It was shown that this instability directly correlated with the number of copies of the recombinant expression cassettes present on the expression platform. This was most likely due to some burden associated with the *in situ* production of rHBD3 within the cell. Although previous *In vitro* work conducted within this lab had shown that synthetic HBD3 was not toxic to *Saccharomyces,* production of the peptide within the cell could lead to some other unfavourable intracellular interaction (Kraszewska *et al.*, 2016). This poses new challenges which must be explored before industrial use of this system would be *via*ble.

This expression system did allow another important question to be answered. Is there a linear relationship between recombinant gene copy number and the level of peptide expressed *in situ* from *Saccharomyces pastorianus* and, if so, is there an upper limit to this linearity.

The ELISA analysis conducted to answer this question showed that there is a somewhat linear relationship between gene copy number and peptide production (Fig. 5.5). It also showed however that there is an upper limit to this linearity; although there was, on average, 26 copies of the high copy expression vector per cell, only ~4 times the amount of rHBD3 was found to be produced

with this system in comparison the single gene copy pYAC-Kan vector (Fig. 5.6). Further investigation would be necessary to determine the nature of this apparent fitness cost. For example, reconstructing the vectors using random peptides known not to possess antimicrobial activity could provide valuable data which may uncover the reason.

This analysis also showed that the vast majority, ~90%, or the recombinantly produced peptide was found within the intracellular space and was not secreted out of the cell. There are a number of possible explanations for this secretory bottleneck. Correct folding, disulfide bond formation and vesicle trafficking are required for efficient protein secretion (Hou *et al.*, 2012).

This secretory bottleneck could also partially explain the upper limit in the linearity between expression gene copy number and amount of peptide produced *in situ*. If the translation and translocation machinery of the cell is overloaded, then increasing gene dosage will have little if any effect on increased peptide production.

Removal of this bottleneck may also alle*via*te the problem of intracellular instability of the synthetic pYAC-Kan expression platform. As was discussed earlier, the intracellular instability of the vector increased as the expression cassette copy number increased; this suggested that there was some burden to the cells carrying these expression cassettes. If the problem was associated with intracellular accumulation of the recombinant peptide, then increased secretion of the peptide should remove this burden thus increasing the stability of the vector to approximately that of the empty pYAC-Kan vector; this would be sufficiently stable for industrial use.

The second major part of this chapter sought to design and construct a set of vectors for the *in situ* expression of Cp-Thionin, Fabatin and the 3-Arg analogue peptides from *S. pastorianus*. The genes for these peptides did not exist in nature in a *Saccharomyces* compatible codon bias. Accordingly, it was necessary to construct the open reading frames for these genes *In vitro*. Polymerase chain assembly was used to accomplish this and these genes were subsequently inserted into high copy expression plasmids based on the rHBD expression plasmid pRS42H-HBD3 previously constructed by this lab (Fig. 5.8,

Fig. 5.10). This produced the vectors pRS42H-Cp-Thionin, pRS42H-Fabatin and pRS42H-3Arg analogue.

At the time of this analysis, there were no commercially available antibodies directed against these AMP's which meant that the detection of recombinant peptide production by ELISA or Western blot analysis was not possible. Indeed, attempts to visualise HBD3 *via* Western blotting were unsuccessful. This may be due to the low levels of expression observed *via* ELISA analysis. To circumvent the problem of analysing the production of the peptides for which no antibodies existed, conformation of *in situ* peptide expression was carried out using mass spectrometry analysis. This analysis showed that the peptides were indeed being produced by *S. pastorianus* (Fig. 5.11, Fig. 5.12, Fig. 5.13). These data also showed that the peptides partitioned into the same intracellular and extracellular locations as recombinantly expressed HBD3, as demonstrated by the ELISA analysis discussed earlier. Qualitatively, these data also suggest that the same secretory bottleneck may exist with these newly constructed expression vectors as was observed with the recombinant HBD3 expression systems.

The overall goal for this section of the study was to produce a set of expression vectors which could be used with *S. pastorianus* to control bacterial growth in yeast based foods. To examine if this had been accomplished, the beer spoiling bacterium *Lactobacillus brevis* was used to carry out *In Vivo* analysis of the antimicrobial activity of these expression vectors (Fig. 5.14(A,B,C,D). This analysis was carried out in both YEPD and brewers' wort at both 30°C and 10°C. It was felt that the analysis conducted at 10°C in brewers' wort was the most industrially relevant choice of conditions in relation to the use of these strains in the brewing industry. It was found that, in all conditions, the recombinantly expressed HBD3 was the most effective at controlling bacterial growth followed by the 3-Arg analogue peptide expressing yeast. Neither the Cp-Thionin nor the Fabatin expressing strains produced a significant level of bacterial control. A direct correlation could be observed between these *In Vivo* data and the *In vitro* antimicrobial analysis discussed in previous sections of this study. If this correlation were to hold true for the 5-Arg analogue peptide (discussed in chapter 4) then that peptide may provide a similar level of *In Vivo*

antimicrobial activity to that of the HBD3 expression system and ultimately may provide a *via*ble alternative to this human derived peptide for use in the food industry.

The analysis showed that the most effective control of bacterial growth, for all peptide expressing strains, was observed to be in brewers' wort at 10°C. There are a number of possible explanations for this. First, the pH of brewers' wort is significantly lower than that of YEPD (He *et al.*, 2014). As has been shown in previous sections of this study, synthetic versions of these peptides have significantly better antimicrobial activity in low pH conditions. Second, the sugar content of brewers' wort is higher than that of YEPD. This increases the osmotic potential of the medium above that of YEPD which may act in a synergistic fashion with the AMP's, by stressing the bacteria and increase their effectiveness at controlling microbial growth (Chirife *et al.*, 1983). The third possible explanation for this observation surrounds the composition of brewers' wort itself. Brewers' wort is a complex mixture of sugars and other components, including native AMP's, derived from barley grains (Szilagyi *et al.*, 2018, Correa de Carvalho *et al.*, 2018, Colilla *et al.*, 1990, Mendez *et al.*, 1990). These native AMP's may also act in synergy with the peptides expressed from *S. pastorianus*.

In conclusion, it was demonstrated that a synthetic peptide expression platform, based on a yeast artificial chromosome which was modular in nature could be constructed. It was found however that this expression platform was unstable while no selective pressure was applied and that this instability was related to a cellular burden associated with the expression of recombinant antimicrobial peptide. It was also shown that there was a somewhat linear relationship between gene dosage and *in situ* antimicrobial production but that there was a limit to this linear relationship which may be associated with the secretion bottleneck also observed. It was also shown by mass spectrometry analysis that the genes for Cp-Thionin, Fabatin and the 3-Arg analogue peptides could be created and used to construct expression vectors which were compatible with *in situ* peptide production by *S. pastorianus*. It was also demonstrated that these recombinantly expressed peptides partitioned to the same cellular locations observed for the rHBD3 expression system and that their secretion may be

plagued by the same bottleneck. It was seen that, of the newly constructed expression vectors, *S. pastorianus* harbouring the pRS42H-3Arg analogue system was the most effective at controlling bacterial growth *In Vivo*. Also, it was shown that most industrially relevant assay conditions produced the most efficacious response in an *In Vivo* setting.

Overall this work has shown that plant based antimicrobial peptide expression systems can be created which can control microbial contamination but that, at present, classical genome integrated expression platforms may still be the most appropriate systems for use in an industrial setting. It has also shown however that expression of AMP's from *S. pastorianus* may not be efficient enough, in the current form, to control microbial contamination in an industrial setting. Indeed, production of AMP's in an industrial setting is more often carried out using other yeasts such as *Pichia pastoris* which offer much higher levels of peptide production (Garrigues *et al.*). The use of *S. pastorianus* should still be developed in the future however as it offers the advantage of being used directly to control microbial contamination in a brewery setting.

Chapter 6:

General Discussion

6.1 General Discussion

The identification and characterization of new AMP's offers many novel avenues for the control of food borne pathogens and spoilage organisms. The development of novel therapeutics such as these would provide a *via*ble alternative to conventional food preservation technologies, the use of which is costly and chemical additives can pose health risks (Anon, 2019, Joardder and Masud, 2019). Human beta-defensin-3 (HBD3) had previously been shown by this lab to be an effective control measure for the beer spoilage bacterium *Lactobacillus brevis* both *In vitro* and *In Vivo* (James *et al.*, 2014). The use of a biotechnology based delivery system such as this to produce peptides *in situ* from *Saccharomyces pastorianus* would provide a highly cost effective platform to control microbial spoilage in a brewery setting. In addition to this, spent yeast, which has been engineered to produce AMP's, could be used as animal feed as a cost effective alternative to classic prophylactic antibiotic use in agriculture sector which is now banned (Casewell *et al.*, 2003, Ocheme Julius *et al.*, 2017).

However, although HBD3 provides effective microbial control, it was felt that given this peptide is of human origin the public perception surrounding its use as a food additive may not be widely accepted. Instead, this study sought to discover plant based peptides which provide similar levels of microbial control *In Vivo* to that of HBD3 as the public has long accepted plant based food additives (Karunaratne and Pamunuwa, 2017).

There are many obstacles to the discovery of novel peptides with effective antimicrobial activity. The identification of peptides based on previously reported activity is not possible due to the lack of standardised comparative analysis. The fact that defensin-like peptides form part of the innate immune systems of all higher eukaryotes and have been evolving for a long time means that they are quite divergent at the primary sequence level (Lehrer *et al.*, 1991, Machado and Ottolini, 2015).

Although defensin-like peptides are highly divergent at the primary sequence level, they are quite conserved at the tertiary structural level (Antcheva *et al.*, 2013). This offered a novel avenue by which to discover peptides with similar antimicrobial activity to that of the HBD3 benchmark used throughout this study.

The peptides uncovered by this process could then be used to identify the peptide characteristics which are critical for effective antimicrobial activity which could then be used to rationally design a set of peptides with increased antimicrobial activity.

The overall goal would then be to synthesis the genes for these peptides for their expression *in situ* from *Saccharomyces pastorianus* with the aim of controlling the growth of the beer spoilage organism *Lactobacillus brevis*. This bacterium was chosen because it is the most important and persistent brewery contaminant (Bokulich and Bamforth, 2013). To completely characterise the antimicrobial activity of these peptides, other bacteria and fungi should be tested in the future.

The initial bioinformatic discovery process uncovered 53 plant derived defensin-like AMP's (Table 3.1). A number of AMP families such as the "Knottins", "Snakins" and "Scorpion-like toxins" were known to be cytotoxic to mammalian cells (Harrison *et al.*, 2014). Due to this, it was decided to remove any candidates which were structurally similar to these unwanted motifs. This filtering process reduced the candidate peptide list to 34 molecules.

The final stage of the discovery process involved tertiary structural comparisons between the structures of the candidate peptides and that of the benchmark HBD3 structure. Although the classic defensin fold is well conserved evolutionarily, there are some subtle global variations with regard to the orientation of these secondary structural elements (Antcheva *et al.*, 2013). This presented some problems with regard to direct structural comparisons. To circumvent this, an alignment method which focused on global rather than local alignment was employed (Xu and Zhang, 2010, Zhang and Skolnick, 2004). This process identified 3 candidate peptides which were structurally similar to HBD3; Cp-Thionin-2, Fabatin-2 and the Mungbean defensin PDF-1 (Fig.3.1).

In vitro antimicrobial analysis revealed that only Cp-Thionin-2 and Fabatin-2 were effective at reducing the survival of *L. brevis* under physiologically relevant conditions (Fig. 3.2). It had been shown previously that the antimicrobial activity of many defensin-like peptides, including HBD3, was sensitive to high osmotic potential in the assay environment (Kraszewska *et al.*, 2016). For peptides

whose primary mode of action is membrane permeation, it was believed that the initial interaction between defensin-like peptides and the bacterial cell surface was mediated by the binding of the cationic peptide to the anionic head groups of the cell membrane lipids (Sudheendra *et al.*, 2015). The presence of ionic species was shown previously to disrupt this interaction and reduce the antimicrobial activity of these peptides (Kandasamy and Larson, 2006).

To determine if this was the case for these candidate peptides, the assay was conducted in a low ionic buffer where it was seen that all of the peptides had increased antimicrobial activity under these conditions (Fig. 3.2). This suggested that cationic charge was an important determinant of antimicrobial activity.

To further probe the potential relationship between peptide charge and antimicrobial activity, these assays were conducted in high and low pH environments. Under low pH conditions, the peptides were predicted to have increased cationic character while the inverse was true of high pH conditions (Table 3.2). It was found that, under low pH conditions the antimicrobial activity of all tested peptides was increased above that of the activity observed at neutral pH (Fig. 3.3(A,B)). Conversely, the antimicrobial activity was found to be decreased under high pH assay conditions (Fig. (3.4(A,B)). This suggested that net cationic charge was indeed an important determinant of antimicrobial activity against *L. brevis*. In all assays conducted so far, the most effective antimicrobial activity was observed for HBD3 followed by Cp-Thionin and Fabatin. The Mungbean defensin was found to have significantly lower activity. When arranged by cationic charge, a clear positive correlation could be observed between positive charge and antimicrobial activity; this reinforced the hypothesis that charge was the most important physiochemical determinant of antimicrobial activity.

To investigate if the tertiary structure played any role in the antimicrobial activity of these peptides, the assays were conducted in the presence of DTT which reduced the stabilising disulfide bonds (Fig. 3.5). It was found that although not the most important determinant of antimicrobial activity, tertiary structure was still important for effective antimicrobial activity. It should be noted however that it had been observed previously that reduction of peptide disulfide bonds using

DTT treatment caused the peptides to become susceptible to degradation (Marcos, 2019, Pers. Comm.). To fully determine if the observed reduction in antimicrobial activity was due to the reduction of the disulfide bonds and not peptide degradation, the peptide stability would need to be assessed in the future.

It was also necessary to determine if the candidate peptides were thermostable as many food production processes involve thermal treatment and the intended use of these peptides was food preservation (Bressani, 2003). It was found that the most thermolabile peptide was HBD3 (Fig. 3.6). The most thermostable was found to be Mungbean defensin followed by Cp-Thionin and Fabatin. Due to the significantly increased concentration of Mungbean peptide required to produce an antimicrobial effect, it was decided that Cp-Thionin would be the most suitable for large scale industrial use.

Given the positive correlation observed between peptide charge and antimicrobial activity (Table 3.3), the first hypothesis to be tested using the rational design process was that the activity of a defensin-like peptide could be increased by increasing the cationic charge of the peptide. The Mungbean defensin was chosen as the lead backbone upon which to make changes. The rationale behind this decision was based on the fact that no activity, antimicrobial or otherwise, had previously been observed for the Mungbean defensin (Lin *et al.*, 2013). Using this peptide as a parental scaffold sequence would allow the elucidation of the peptide characteristics required for antimicrobial activity without any interference from other residues which may possess antimicrobial activity; the antimicrobial activity caused by these residues on peptides of known activity may mask any changes in activity caused by the creation of analogue peptides. The initial rationally designed analogue peptides replaced 3 and 5 negatively charged residues to create the 3-Arg and 5-Arg analogues respectively (Fig. 4.2).

The antimicrobial activity of these peptides was found to be significantly increased above that of the lead Mungbean defensin molecule (Fig. 4.9(A,B)). It was also observed that the higher charged, 5-Arg, analogue was the most effective analogue peptide at reducing *L. brevis* survival. The same salt sensitivity described previously in section 3.2.2.1 was observed with these

analogue peptides also. The antimicrobial activities of the analogue peptides were also found to react similarly to assay environment pH as was seen in section 3.2.2.2 and it was found that in low ionic strength buffer at pH4, the 5-Arg analogue peptide had more effective activity than HBD3 (Fig 4.9(B)). This gave weight to the hypothesis that a peptide could be designed which had increased antimicrobial activity in comparison to the lead molecule simply by increasing the positive charge of the molecule. However it was also found that increasing the charge alone was not sufficient to overcome the effect of competing ions in the assay environment as the Mungbean defensin in low pH environment had a greater charge than either Fabatin or Cp-Thionin at neutral pH but it possessed no activity at physiologically relevant salt concentrations.

The second approach to the rational design probed whether one could increase the antimicrobial activity of a peptide by increasing the structural fluidity of the molecule, allowing it to interact with the bacterial surface in an “Induced Fit” manner?

To answer this question, 3 further peptides were designed, based on the 5-Arg analogue peptide. The first two peptides attempted to reduce the overall structural rigidity of the molecule by removing cysteine residues. The first such peptide had all cysteine residues replaced; this formed the All Cysteine Knockout analogue. The second such peptide analogue to be designed replaced all of the internal cysteine residues, leaving only the two cysteine residues which formed the disulfide bridge between the N and C-termini; this formed the Internal Cysteine Knockout analogue (Fig.4.3). The third analogue peptide based on this rationale was designed to more closely resemble the structure of the N-terminus of HBD3; this was the N-terminal fluid analogue. Although the modelled structures of the All Cysteine and internal cysteine knockout analogues were not found to de*via*te significantly from that of the parental 5-Arg analogue structure, the tertiary structure, as determined by *in silico* modelling, for the N-terminal fluid analogue was found to be vastly different; this molecule was found to have a strong alpha helix on both the N- and C-termini (Fig. 4.3). The modelling process also predicted that no intramolecular disulfide bonds were formed.

When the antimicrobial activity of these peptides was examined, it was seen that all of the alterations made to create the analogue peptides reduced the effectiveness of the peptides at reducing bacterial survival and increased the salt sensitivity of the antimicrobial activity above that of the parental molecule (Fig. 4.9(A,B)). The antimicrobial activity of the all cysteine knockout analogue and the N-terminal fluid analogue, in both high and low osmotic conditions, was seen to increase when the pH of the assay environment was decreased (Fig. 4.10(A,B)). Interestingly, it was observed that, in comparison to the assay conducted at pH7, the internal cysteine knockout analogue had increased antimicrobial activity at low pH in low osmotic conditions but reduced activity in the high osmotic conditions. This molecule had reduced activity overall in the higher pH8 assays (Fig. 4.11(A,B)). This may suggest that the presence of any ions disrupts the antimicrobial activity of this peptide.

The third subset of analogue peptides to be designed sought to investigate the minimal peptide size to provide effective antimicrobial activity against *L. brevis* to that seen with the parental molecule. These again were based on the 5-Arg analogue peptide. The first of these analogues was designed to have the N-terminal proximal beta strand of the parental molecule deleted while the second had both the N- and C-terminal beta strands deleted; these were designated 1 Strand deletion analogue and 2 Strand deletion analogue respectively (Fig. 4.6).

It was found that the activities of this set of analogue peptides was lower than that of the parental 5-Arg peptide (Fig. 4.9(A,B)). Indeed it was found that the 2 Strand deletion analogue was the most salt sensitive peptide designed in this study.

When the effect of the assay environment pH was examined with these analogue peptides, it was found that for the 1 Strand deletion analogue, the antimicrobial activity was increased in low pH and decreased at high pH in both high and low osmotic conditions (Fig. 4.10(A,B) and 4.11(A,B)). Interestingly, as was observed with the internal cysteine knockout analogue, the antimicrobial activity of the 2 Strand deletion analogue was decreased in low pH, under high osmotic conditions, in comparison with neutral pH. This again suggested that any ionic species in the assay environment reduced the antimicrobial activity for this subset of peptides. Another explanation may be that the two stages to

membrane insertion of the peptides, initial ionic interaction and subsequent hydrophobic insertion into the lipid bilayer are affected independently by net charge and peptide structural changes respectively.

Overall this analysis showed that small analogue peptides with antimicrobial activity similar to that of the parental molecule could not be designed.

When the importance of disulfide bond formation was investigated it was found that again correct formation of these bonds was necessary for effective antimicrobial activity (Fig. 4.12). Unsurprisingly, a clear negative correlation was observed between the number of cysteine residues present in the peptide sequence and the antimicrobial activity in the presence of the reducing agent DTT. These data also suggested that analogue peptides had correctly formed their disulfide bridges. This was an important observation as no experimental evidence of this had been previously been seen throughout this study. However, again, the stability of the peptides would need to be fully assessed in order to firmly stand by this statement. The data, reported throughout the primary literature to date, were not in agreement on the importance of disulfide bonds for effective antimicrobial activity (Ma *et al.*, 2016, Haag *et al.*, 2012). In direct contrast to what has been observed throughout this work it had been previously reported that HBD3 did not require disulfide bonds for activity against *Streptococcus pneumonia* and *E. coli* (Kluver *et al.*, 2005). It should be noted however that the concentration of HBD3 used in that study was significantly higher than was used in this study. The fact that this study challenged a different bacterium than the ones used in the reported study could also account for the discrepancy in results. Overall the results of this study show very clearly that correct disulfide bond formation is required for effective antimicrobial activity.

A clear negative correlation between the number of disulfide bonds and the level of retained antimicrobial activity post-thermal treatment was observed (Fig. 4.13). The generally accepted hypothesis surrounding the thermostability of larger proteins states that disulfide bridges increases the thermostability of the protein activity (Bulaj, 2005, Matsumura *et al.*, 1989, Wetzel *et al.*, 1988). These results contradicted this hypothesis.

It has been shown here that the most thermotolerant peptides were the peptides with few or no disulfide bonds; these peptides could easily unfold during thermal treatment and were not structurally restricted while refolding. The next most thermotolerant peptides were the ones with four disulfide bonds which could resist thermally induced unfolding to a greater degree than the peptides with two or three disulfide bonds; resisting unfolding in this manner meant that they did not require a high degree of refolding. Peptides such as HBD3 with three disulfide bonds could unfold to a high degree but may become trapped in a local energy minimum upon refolding. This may suggest that there is a fine balancing act between the number of disulfide bonds which a peptide has, its ability to resist thermally induced unfolding and the propensity for misfolding when it cools down.

Another explanation could be that there may be some degree of peptide degradation upon thermal treatment and the ability to resist this may be linked to some other hidden characteristic of the peptide. To examine this in the future, as with the DTT treatment assays, the peptide stability would need to be investigated to uncover if the peptides are being degraded or if they remain misfolded after thermal treatment.

In conclusion, this part of the study demonstrated that designing analogue peptides with increased positive charge could increase the antimicrobial activity above that of the parental molecule. It also suggested that reducing the structural rigidity of the parental molecule reduced the antimicrobial activity but increased the thermotolerance of the molecule. It was also showed that small antimicrobial peptide analogues could be designed which had antimicrobial activity but not at the same level as the parental molecule. Further development of these peptides by altering characteristics such as hydrophobicity, amphipathicity or helicity may uncover peptides with increased antimicrobial activity beyond what was observed with these analogue peptides.

However it has been observed in the past that modifications such as these produced peptides that not only had increased antimicrobial activity but also possessed increased cytotoxicity (Edwards *et al.*, 2016, Inui Kishi *et al.*, 2018). Given that the overall goal of this study was to engineer peptides for food

preservation on an industrial scale, the cytotoxicity would need to be fully investigated.

The ultimate goal of this study was to create a set of *Saccharomyces* strains which would be used for the *in situ* production of recombinant defensin-like peptides to control bacterial contamination in yeast based foods, particularly brewery contaminants. As described in section 5.1, from personal experience, classic synthetic expression systems based on genome integrated cassettes are cumbersome and time consuming to construct. On the other hand plasmid based systems require selective pressure for their intracellular maintenance. This selective pressure generally comes in the form of antibiotic or auxotrophic selection. The former is cost prohibitive and ethically unsound for large scale use in the face of growing antibiotic resistance while the latter is not feasible as most industrial yeasts lack auxotrophies (Casewell *et al.*, 2003).

To circumvent these problems it was attempted to design a synthetic expression platform which was both modular in nature and would be stably maintained within the cell without the need for selective pressure. This platform was based on the, single copy, yeast artificial chromosome pYAC4 (Fig. 5.1(A)). This platform was intended for use in *Saccharomyces pastorianus* which lacked the auxotrophies necessary for the selection of initial genetic transformants. Because of this it was necessary to first create the vector pYAC-Kan by integrating an antibiotic resistance cassette conferring resistance to G418 into the TRP1 gene of pYAC4 (5.1(B)). After the initial selection of successful transformants it was intended that the intracellular stability of the vector would be mediated by the exposed telomere sequences negating the need for further antibiotic use.

To make this expression platform modular in nature, a primer system was designed which was used to amplify a rHBD3 expression cassette which had previously been constructed in this lab. These primers would add DNA tails to the PCR product which were homologous to the pYAC-Kan vector allowing *Saccharomyces* to natively carry out homologous recombination to construct the expression vectors (Fig. 5.2(A)). The primers also contained a unique NotI restriction site which was used to linearise the vector after each round of cassette integration allowing the subsequent integration of the next cassette

(Fig. 5.3(A,B)). After each round of integration the NotI restriction site was reformed downstream of the last expression cassette which allowed recycling of the NotI site (Fig. 5.3(B)). The primers were also designed in such a way that after each cassette integration, a unique barcode was formed between each cassette which allowed verification of cassette integration *via* PCR (Fig. 5.4).

This sequential strategy was used to construct pYAC-Kan 1xHBD3, pYAC-Kan 2xHBD3 and pYAC-Kan 3xHBD3 (Fig. 5.4(A,B,C)). The successful construction of these vectors showed that a modular expression platform could be created. Unfortunately it was found that these vectors, when carrying rHBD3 expression cassettes, were not stably maintained within *S. pastorianus* while no selective pressure was applied (Fig. 5.7(A,B)). A negative correlation could be observed between vector stability and the number of rHBD3 expression integrated into the vector. This suggested that some cellular burden existed with regard to *in situ* production of rHBD3 even though it had been shown previously that synthetic HBD3 had no *In vitro* antimicrobial activity against *Saccharomyces* (Kraszewska *et al.*, 2016). However, this study did not determine if HBD3 interacted with intracellular components of the yeast. This, and other challenges, would need to be analysed in the future before the expression platform would be suitable for industrial use.

These vectors did allow the question surrounding the possible linear relationship between the level of peptide expression and the expression cassette copy number to be probed. Understanding this relationship would uncover the most effective number of cassettes which were required to produce the AMP's at a level sufficient to control microbial contamination in an industrial setting. It was found that there was a somewhat linear relationship but that there was an upper limit to this linear relationship (Fig. 5.5). This analysis also showed that there was some secretion bottleneck with ~90% of the recombinantly produced peptide being found inside the cell. If the cellular translation and translocation machinery of the cell were becoming overloaded by the production of rHBD3, this bottleneck could partially explain both upper limit to the gene dosage/peptide production relationship and the intracellular instability of the expression platform. Removing this bottleneck in the future may

alle*via*te some of the burdens barring further development of the platform for industrial use.

Although this system could not be used to stably express recombinant versions of the newly discovered and designed peptides discussed earlier, they could be expressed from standard plasmid based systems. The genes for Cp-Thionin, Fabatin and the 5-Arg analogue did not exist in nature in a codon bias compatible with expression from *S. pastorianus*.

First, the genes for their open reading frames were synthetically constructed using polymerase chain assemble before being integrated into a donor expression plasmid to create pRS42H-Cp-Thionin, pRS42H-Fabatin and pRS42H-3Arg analogue (Fig. 5.8 and 5.10).

Given that there were no commercially available antibodies against the above peptides, it was necessary to determine if the peptides were being produced *in situ* from *S. pastorianus* by mass spectrometry. This analysis confirmed that the peptides were being expressed *in situ* (Fig. 5.11, 5.12 and 5.13). Further, this analysis also suggested that the same secretory bottleneck described earlier existed for these expression platforms also.

To examine if *S. pastorianus* expressing the recombinant peptides *in situ* could control bacterial contamination, a co-culture assay designed to challenge the survival of *L. brevis* was conducted. This assay was conducted in YEPD and brewers' wort at 30°C and the more industrially relevant temperature of 10°C (the average temperature at which many beer fermentations are carried out) (Fig. 5.14(A,B), Fig. 5.15 (A,B)). Under these conditions, the Cp-Thionin and Fabatin expressing *S. pastorianus* strains showed no significant control of bacterial growth. The 3-Arg analogue peptide expressing strain did control bacterial growth but with significantly lower effectiveness than the control afforded by the rHBD3 expressing strain. The greatest level of bacterial reduction, with all recombinant peptide expressing strains, was observed in brewers' wort at 10°C.

There are a number of possible explanations for this. First, the pH of brewers' wort is significantly lower than that of YEPD (He *et al.*, 2014). As has been

shown in previous sections of this study, synthetic versions of these peptides have significantly better antimicrobial activity in low pH conditions.

Second, the sugar content of brewers' wort is higher than that of YEPD (He *et al.*, 2014). This increases the osmotic potential of the medium above that of YEPD which may act in a synergistic fashion with the AMP's, by stressing the bacteria and increase their effectiveness at controlling microbial growth (Chirife *et al.*, 1983).

The third possible explanation for this observation surrounds the composition of brewers' wort itself. Brewers' wort is a complex mixture of sugars and other components, including native AMP's, derived from barley grains (Szilagyi *et al.*, 2018, Correa de Carvalho *et al.*, 2018, Colilla *et al.*, 1990, Mendez *et al.*, 1990). These native AMP's may also act in synergy with the peptides expressed from *S. pastorianus*. The general trend observed in the data from these *In Vivo* co-culture assays correlated with the data from the *In vitro* antimicrobial assay.

This would suggest that expression of the more potent 5-Arg analogue peptide from *S. pastorianus* may provide an effective level of control of the growth of *L. brevis* and may be a *via*ble alternative to HBD3 in an industrially setting. Future work may uncover if this is indeed the case.

In conclusion this work has shown that, a bioinformatic discovery approach to finding active plant derived defensin-like peptides based solely on tertiary structural comparisons is not an effective approach due to the fact that net positive charge is a major determinant of antimicrobial activity. It was shown however that tertiary structure is crucial for effective antimicrobial activity. It was also shown that a rational design based approach can be used to design peptides which are more effective than the parental molecule by increasing the positive character of the analogue peptides. It was seen that designing analogue peptides which either reduced the structural rigidity or the size of the parental molecule reduced the antimicrobial ability of these peptides below that of the lead molecule. This work also uncovered a negative correlation between the thermotolerance of antimicrobial activity and the number of disulfide bonds the peptide contained. It was found that a synthetic expression platform which was modular in nature could be constructed but was ultimately unstable without

selective pressure. This platform uncovered a somewhat linear relationship between gene copy and the level of recombinantly produced peptide but also showed that there was a limit to this linearity.

Finally, it was shown that the rationally designed 3-Arg analogue peptide could be expressed from the yeast *S. pastorianus* and could be used to effectively control contamination by *L. brevis* in an industrially relevant setting.

Overall, this work has shown that future development of recombinantly expressed AMP's could provide a cost effective and safe alternative to current food preservation strategies for industrial scale use.

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