Ollscoil na hÉireann

National University of Ireland, Cork

Coláiste na hOllscoile, Corcaigh

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A STUDY ON THE APPLICATION OF SYNTHETIC ANTIMICROBIAL PEPTIDES DERIVED FROM PLANTS FOR THE REDUCTION OF YEAST SPOILAGE IN FOOD

Thesis presented by

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BSc, MSc Food Microbiology

for the degree of

Doctor of Philosophy – PhD in Food Science and Technology

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February 2021

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

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Signature

Date: 26/02/2021

Acknowledgements

I would like to express my utmost gratitude to my supervisor **Prof. Elke Arendt**. Nearly 4 years ago, I was given the opportunity to join this incredible research group. There are not enough words to express just how grateful I am for this. Thank you for giving me a chance and for believing in me from the very beginning. I was able to grow as a person and as a scientist because of the opportunities you gave me. Thank you for allowing me to be free with my research and providing me with the tools to explore, to learn and to become the person I am today. I always knew that when things got too tough for me, you would always be there to make everything better again. You have always supported me and believed in me from the very beginning, even when there were times that I didn't believe in myself. I am so thankful for all the doors that you have opened for me. Thank you for being the best supervisor.

My first year and a half of my PhD would have been a lot more challenging if it hadn't been for **Dr. Thibaut Thery**. Thibaut, thank you for the countless times that you had to explain things to me. When I first started working in this research group, I had so much to learn. You took me under your wing and taught me so much from the very beginning. Thank you for not giving up on me and for helping me develop my love for science. Watching your brilliant mind at work helped me understand that there is so much more beyond what we already know. I am so grateful and so lucky to have worked alongside you and to have learned so much from you. Although it has been over 2 years since you left the group, there is a part of me that is still sad that you left so soon.

To my friend, colleague and postdoc supervisor, **Dr. Kieran Lynch**, thank you. Thank you for being the person that I needed during these years. I truly believe that this PhD

thesis would not have been possible without all your help. Thank you for spending hours and hours of your time with me, helping me improve my writing and become a better scientist. Thank you for all the discussions that we had together. I will always look back on the great times we had together in the group and look forward to the many more great times we will have together. I am eternally grateful for everything you have done for me.

Thank you to my dear friends **Rozi** and **Lilit**, both brilliant scientists and even better friends. We made so many memories together and I cannot wait to make more in the near future. And to all the friends I made throughout my time in this research group. I have made so many great memories because of you. Thank you to my Italian counterpart **Giulia** for being the best partner a girl could ask for. I am so grateful that I was able to do this project alongside you and that we found each other this way. Thank you to **Dr. Aylin Sahin** for always being there when I needed help and giving me the courage to always ask for help when I need it.

I want to thank **Prof. Aidan Coffey**, for allowing me to perform all those experiments in your lab. You always made me feel welcome in your research group and I always enjoyed going to CIT because of that.

A huge thank you to the technical and administrative staff in the department of food and nutritional sciences. Any time I was faced with a problem, big or small, I could always count on all of you to help me.

To my beloved family, I am indebted to you for all the support you have given me to get to where I am today. **Mum** and **Dad**, even when times are tough, you have always taught me to persevere and fight for what I deserve in life. I have gotten this far because of you. Thank you to my siblings **Sarah** and **Aissa**, even from far away, you made

sure I can always count on you for anything. To my dear friends **Barry** and **Rachael**, your friendship means the world to me and has helped me during some hard times. Thank you for always being there for me.

Thank you so much to **Robert**, my rock during the three years that we have been together. You have helped me a lot more than you realise. Thank you for listening to my problems (big or small) and for always having a happy house for me to come home to. It is my turn to spoil you now.

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Abbreviations

ANOVA	Analysis of variance
ADP3	Antimicrobial peptide database
ANN	Artificial neural networks
CLSM	Confocal laser scanning microscope
CD	Circular dichroism
CAP	Controlled atmosphere packaging
CSαβ-motif	Cysteine stabilised alpha/beta motif
D-lp1	Defensin-like protein 1
EO	Essential oils
FAO	Food and agriculture organisation
GASA	Gibberellic acid-stimulated arabidopsis
GRAS	Generally regarded as safe
HDMS	Hexamethyldisilazane
IEXC	Ion exchange chromatography
LTP	Lipid transfer proteins
LPPS	Liquid-phase peptide synthesis
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
NCCLS	National committee for clinical laboratory standards
PBS	Phosphate-buffered saline
QSAR	Quantitative structure-activity relationships
RP-HPLC	Reverse-phase high performance liquid chromatography
RF	Random forests
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SWOT	Strengths, weaknesses, opportunities and threats
SD	Sabouraud dextrose
SEM	Scanning electron microscope
SN-1	Snakin-1
SDS	Sodium dodecyl sulfate
SPPS	Solid-phase peptide synthesis
SVM	Support vector machines
YPD	Yeast extract-peptone-dextrose
YMM	Yeast minimal media
Z. bailii	*Zygosaccharomyces bailii*
β-di-phenylalanine	β-di-phenylalanine

Abstract

Food spoilage caused by the undesirable growth of microorganisms contributes to the crisis of food waste and food loss worldwide. Bacterial and fungal microorganisms can grow and spread in food, resulting in the spoilage of many food products intended for human consumption. Food preservatives and various preservation techniques that have been developed of decades within the food industry aim to slow the rate of food spoilage, therefore increasing the shelf-life of food products and control the level of food waste. Although preservatives have been successful in reducing spoilage and associated food waste, spoilage microbes persist, reducing the quality of food, making them inedible and further contributing to food waste. In recent decades there has been an increasing demand from consumers for food products that contain less chemicals and more "natural" forms of food preservatives. The use of antimicrobial peptides (AMPs) for this purpose is the basis for the research that was performed in this thesis. Plants produce AMPs as part of their immune system, possessing strong antimicrobial activity. This thesis aims to explore the antimicrobial activity of various plant AMPs that have been synthesised and tested for their inhibitory activity against food spoilage yeast. A literature review is presented in chapter 2, that explores the diversity of plant AMPs, their spectrum of activity, and their potential as novel food preservatives. Chapter 3 reviews the potential of synthetic modified peptides for the purpose of reducing food waste through their use as novel preservative agents in food. Two different families of plant AMPs have been examined for their antiyeast activity against common food spoilage yeasts in chapters 4, 5 and 6. Chapter 4 and 5 investigate three AMPs (radish peptides Rs-AFP1, Rs-AFP2 and barley peptide D-lp1) from the family of plant defensins, as well as their characteristics (mechanism of action, safety, stability) and applicability in various food products to reduce the growth of spoilage yeast. Both radish defensins were successful in reducing the growth of the yeast, with Rs-AFP2 proving more effective. The barley defensin showed higher antiyeast activity than the radish peptides. All three peptides were successfully incorporated into various food matrices, supporting and serving as a proof of concept for their potential use as novel food preservative agents. A different family of plant AMPs (snakin) was studied in chapter 5 to examine its potential as a food preservative, and to gain an understanding of the properties and characteristics that peptides deriving from different families of plant AMPs may have (defensin *versus* snakin peptides). The snakin peptide originated from potato tubers, and although was demonstrated to reduce the growth of the yeast and to have potential in different beverage matrices, it was less effective than the defensin peptides. Chapter 7 explores the antiyeast activity of a peptide (KK-14) whose sequence was designed based on known characteristics of plant AMPs that have been associated with antimicrobial activity. This peptide was further modified to generate three peptide derivatives. These derivatives displayed stronger activity against the yeast than the original KK-14 peptide and could be regarded as a potential food preservative. The thesis discussion also explores the various factors that must be considered in any discussion around the potential and future use of synthesised peptides as novel food preservative agents.

Chapter 1

Introduction

1.1 Introduction

Planet earth is home to nearly 8 billion people (Worldometer, 2020) and about 8.7 million animal species that live alongside us (Mora *et al.*, 2011). Since the late 18th century there has been a rapid increase in the global population that can be traced to the start of the industrial revolution, to improved agriculture and thus to improved food production. As a result, a decline in death rates slowly began to influence the overall global population over time (Ezeh, Bongaarts and Mberu, 2012). Due to this, there has been an increased demand to feed this rising population. Although technological advances in food production have made progress to sustain Earth's inhabitants, it is still not sufficient to sustain the expected rise in the global population in the coming decades. It is estimated that by the year 2050, an increase is expected from 7.8 billion (in 2020) to 9.9 billion people (AFP, 2013). Although the current state of food production may be sufficient to sustain the 7.8 billion people on this planet at present, a real challenge faced by society is the increasing rate of food waste and food loss that is prevalent throughout the whole food chain, from the farm through to the consumer. (FAO, 2019). In order to feed the growing population, food waste and food loss must be confronted as a real issue. In addition, the persistent environmental strain on the planet to sustain even the current food production levels creates a further urgency to make food waste a problem of the past. According to the UN Food and Agriculture Organisation (FAO), at least 1.3 billion tonnes of food is wasted every year worldwide. One of the main causes of this waste and loss of food is spoilage caused by microorganisms (Raak *et al.*, 2017). Warding off spoilage by microorganisms including bacteria, fungal (mould) and yeast species is a continual challenge for food industries.

The establishment of different preservation techniques have helped to slow down food spoilage in various food products. Even since ancient times the importance of using specific techniques to control and slow the growth of spoilage microorganisms was recognised. One of the oldest and earliest recognised preservation techniques was fermentation and salting, which have been used since antiquity (Farnworth, 2003; Joardder *et al.*, 2019). Preservation techniques such as freezing, thermal treatment (for example pasteurisation or sterilization), pickling, irradiation, and the addition of chemicals (such as sodium benzoates, nitrates and sulfites) are various methods used today in the food industry to preserve foods for a longer shelf life and for safe consumption (Rahman, 2007). Certain preservation techniques are unsuitable for certain types of food products as they may cause deterioration of the food constituents or nutritional value. For example, this may be why chemicals are used over the heat treatment for certain types of food.

Despite a thorough understanding of the mechanism of action of such techniques and their widespread use today, the problem of microbial spoilage persists. In addition, increasing consumer awareness and a negative perception of some preservatives, in particular chemical additives, has, in recent decades called for a search for more natural preservatives, alongside a call for application at increasing lower levels. These changes surrounding food preservatives are driving demand from the food industry for preservatives derived from more natural sources.

Spoilage of all food is an inevitable and natural process. The food will eventually decay and return to the earth from which it came. This decaying or rotting process is naturally driven by microorganisms, for example moulds (endogenous enzymes in the food itself are also responsible for this decaying process). The challenge for food manufacturers is to delay this process so that the food is edible and safe for

consumption for as long as possible, therefore increasing the shelf life of the product. The type of microbial spoilage is dependent on the food substrate and environmental conditions. Foodborne bacterial pathogens include species such as *Escherichia coli*, *Salmonella*, *Bacillus*, *Clostridium*, *Listeria*, *Staphylococcus* and *Campylobacter*, amongst others (Bintsis, 2017). Some highly heat-resistant pathogens such as *Bacillus subtilus* and *Clostridium botulinum* can form spores that are able to survive conventional heat treatments and can therefore grow in foods such as raw/cooked rice (Soni *et al.*, 2016) and canned foods (Johnson, 2019), respectively. On the other side of the spectrum, certain bacterial pathogens such as *Listeria monocytogenes* are able to grow at low temperatures of less than 10°C, making foods such as ready-to-eat products a target for spoilage (Shamloo *et al.*, 2019). A significant amount of annual revenue is also lost due to spoilage caused by fungal pathogens. The extent of this economic loss is not known, however, it is estimated that about 25% of all foods produced globally are lost due to microbial spoilage (Gram *et al.*, 2002), of which between 5 and 8% is caused by fungal spoilage (Pitt and Hocking, 2009). Moreover, the production of mycotoxins by some moulds (such as *Fusarium* or *Aspergillus* species) raises food safety issues and can result in various illnesses in humans (both acute and chronic poisoning can occur) (Bennett and Klich, 2003; Adeyeye, 2016). Fresh fruit and vegetables, cereal grains, meats and dairy products are all susceptible to mould spoilage (Adeyeye, 2016; Rico-Munoz, Samson and Houbraken, 2019). Spoilage caused by fungi, particularly yeast, are addressed by the topic of this thesis.

Yeast are unicellular fungal organisms that are responsible for a significant amount of food waste due to their persistent growth in/on various food matrices. Yeast species have been associated with the spoilage of a wide variety of products including fresh fruit and vegetables, dairy, meat and fish, and high salt and sugar products (Nychas

and Panagou, 2011). *Zygosaccharomyces* species such as *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* are frequently found to spoil high sugar/salt products (fruit juices or pickled gherkins) (Martorell *et al.*, 2007). Their ability to withstand these conditions as well as the low pH regularly found in these products contributes to their growth in such foods. *Zygosaccharomyces* species are common spoilers of carbonated drinks due to their ability to tolerate the high carbon dioxide concentrations that would ordinarily inhibit the growth of many mould and bacterial species (Stratford and Capell, 2003). The low temperatures that refrigerated products are stored at can combat the growth of most microorganisms, including most yeast. However, some yeast species such as *Debaryomyces hansenii* can survive at temperatures as low as -12°C (Aggarwal and Mondal, 2009). *D. hansenii* has been isolated from commonly refrigerated products such as semi-hard/soft cheeses. More commonly however, *D. hansenii* is observed to grow in the high sugar conditions found in fruit syrups or jams (Fleet, 1992). *Kluyveromyces* species, such as *Kluyveromyces lactis* and *Kluyveromyces marxianus*, have the ability to hydrolyse the proteins and fats found in dairy products as well as ferment lactose (Valderrama *et al.*, 1999). *S. cerevisiae* is the major 'workhorse' in the production of fermented grain-based foods such as baked products and alcoholic beverages; however soft drinks and fruit juices are also victim to spoilage by this yeast (Wareing and Davenport, 2005). In alcoholic beverages such as wine where *S. cerevisiae* is the main contributor for these fermentations, spoilage can arise if the yeast is not properly eliminated. Its resistance to the high ethanol concentration makes it a common spoiler in such an environment where the residual sugars present can support yeast growth (via the continued fermentation of the sugars) (Martorell, Querol and Fernández-Espinar, 2005). The growth of these

microorganisms, whether bacterial or fungal (mould and yeast), results in the spoilage, and subsequently, in the waste and loss of numerous food products.

The use of compounds derived from plants have been explored for the attempt to resolve the concern that consumers have developed over the addition of chemical preservatives in food products. These plant-derived compounds are known as phytochemicals. They are chemical compounds produced by plants that aid them in resisting microbial infections and that deter feeding insects. Essential oils are the most well-known phytochemicals that have been well characterised for their preservation properties. Amongst other attributes, their antimicrobial (Nedorostova *et al.*, 2009) and antifungal properties (Nazzaro *et al.*, 2017) have increased their desirability and popularity as preservative agents in foods (Pandey *et al.*, 2017). Phenolic compounds such as flavonoids, flavanols, flavones and catechins have also proven successful in reducing microbial spoilage and increasing the shelf life of food products (Martillanes *et al.*, 2017). Exploiting plants for their antimicrobial compounds such as these has given us the incentive to dig deeper into the plants' innate immune response. Such research led to the discovery that plants produce antimicrobial peptides (also known as host defence peptides). These antimicrobial peptides (AMPs) can be found in virtually all organisms. In plants they act as the first line of defence against invading pathogens (Salas *et al.*, 2015). Their antimicrobial properties can be exploited in the search for novel, natural, plant-derived food preservatives. Within this group of plant AMPs, there are various families that have shown strong antimicrobial activity. AMPs are produced in different areas of a plant and can be isolated from the seeds, flowers, stems, leaves and even the roots. Defensins, thionins, cyclotides, snakins, lipid transfer proteins, hevein-type and knottin-type peptides are the main families of plant AMPs. Peptides within each family are homologous in their amino acid sequences and are

grouped based on their sequence structure (Nawrot *et al.*, 2014). The antipathogenic properties of AMPs from sources other than plants have been extensively studied for their application in pharmaceutical (Adem Bahar and Ren, 2013) and agricultural (Keymanesh, Soltani and Sardari, 2009) applications, while less attention has been given to the application of plant AMPs in food products for the purpose of preventing microbial spoilage. Studies that consider plant AMPs for their antimicrobial properties in such applications have placed a great focus on their safety as potential preservatives. The safety of any food preservative agent must be paramount when intended for human consumption. Their mechanism of action and their affinity to microbial membranes compared to eukaryotic cell membranes make them ideal for their integration into food products.

Research in recent decades has looked at the extraction and purification of natural AMPs for pharmaceutical and biotechnological applications through the development of novel antibiotics and pesticides, and the development of transgenic plants (Nawrot *et al.*, 2014). The extraction and purification of AMPs from plants involves numerous steps that can be time consuming and an overall elaborate process. AMPs from plants can be extracted using a buffer of a specific pH, the extract then being subjected to various centrifugation steps, dialysis, ion exchange chromatography, high performance liquid chromatography, gel electrophoresis and protein analysis (Tang *et al.*, 2018). This process can be labour intensive, with the production of small quantities of the peptide of interest. An alternative method for peptide production is through their synthesis using various methods (either biological or chemical synthesis). The chemical synthesis of peptides is a rapid alternative method to obtain pure and highly concentrated peptides. It has been applied to develop various potent AMPs (Appendini and Hotchkiss, 2000; Laverty *et al.*, 2010; Harris *et al.*, 2014; Thery *et al.*, 2019) and

continues to be used as one of the main approaches for peptide synthesis. Solid phase peptide synthesis (SPPS) is the more common method of chemical peptide synthesis, compared to the older variation of peptide production called liquid phase peptide synthesis (LPPS) (Tymecka and Misicka, 2020).

The aim of this thesis was to explore and compare the characteristics of chemically synthesised plant AMPs for their application as novel preservative agents against food spoilage yeast. The origin of plant AMPs are discussed **(chapter 2)** with the different families of plant AMPs being reviewed with regard to their ability to reduce spoilage in food and agriculturally significant crops. The impact that spoilage microorganisms can impart on food is highlighted in this chapter, alongside a review of the current preservative methods that are employed in the food industry. Each family of plant AMP are discussed for their contribution in reducing the growth of spoilage microorganisms, with particular emphasis on plant defensins and the safety aspect of these peptides.

The alternative development of plant peptides is debated in **chapter 3,** where their synthetic production is thoroughly discussed. The generation of peptide sequences based on known characteristics of plant AMPs is discussed, including the various factors that are important to consider when developing a novel, de-novo AMP based on plant peptide sequences. The methods of peptide production based on biological and chemical synthesis are emphasised, highlighting the significance of chemical synthesis within this chapter. The critical importance a potent, safe, and stable peptide is discussed and considered as part of the production of peptides for the purpose of applying them as potential food preservatives. These properties are investigated throughout this thesis, with great emphasis placed on the inhibitory activity, mechanism of action, safety, and application in food products.

Chapter 4 examines the antiyeast activity of two well-known peptides (Rs-AFP1 and Rs-AFP2) whose sequences were chemically synthesised based on peptides previously found in radish seeds. Their antiyeast activity, safety, mechanism of action, and stability properties were studied. The more potent radish peptide (Rs-AFP2) was closely examined and the characteristics that afforded these strong antiyeast properties were selected and applied as the basis for the subsequent peptide that was tested in **chapter 5.** The greater number of positive charged residues and the higher net charge within Rs-AFP2's sequence were the differentiating characteristics that resulted in its higher antimicrobial activity (compared to Rs-AFP1). Therefore, a peptide with a higher count of positively charged residues and a higher net charge was chosen for evaluation in this next chapter. In order to obtain a defensin with more potent and stable properties (in terms of salt and pH stability) than those naturally present in the radish peptides, the antiyeast activity of a synthetic peptide derived from barley endosperm was analysed. This barley peptide displayed increased antiyeast, stability and safety characteristics compared to the properties observed in the radish peptide Rs-AFP2. For both the radish and barley peptides, the cheap production cost and bioavailability of these plants make them suitable candidates for their further development into novel preservatives. In particular, the barley plant is the fourth most important and readily grown crop globally (Briggs and Briggs, 1978). Due to its ease of cultivation and bioavailability, the analysis of a barley derived AMP can be advantageous for its development as a novel preservative.

In order to evaluate the antiyeast activity of another family of plant AMPs, a synthetic peptide in the snakin family was chemically synthesised and its inhibitory properties characterised, alongside its stability and safety properties for its application as a food preservative **(chapter 6).** This peptide was synthesised from the AMP originally

purified from potato tubers (Sn-1). The basis for choosing Sn-1 for further analysis was on account of its origin. The bioavailability and cheap production cost of potatoes around the world, and in particular in Ireland, feeds into the objective of further developing this peptide with the aim to apply it as a potential food preservative.

As discussed in chapter 3**,** the study and generation of peptides based on known AMPs of plant origin can ensue the development of AMPs with greater antimicrobial properties, better stability, and increased safety. **Chapter 7** explores this approach and characterises a novel peptide whose sequence was generated from known plant AMPs and then further modified to generate three additional synthetic derivative peptides. Their characterisation in terms of their ability to reduce the growth of food spoilage yeast and their safety and stability were investigated for the purpose of developing enhanced, yet safe and stable preservative agents. The modification of this novel peptide was explored in the attempt to understand whether such modifications can lead to an enhanced antimicrobial activity, therefore enabling the use of lower amounts of peptide to achieve the same preservative power. In addition, this approach permitted the study of these novel peptides in the aim to compare their antiyeast activity and other properties to wild type natural peptides based on and derived from the genome of plants (explored in chapters 4, 5 and 6). Chapter 7 also presents the argument of whether the slight increase in antiyeast activity observed with these novel peptides is sufficient to deem them a better alternative to the wild-type natural amino acid sequences (examined in chapters 4, 5 and 6) for their application as potential food preservatives.

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Chapter 2

Plant compounds for the potential reduction of food waste – a focus on antimicrobial peptides

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Published in Critical Reviews in Food Science and Nutrition as:

Shwaiki, L. N., Arendt, E. K. and Lynch, K. M. (2021) 'Plant compounds for the potential reduction of food waste–a focus on antimicrobial peptides', Critical Reviews in Food Science and Nutrition. Bellwether Publishing, Ltd. doi: 10.1080/10408398.2021.1873733.

2.1 Abstract

A large portion of global food waste is caused by microbial spoilage. The modern approach to preserve food is to apply different hurdles for microbial pathogens to overcome. These vary from thermal processes and chemical additives, to the application of irradiation and modified atmosphere packaging. Even though such preservative techniques exist, loss of food to spoilage still prevails. Plant compounds and peptides represent an untapped source of potential novel natural food preservatives. Of these, antimicrobial peptides (AMPs) are very promising for exploitation. Antimicrobial peptides are a significant component of a plant's innate defense system. Numerous studies have demonstrated the potential application of these AMPs; however, more studies, particularly in the area of food preservation are warranted. This review examines the literature on the application of AMPs and other plant compounds for the purpose of reducing food losses and waste (including crop protection). A focus is placed on the plant defensins, their natural extraction and synthetic production, and their safety and application in food preservation. In addition, current challenges and impediments to their full exploitation are discussed.

2.2 Introduction

Food waste is a well-known challenge in modern society, either as a result of spoilage or due to consumers' mindset that food past their best by date should be discarded (regardless of whether the food is still safe to consume) (Neff, Spiker and Truant, 2015). The spoilage of food can be caused by a number of factors at different stages of production; ranging from physical damage, natural enzyme activity, damage from the presence of insects, and changes caused by chemicals or due to chemical reactions. One of the most well-known causes of spoilage however, is the undesirable growth of microorganisms (Abdel-Aziz *et al.*, 2016). Microbial food spoilage is a well-understood and researched phenomenon in many aspects of food production because of the variety of spoilers that can arise from certain favourable conditions, ranging from bacterial to fungal and yeast spoilage (de W. Blackburn, 2010). Preservatives have been incorporated into foods for centuries, however the type of preservation was much less complex than what it is today. The use of salt dates back to Roman times where it was described as the first preservative, with spices following not far behind (Toussaint-Samat, 2009). Today, preserving food has advanced and diversified in terms of the methods used, such as applying low temperatures, heat, vacuum packaging, reducing the water content, acidification and the most well-known, chemical preservation (Harrison, 1906). For many consumers, the use of chemicals as food additives has become unappealing, even if the purpose of such chemicals is to increase shelf life, reduce microbial spoilage, and increase safety for consumers (IFT, no date). More natural forms of preservatives such as organic acids and essential oils, have been successfully introduced. However, there remains a desire from consumers for more naturally occurring forms of food preservatives to combat food spoilage and food waste (Theron and Lues, 2011; Das, 2016). Consumers' perception of chemical

preservatives is stigmatised by the lack of information on the topic (Shim *et al.*, 2011), creating a demand for what they consider to be healthier and safer alternatives. Essential oils and organic acids have made their way into the market as natural alternatives, however even with their introduction to the market, food spoilage still prevails. New forms of natural preservative need to be investigated and developed in the fight against food spoilage and waste.

2.3 Food wastage

Food waste is a growing problem in our modern society. Recently, the Food and Agriculture Organisation (FAO) created a distinction between food loss and food waste. Both food loss and food waste are the "decrease in quantity or quality of food along the food supply chain". They further define food loss as "occurring along the food supply chain from harvest/slaughter/catch up to, but not including the retail level", while food waste as "occurring at the retail and consumption level" (FAO, 2019). The loss of food can be observed from as far back as the mid-twentieth century, albeit the extent of waste is unlike it is today, the problem of throwing out food fit for consumption is deep-rooted. The increase in food production post-World War 2 was the beginning of this endemic, as the production and manufacturing of food became more efficient (Zhang and Wynne 2012; Evans, Campbell, and Murcott 2013). The more plentiful availability of food and increased nourishment of particularly developed nations resulted in an increase in consumers' expectations of what food should resemble and a reduction in the 'value' placed on food by consumers. This industrial and societal change led to increased loss of produce, for example, of that which may be deemed to standard for selling in retail. This has been followed in recent decades by increased food waste at the consumer level. In Europe and North America, per capita consumer waste was estimated to be between 95–115 kg a year, and 6–11 kg in less developed countries (Bellù, 2018). In the developed world, consumer behaviour and mindsets lead to over-estimation of food portions, over-shopping, lack of attention to the expiration date of foods, and refusal to consume food past its best before date (Williams *et al.*, 2012; Qi and Roe, 2016; Ishangulyyev, Kim and Lee, 2019). In less developed countries, the lack of infrastructure, transportation and

storage are leading factors contributing to food waste (Ishangulyyev, Kim and Lee, 2019).

Aside from direct food loss and waste of the raw material or product itself, the production of food leads to other resources and energy losses. The large quantity of water required results in the production of high carbon dioxide emissions (Bond, M., Meacham, T., Bhunnoo, R. and Benton, 2013). For example, Spanish tomatoes require the use of 71 million m^3 of water per year to produce the 955,000 tonnes of the fruit that the EU and UK consumes (Chapagain and James, 2013). Therefore, throwing away food only to produce more food negatively impacts the planet and can lead to increased global warming and climate change (Abeliotis *et al.*, 2015). Due to the rise in global food waste, there is a growing obligation to reduce the amount of food that is thrown away or lost. One way to do this is through the prevention of food spoilage and extension of the shelf life of foods. This could be done in different ways, with the right method breaking this cycle of constant food waste.

2.4 Microbial food spoilage

2.4.1. Bacteria

Microbial food waste is caused by the spoilage of food products resulting from the growth of microorganisms, causing undesirable change and making the product unfit for consumption. Spoilage can by triggered by the growth of bacteria, fungi or yeast (Gram *et al.*, 2002). Bacterial spoilage has been reported to be induced by a diverse number of bacterial species on or in a wide range of products. Refrigerated meat products are typically spoilt by *Carnobacterium* spp., *Salmonella* enterica strains, *Acinetobacter, Moraxella, Micrococcus, Lactobacillus, Leuconostoc, Aeromonas* and *Pseudomonas* species, to name a few (Borch, Kant-Muermans and Blixt, 1996; Erkmen and T. F. Bozoglu, 2016). Contamination of meat results in off-flavours and unpleasant odours, but also discolouration, and gas and slime production (Borch, Kant-Muermans and Blixt, 1996; Erkmen and T. F. Bozoglu, 2016). Psychrotrophs such as *Pseudomonas*, *Bacillus*, *Aerococcus*, and *Lactococcus* species are notorious for their spoilage of raw milk, while coliforms and lactic acid bacteria (lactococci) spoil buttermilk and sour cream. Lactobacilli and *Leuconostoc* can cause the development of gas and off-flavours in ripened cheese (Ledenbach and Marshall, 2009). Beverages such as bottled water and carbonated (non-alcoholic) soft drinks can also become contaminated by bacteria. In bottled water, the different stages of production can result in the contamination of the beverage. *Pseudomonas, Flavobacterium, Acinetobacter, Moraxella, Chromobacterium, Bacillus, Enterobacter, Klebsiella, Actinomyces*, and *Streptomyces* species can all be involved. In carbonated soft drinks the presence of lactic acid bacteria such as *Leuconostoc mesenteroides* and *Lactobacillus paracasei,* and acetic acid bacteria, namely *Acetobacter* and *Gluconobacter* spp., cause reduction in a beverage's overall quality.

Lactic acid bacteria can change the appearance of the drink and introduce an astringent flavour, which can lead to a loss of carbonation. Acetic acid bacteria produce a somewhat hazy and acidic product, with vinegary off-flavours (Lawlor *et al.*, 2009). In wine, *Lactobacillus* and *Pediococcus* species can contribute to the production of undesirable volatile compounds leading to bitter, acidic, oily, slimy and strong buttery properties (Bartowsky, 2009).

2.4.2. Filamentous fungi (moulds)

The role of fungal species such as the *Penicillium* mould paved the way for what was to be known as modern medicine. The accidental discovery of penicillin in the late 1920s resulted in the saving of thousands of lives, as once deadly bacterial infections ceased to be the main cause of preventable deaths (Ligon, 2004). Although this discovery was beneficial for their exploitation, fungi are notorious for their prevalence in foods and beverages. Their fastidious growth has led to major food and economic losses over the years (Snyder and Worobo, 2018). Fungi are able to spoil a wide variety of food types, ranging from fresh and perishable foods such as fruit, vegetables, dairy, meat and cereal products, to the more problematic spoilage of stored, processed and preserved foods (Pitt and Hocking, 2009). In dairy products, moulds cause spoilage by growing in large numbers, producing a "fluffy" growth due to mycelium production and causing discolouration of the product. Foods such as butter, cheese, yogurt and milk are spoiled by fungal species in the genus *Penicillium*, *Aspergillus*, *Cladosporium*, *Mucor* and *Fusarium*, to name a few (Pal, 2014; Garnier, Valence and Mounier, 2017). Bread and bakery products can also be victims of spoilage with factors such as the type of bread and temperature of storage effecting mould growth.

Yeast-raised wheat breads are spoilt by *Penicillium*, *Aspergillus*, *Neurospora* and *Cladosporium* mould, while sourdough breads have been found to be affected by *Penicillium roqueforti* (Vagelas *et al.*, 2011; Legan, 2018). *Rhizopus*, *Aspergillus*, *Penicillium* and *Eurotium* genera are some of the known spoilers of bakery products (Saranraj and Geetha, 2012). Spoilage of bakery products, including bread, occurs after the baking process as the heat applied during baking/cooking will generally kill mould spores (Axel, Zannini and Arendt, 2017). As a result, spoilage by mould is commonly caused either during cooling, wrapping or from any contaminated surfaces or appliances (Saranraj and Geetha, 2012). Cereal grains are another major group associated with fungal spoilage. The occurrence of filamentous fungi belonging to the genera *Aspergillus* and *Penicillium* poses a major threat to cereal crops during storage (Schmidt, Zannini and Arendt, 2018). Such fungal species are able to produce metabolites known as mycotoxins. These naturally occurring compounds can result in the devastating loss of numerous food groups such as cereals, nuts, spices, and fruit. Mycotoxins present in food can have major implications on the food industry as food loss is inevitable (Berthiller *et al.*, 2013). Moreover, the effect of mycotoxins, for example Aflatoxins and Ochratoxin A produced by several species of *Aspergillus* and *Penicillium*, can lead to harmful and life-threatening consequences to both human and animal health (Bhat, Rai and Karim, 2010).

2.4.3 Yeast

As seen with bacterial and fungal spoilage, the prevalence of food spoilage yeast also varies depending on the food type. The visual effect of spoilage differs depending on the yeast and food in question. Yeast such as *Zygosaccharomyces*, *Kluyveromyces*,

Saccharomyces, *Debaryomyces*, *Pichia*, *Brettanomyces* and *Candida* are some well-known spoilers of both food and beverages (Querol and Fleet, 2006). *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* are the most recognised *Zygosaccharomyces* species that are associated with high salt and sugar content foods, such as fruit juices, jams and preserves, salad dressings, sugar syrups and wine (Barnett, Payne and Yarrow, 2000; Pitt and Hocking, 2009; Nychas and Panagou, 2011). Their ability to withstand high levels of preservatives and low pH makes them notorious as food spoilers (Stratford *et al.*, 2013). The most noticeable effect of spoilage by these species is the production of excess gas. This is due to fermentation of the sugars by the yeast during the shelf life of the product (Martorell *et al.*, 2007). This leads to expansion and swelling of the container and even to explosions if conditions are particularly favourable for the yeast (Alonso *et al.*, 2015; Escott *et al.*, 2018).

Kluyveromyces lactis and *Kluyveromyces marxians* are spoilers of dairy products. Their ability to hydrolyse proteins or fats and ferment lactose make them perfect perpetrators for the spoilage of dairy products like milk, yogurts, cream and soft cheeses (Lourens-Hattingh and Viljoen, 2002; Garnier, Valence and Mounier, 2017). Depending on the substrate, off flavours, discolouration, swelling, change in texture and the formation of gas can occur (Büchl and Seiler, 2011). Their tolerance to high temperatures and their ability to grow in such a wide range of substrates in a short period make them infamous in the dairy industry as common spoilers (Tabanelli *et al.*, 2016).

Saccharomyces species, in particular *Saccharomyces cerevisiae* strains, are recognised for their positive impact in the food industry as producers of fermented beverages such

as beer and wine and leavened baked goods (Berry and Slaughter, 2003; Stewart, 2016; Walker and Stewart, 2016). Nevertheless, product spoilage by *Saccharomyces* species can occur. *Saccharomyces cerevisiae* var. *diastaticus* (Boulton and Quain, 2001) can ferment residual carbohydrates in beer (Meier-Dörnberg *et al.*, 2018). Other species such as *Saccharomyces barnetti, Saccharomyces castelli, Saccharomyces dairenensis* and *Saccharomyces turicensis* have been found to spoil food and beverages (de W. Blackburn, 2010).

Debaryomyces and other species such as *Yarrowia* and *Rhodotorula*, are often found in high numbers in spoiled meats and cheeses (Gardini *et al.*, 2001; Pitt and Hocking, 2009). *Debaryomyces hansenii* and *Yarrowia lipolytica* can cause spoilage by developing off-flavours and bitter compounds in Cheddar and Gouda. These characteristics are caused by the breakdown of lipids and proteins and the fermentation of lactose by the yeast, respectively (Szołtysik *et al.*, 2013). In mould-ripened cheeses, brown defects can be a sign of spoilage (Ross *et al.*, 2000). Several *Debaryomyces* strains have been isolated from fermenting cucumber brines and other fermented foods of high osmolarity and salt/sugar content (Johnson and Echavarri-Erasun, 2011).

Pichia is a film-forming yeast (Malfeito-Ferreira, 2019). *Pichia anomala* has been reported to spoil wine by producing esters and acetaldehydes including ethyl acetate (Escott *et al.*, 2017). It has also been isolated from fruit juices/concentrates (Combina *et al.*, 2008), yoghurts (Caggia *et al.*, 2001) and soft drinks (Ancasi, Carrillo and Benítez Ahrendts, 2006). In soft drinks, carbon dioxide, esters, ethanol and acetaldehyde production can occur resulting in yeasty, solvent and/or vinegar flavours (Kregiel, 2015). *Brettanomyces* is a yeast that has been used for the production of beer for many years for its secondary fermentation and development of flavours (Serra

Colomer, Funch and Forster, 2019). Cider (Morrissey *et al.*, 2004), wine (Oelofse, Pretorius and du Toit, 2008) and kombucha (Teoh, Heard and Cox, 2004) production are also influenced by this yeast (Oro *et al.*, 2019). Such yeast species in these products are used for the fermentation of their primary ingredient - grapes, apples, and sugared tea in the case of beverages such as cider, wine and kombucha, respectively. The alcoholic fermentation of grapes and apples are a result of the impact of yeast species breaking down the sugars in these fruits (Bisson, 2004; Valles *et al.*, 2007). In kombucha, the yeast influences the overall flavour and acidity of the beverage, an essential component of a typical kombucha culture consisting of both lactic acid bacteria and yeast. However, for many wineries and some breweries, *Brettanomyces* can be a major cause of spoilage as they can produce secondary flavours not intended for the beverage. In wine production, the presence of *Brettanomyces bruxellensis* can result in the release of volatile phenols, causing a drastic change in the flavour of the wine (Steensels *et al.*, 2015; Malfeito-Ferreira, 2018).

Candida species can spoil many types of food products. Dairy products such as cheese, milk, and yoghurts susceptible to spoilage with *Candida* species. Like *Kluyveromyces*, they have the ability to hydrolyse fats and proteins and can ferment lactose, which allows them to grow in dairy products to such extents that can lead to spoilage (Fleet, 1992). Fresh red and poultry meats are also affected. *Candida zeylanoides* has been isolated from both processed and refrigerated poultry (Deak, Chen and Beuchat, 2000). Dairy products, such as fresh unripe, hard and soft cheeses can also be susceptible to spoilage by this yeast (Garnier, Valence and Mounier, 2017). As with most yeast, *Candida* spoilage results in off-flavours in yogurts, typically yeasty and bitter, and the production of carbon dioxide in containers that can lead to explosions (Batt and Tortorello, 2014).

Rhodotorula species, *R. mucilaginosa* and *R. glutinis* are examples of some of the more well-known species of this genus. They can grow at refrigeration temperatures; therefore, dairy products like cream, butter, yogurt, and cheese can be affected. Shellfish, crustaceans, fresh and processed meats are also vulnerable to spoilage (Pitt and Hocking, 2009). The off-odour from turkey carcasses can be explained by the growth of *R. glutinis* (Albertyn, Pohl and Viljoen, 2014).

2.5 Food Preservatives

2.5.1 Common preservation techniques

The preservation of food is critical for the prevention of product spoilage. Preservation increases shelf life through the addition of hurdles, which spoilage microorganisms must overcome in order to grow and replicate (Sancho-Madriz, 2003). A long shelf life is indicated by a long period that a product is suitable for consumption (Kilcast and Subramaniam, 2000). Honey can have a shelf life of 2-3 years, depending on the type of honey (Prakash *et al.*, 2015). Fresh fruits and vegetables (Barth *et al.*, 2009), meat (Ghaly, Dave, and Ghaly 2011), dairy (Muir, 1996) and baked products (Smith *et al.*, 2004a) have a short shelf life, with the spoilage or contamination of these products after a significantly short amount of time. Therefore, different techniques are applied to reduce this spoilage. Reducing the temperature via cooling, freezing or chilling retards the growth of microorganisms based on the fact that most microorganisms cannot grow and replicate at such low temperatures (Jay, 1998b). Drying or dehydration of food reduces the water activity enough to delay or prevent unwanted growth (Harrison, 1906). Weak acids such as benzoic and sorbic (and their salts) are commonly used for the reduction of substrate pH causing inhibition directly or indirectly through the low pH (Jay, 1998a). Reducing the pH of a substrate can lead to the disruption of microbial enzymatic and metabolic reactions caused by the sensitivity to high hydrogen ion concentrations found in these environments. Weak acids can reduce the pH of a substrate and thus prevent the growth of spoilage microorganisms in certain foods (Jin and Kirk 2018). An alternative process that reduces the pH of a substrate is lactic acid fermentation. This process results in the production of edible and nutritious food products. Lactic acid bacteria fermentation is an ancient preservation technique and traditionally has been applied to a range of

substrates ranging from vegetables such as cabbage (sauerkraut and kimchi) (Park, Kim and Jeong, 2017; Zabat *et al.*, 2018), cucumber (pickles) (Behera *et al.*, 2020) and radish (Joshi and Sharma, 2009), to dairy products for the production of yogurt (Chen *et al*. 2017) and cheese (Marcelino, 2013). Meats can also be fermented; for example fermented sausages – peperoni and chorizo (Krockel, 2013; Cui and Fan, 2019). The process of lactic acid fermentation combines the microorganism's ability to grow at such low pH, unparalleled by spoilage microorganisms, most of which cannot survive in such harsh conditions. This results in the production of a food with a long shelf life in addition to nutritional and potentially probiotic benefits (Jaiswal, Gupta and Abu-Ghannam, 2012; Ghosh *et al.*, 2019).

Using thermal treatment is another common method of food preservation. Pasteurization is a heat transfer mechanism employed to kill pathogens in products such as milk, to increase product shelf life (Swart, Blignaut and Jooste, 2003). Applying this process can result in a reduction in sensory and nutritional qualities, restricting its use for certain applications (Lado and Yousef, 2002). Fruit juices are also subjected to thermal pasteurization, with consequences for the properties of the beverage (Lee and Coates, 1999, 2003). In pasteurization, thermal temperatures of below 100°C are used (Ball 1943; Silva and Gibbs 2009). Sterilization is a similar process. However, this process employs a much higher temperature (above 100°C) for longer periods, resulting in the complete destruction of all microorganisms and any spores that may be present (Ramesh, 2003). Ultra-high temperature sterilization preserves low viscous products due to heating of the product to temperatures above 100°C for a much shorter time (Penfield and Campbell, 1990).

The practice of applying irradiation to treat certain food products in order to eliminate or reduce food pathogens can be divided into three processes: radappertization, radurization and radicidation. For each process a mild dose of ionizing radiation is applied, with the major difference between them being the extent of radiation used. For radappertization, 20 to 30 kilograys are used, while for radurization and radicidation, 1 to 10 and 1 kilograys are used, respectively (Singh and Desrosier, 2018). Meat, fish, poultry and different fruit and vegetables are candidates for such preservation techniques (Josephson and Peterson, 2018).

Modified atmospheric packaging (MAP) utilises a gaseous mixture to replace the air surrounding a product, thus delaying microbial spoilage and/or natural degradation. The composition of the gas used here is constantly changing due to the chemical reactions taking place in the food (Sebranek and Houser, 2017; Cachon, Girardon and Voilley, 2019). MAP is incorporated of oxygen (O2), carbon dioxide (CO2) and Nitrogen (N2) (McMillin, 2008). Unlike thermal processes, the sensory and nutritional qualities of the product are maintained, making it a favourable mechanism for the preservation of packaged foods such as fresh meats and fruit (Muhlisin *et al.*, 2014; Kirtil and Oztop, 2016). Controlled atmosphere packaging (CAP) differs from MAP as the composition and concertation of the gas here is fixed and monitored (Gill, 1990). Similarly to MAP, fresh meats are the most common products subjected to CAP.

Chemical preservatives such as benzoates (sodium benzoate and benzoic acid), nitrates and nitrites (sodium nitrate and sodium nitrite), sulphites (sulfur dioxide) and sorbates (sodium sorbate, potassium sorbate) are all common additives in foods today (Caballero, 2003). Sodium benzoate is most commonly found in soft drinks and just like the other food preservative mentioned, are “generally regarded as safe” (GRAS)

for consumption (Lennerz *et al.*, 2015). Sodium nitrate and nitrite are salts added to red meats, salami and some fish that not only acts as a preservative, but also give a distinct flavour to the product (Epley, Addis and Warthesen, 1992; Leth *et al.*, 2008). The combination of the antioxidant properties and preservative effect of sulfur dioxide makes it a suitable agent for application to dried fruit, burger meats, dried sausages, alcoholifc beverages and soft drinks (FSAI, 2018). The sodium and potassium salt of sorbic acid is used on dairy products (Mazdeh *et al.*, 2017), meat (Hoang and Vu, 2016), and in ketchup and mayonnaise (Sharafati Chaleshtori, Arian and Sharafati Chaleshtori, 2018), and jams (Khan *et al.*, 2014).

Food preservation can be summed up as a well-structured mechanism that utilises different agents and procedures to increase the shelf life of a product. It does this by hindering the spread and development of microbial growth. However, even in the presence of such preservative measures, spoilage can still occur. Not only this, but in recent years there has been a negative connotation associated with use of chemical preservatives (Rai *et al.*, 2016), thus increasing the demand for more natural but equally effective forms of preservatives to be introduced. Table 1 provides some examples of preservative methods applied to prevent the spoilage of different food groups and related common spoilage pathogens.

Table 1: Preservative techniques employed to prevent the microbial spoilage of different food products. Common food spoilers encountered in these food types are also mentioned.

Food type	Food products	Common spoilage pathogens	Common preservation method	Reference
Dairy	Raw milk	*Mycobacterium avium* subsp. *paratuberculosis*	Pasteurization	(McDonald *et al.*, 2005)
	Cheese	*Listeria monocytogenes*	Modified atmosphere packaging (MAP); pasteurisation; chemical preservatives	(Brown, Forauer and D'Amico, 2018) (Jalilzadeh, Tunçtürk and Hesari, 2015)
	Cottage Cheese	*Pseydomonas* spp., *Enterobacter agglomerans*	Reduction of pH; heat treatment; MAP; chemical preservatives	(Ho, Howes and Bhandari, 2016)
	Yogurt	*Kluyveromyces* and *Candida spp.*	Addition of chemical preservatives; pasteurization	(Serrano, López, and Revilla 1991) (Suriyarachchi and Fleet, 1981) (Tamime and Deeth, 1980)

Meats	Refrigerated Beef and Pork	*Carnobacterium, Pseudomonas* and *Lactobacillus spp., Enterobacteriaceae*	Addition of chemicals; chilling, freezing; fermentation; irradiation	(Ghaly, Dave, and Ghaly 2011) (Rantsiou and Cocolin, 2007) (Farkas and Mohácsi-Farkas, 2011)
Fish and seafood	Iced fresh fish	*Shewanella putrefaciens* and *Pseudomonas spp.*	Freezing; addition of chemical preservatives; fermentation; drying; canning	(Ghaly *et al*. 2010) (Barth *et al.*, 2009)
Fresh Fruit and Vegetables	Fruit and Vegetables	*Botrytis, Alternaria, Fusarium, Rhizopus, Penicillium* spp.	Vacuum packaging (VP); MAP; fermentation; chemical preservatives; irradiation	(Erkmen and F. T. Bozoglu, 2016) (Farkas and Mohácsi-Farkas, 2011)
Baked products	Bread	*Penecillium* and *Aspergillus* spp.	Pasteurization; chemical preservatives; MAP	(Magan, Aldred and Arroyo, 2012) (Smith *et al.*, 2004b)
Beverages	Beer	*Lactobacillus* spp., *Saccharomyces* spp.	Pasteurization; chemical preservatives	(Wray, 2015)

	Fruit juices	*Zygossacharomyces* spp., *Escherichia coli*	Pasteurization; ultrasound and irradiation; chemical preservatives	(Rai Aneja *et al.*, 2014)
	Wine	*Lactobacillus, Zygosaccharomyces, Candida* and *Pediococcus* spp.	Chemical preservatives; pasteurization	(du Toit and Pretorius, 2019) (MacDonald *et al.*, 2017)

2.5.2 Natural plant compounds as food preservatives

Plants contain a large number of compounds and mechanisms that represent untapped potential for exploitation by the food industry. By studying their potential as food additives and preservatives, plant compounds could play an important role in reducing food waste.

Essential oils (EO) extracted from aromatic plants, are an example of an interesting group of compounds which have been extensively researched for the purpose of food preservation. Meat such as lamb (Karabagias, Badeka and Kontominas, 2011), chicken (Fratianni *et al.*, 2010) and minced beef (Skandamis and Nychas, 2001), have been positively influenced by EOs used to reduce spoilage. Their use has been reported in cooked meat products (Adelakun, Oyelade and Olanipekun, 2016), but as in other products such as fruit juices, they are often applied in combination with other preservative technologies (Espina *et al.*, 2013, 2014). Essential oils have also been applied to great benefit on fresh fruit and vegetables. Studies have found that the freshness of fruit during storage can be prolonged by incorporating them (Serrano *et al*. 2008; Sivakumar and Bautista-Baños 2014; Tripathi and Dubey 2004), enabling spoilage and decomposition to be delayed. Dairy products like yogurt (AlOtaibi and ElDemerdash, 2008) and milks (Muttagi, Chavan and Bhatt, 2019) similarly benefitted from EO application. In addition, antimicrobial films containing EOs have been used for the preservation of packaged foods (Ha, Kim and Lee, 2001; Seydim and Sarikus, 2006; López *et al.*, 2007).

Like plant AMPs and EOs (Butnariu and Sarac, 2018), phenolic compounds (also known as polyphenols) are plant secondary metabolites involved in the defense (Martillanes *et al.*, 2017). These compounds can be extracted from different fruits and

herbs. Some studies have looked at the incorporation of polyphenols into active packaging to extend the shelf life of beef (Barbosa-Pereira *et al.*, 2014), lamb (Camo, Beltrán and Roncalés, 2008) and mortadella-type sausages (Moradi *et al.*, 2011). Propolis, a natural resin found in certain parts of plants, buds and exudates (Marcucci, 1995), is a compound that has been researched for its preservative properties. The preservation of raw milk (El-Deeb, 2017), orange juice (Yang *et al.*, 2017) and fresh-cut vegetables (Alvarez *et al.*, 2017) was achieved in the presence of propolis extract, either alone or in combination with other treatments.

2.5.3 Natural plant peptides as novel preservatives

During their lifetime, plants undergo enormous stress due to the constant battle to survive in nature. They have evolved to adapt to this stress by developing different pathways and mechanisms to resist biotic and abiotic factors, such as competing for nutrients with neighbouring plants, combatting harsh weather conditions and resisting infections (de León and Montesano, 2013; Ben Rejeb, Pastor and Mauch-Mani, 2014). Plants have therefore evolved numerous physiological, molecular and cellular adaptations. One mechanism found in plants is the production of antimicrobial peptides (AMPs) as a defense mechanism to combat fungal and microbial pathogens (Broekaert *et al.*, 1997). These AMPs have been studied and examined for their ability to inhibit the growth of different microorganisms, including food spoilage pathogens, and thus hold great promise as potential preservatives in the food industry. Application of such AMPs could represent a revolutionary addition in combatting food spoilage in the food industry. However, the potency of these small peptides is yet to be fully recognised and exploited. Although AMPs have not been widely researched for their

application in food preservation, there are many studies that have examined the inhibitory effect of these peptides against food/plant pathogens. Table 2 shows a list of these AMPs and others that have been tested for their inhibition against food and plant pathogens. Defensins, a major group of plant AMPs will be discussed in more detail in the next section, regarding their antimicrobial/antifungal capacities.

Table 2: Examples of different types of plant AMPs and their inhibition against common plant/food pathogens. The food typically effected by each spoilage pathogen is also included.

Type of AMP	Antimicrobial peptide	Pathogen	Food/Plants	Reference
Thionins	*Triticum aestivum* (Wheat flour) Thionin	*Listeria monocytogenes* and other Listeria *spp.*	Meat, fish, raw vegetables, ready to eat foods	(López-Solanilla *et al.*, 2003)
	Arabidopsis thaliana Thi2.1	*Staphylococcus aureus*	Meat, dairy products	(Loeza-Ángeles *et al.*, 2008)
		Escherichia coli	Raw/uncooked meat, dairy products, contaminated vegetables	
	Triticum aestivum (Wheat) Wα1, Wβ *Hordeum vulgare* (Barley) Bβ (now a defensin)	*Clavibacter michiganensis* subsp. *sepedonicus* and *Pseudomonas solanacearum*	Potato and tobacco crops	(Molina *et al.*, 1993)
		Rosellinia necatrix	Fruits such as apples, apricots, strawberries	
		Colletotrichum lagenarium	Melon and cucumber	
		Fusarium solani	Peas, beans, potatoes, tomatoes	
	Oryza sativa (Rice) Thionin	*Meloidogyne graminicola*	Rice crops	(Ji *et al.*, 2015)
		Pythium graminicola		
		Thielaviopsis paradoxa	Sugar cane	(Bohlmann *et al.*, 1988)

	Hordeum vulgare (Barley) Thionin	*Drechslera teres*	Barley	
	Triticum aestivum (Wheat) Purothionins, *Hordeum vulgare* L. cv. Femina (Barley flour) hordothionin	*Clavibacter michiganensis* subsp. *michiganensis*	Tomatoes	(Florack *et al.*, 1993)
		C. m. subsp. sepedonicus	Potatoes	
		Xanthomonas campestris pv.vesicatoria	Tomatoes, peppers	
	Triticum aestivum L. (Wheat) Purothionin	*Pseudomonas spp.*	Mushrooms, tomatoes, rice, garlic	(Fernandez de Caleya *et al.*, 1972)
		Xanthomonas spp.	Cabbage family	
		Erwinia spp.	Fruit; apples, blackberries, raspberries	
		Corynebacterium spp.	Peppers, corn, potatoes, wheat, sugarcane	
Snakins/GASA	*Solanum tuberosum* (Potato) StSN1	*C. michiganensis* subsp. *Sepedonicus*	Potatoes	(Segura *et al.*, 1999)
		Botrytis cinerea	Wine grapes	
	Medicago sativa (Alfalfa) MsSN1	*Agrobacterium tumefaciens*	Walnuts, grapevines, sugar beets, horseradish, rhubarb	(García *et al.*, 2014)
		Phoma medicaginis var. medicaginis	Alfalfa plant	
	S. tuberosum (Potato) St-SN2	*C. michiganensis*	Potatoes	(Berrocal-Lobo *et al.*, 2002)
		Botrytis cinerea	Wine grapes	
		Fusarium spp.	Peas, beans, potatoes	
		Plectosphaerella cucumerina	Potatoes	
		Aspergillus flavus	Cereal grains, legumes	
		Bipolaris maydis	Maize	
	Solanum lycopersicum (Tomato) SN-2	*F. solani*	Peas, beans, potatoes, tomatoes	(Herbel, Schäfer and Wink, 2015)

		A. tumefaciens	Walnuts, grapevines, sugar beets, horseradish, rhubarb	(Balaji and Smart, 2012)
		E. coli	Raw/uncooked meat, dairy products, contaminated vegetables	
		C. michiganensis subsp. *michiganensis*	Tomatoes	
	Ziziphus jujuba (Jujube) Snakin-Z	*S. aureus*	Meat, dairy products	(Daneshmand, Zare-Zardini and Ebrahimi, 2013)
		Phomopsis azadirachtae	Neem (herb)	
Hevein-type	Amaranthus (Amaranth) Ac-AMP1 & Ac-AMP2	*Alternaria Brassicicola*	Cabbage, cauliflower, broccoli	(Broekaert *et al.*, 1992)
		Ascochyta pisi	Peas, lentils, beans, alfalfa	
		Fusarium culmorum	Wheat	
		B. cinerea	Wine grapes	
		Colletotrichum lindemuthianum	Beans	
		Verticillium dahlia	Verticillium wilt in potatoes, peppers, potatoes, cotton	
	Vaccaria Hispanica (Cowherb) Vaccatide vH2	*Alternaria alternate*	Tomatoes	(Wong *et al.*, 2017)
		Curvularia lunata	Cereal grains	
		Fusarium oxysporum	Tomatoes, banana, cotton, melon	
		Rhizoctonia solani	Potatoes, cucumber, cereals, rice.	
	Triticum kiharae (Wheat) WAMP-1a	*F. solani*	Peas, beans, potatoes, tomatoes	(Odintsova *et al.*, 2009)
		Fusarium oxysporum	Tomatoes, banana, cotton, melon	(Odintsova *et al.*, 2009)
		Bipolaris sorokiniana	Barley, wheat	
		B. cinerea	Wine grapes	(Rogozhin *et al.*, 2015)
		B. sorokiniana	Wheat, barley	

	Stellaria media (Chickweed) SmAMP3	*Aspergillus niger*	Fruit and vegetables, peanuts	
		F. solani	Peas, beans, potatoes, tomatoes	
		A. alternate	Tomatoes	
	Ginkgo biloba (Maidenhair tree) Ginkgotide gB5	*A. niger*	Fruit and vegetables, peanuts	(Wong *et al.*, 2016)
		C. lunata	Cereal grains	
		F. oxysporum	Tomatoes, banana, cotton, melon	
		R. solani	Potatoes, cucumber, cereals, rice	
	Euonymus europaeus (European spindle) Ee-CBP	*A. brassicicola*	Cabbage, cauliflower, broccoli	(Van Den Bergh *et al.*, 2002)
		B. cinerea	Wine grapes	
		Fusarium culmorum	Wheat	
		Fusarium oxysporum f.sp. cubense	Bananas	
		Phoma exigua	Potatoes, cotton	
		Pythium ultimum	Corn, potatoes, wheat, soybeans	
Knottin-type	*Mirabilis jalapa* (Marvel of Peru) Mj-AMP1 and Mj-AMP2	*Cercospora beticola*	Sugar beets, spinach	(Cammue *et al.*, 1992)
		Pyricularia oryzae	Rice	
		Phoma beta	Beets	
		Venturia inaequalis	Apples	
		E. coli	Raw/uncooked meat, dairy products, contaminated vegetables	
		Erwinia carotovora	Carrot, tomatoes, potatoes	
	Phytolacca americana (Pokeweed) PAFP-s	*Fusarium oxysporum*	Banana, sweet potato, tomato	
		Fusarium graminearum	Wheat, corn, barley	

		*Alternaria tenuis (*aka *Alternaria alternata)*	Tomatoes	(Shao *et al*. 1999)
		P. oryzae	Rice	
Cyclotides	*Viola odorata* (Sweet violet) CyO2	*Klebsiella pneumoniae*	Raw meats, vegetables, ready to eat meals	(Pränting *et al*., 2010)
		Salmonella enterica serovar *Typhimurium*	Beef, chicken, fruit, vegetable	
		E. coli	Raw/uncooked meat, dairy products, contaminated vegetables	
		Xanthomonas Oryzae	Rice	(Zarrabi *et al*., 2013)
		Rhizobium cicil	Legumes	

Thionins are a large group of AMPs that were the first major group to be discovered (Hancock, 1997). They can be purified from every plant tissue, ranging from seeds to leaves (Nawrot *et al*., 2014) and have been studied for their antimicrobial effect against food pathogens *in vitro*. Their small sulfur-rich structures are compact and amphipathic, making them potent antimicrobial agents with approximate molecular weights of 5000 kDa. Their structure is typically stabilised by 3 to 4 disulfide bridges, resulting in an overall basic charge (Bohlmann, 1994).

López-Solanilla *et al.*, 2003 successfully purified thionin from wheat flour and reported strong antimicrobial activity against the food-borne pathogen *Listeria monocytogenes* and other *Listeria* species. Food pathogens *Staphylococcus aureus* and *Escherichia coli* were inhibited by thionin extracted from the *Arabidopsis thaliana* plant (Loeza-Ángeles *et al.*, 2008). Other preventative studies relating to the application of thionins are those that examine their effect on plant pathogens. Plant pathogens can cause a large volume of crop losses each year, ultimately leading to high volumes of food losses (Luck *et al*., 2011). This field of research is therefore

crucial for the prevention of food losses globally. Molina *et al.*, 1993 conducted some progressive research on thionins and their effectiveness on inhibiting plant pathogens. The study purified thionins of type 1 and type 2 (differences in the number of amino acids present in their sequence) from the endosperm of wheat and barley, and barley leaves, respectively. It was shown, although with some more than others, that the thionins extracted were inhibitory against the bacterial species *Clavibacter michiganensis* subsp. *sepedonicus* and *Pseudomonas solanacearum* (pathogens of the potato and tobacco crop, respectively), and the fungal pathogens *Rosellinia necatrix* (effecting fruits such as apples, apricots, strawberries), *Colletotrichum lagenarium* (melons and cucumbers pathogen) and *Fusarium solani* (peas, beans and potato pathogen). The purified thionins were also more effective than the tested commercial fungicide Ridomil on a molar basis. Another study, by Ji *et al.*, 2015 established that the thionin genes present in rice are responsible for the defense of the crop. Two root pathogens *Meloidogyne graminicola* and *Pythium graminicola* were successfully inhibited by these thionins, an advancement required to prevent the spoilage of some of the most damaging root pathogens in rice production.

Snakin/GASA (Gibberellic Acid-Stimulated Arabidopsis peptides) peptides are a family of AMPs whose members have also been considered for fighting plant infections. The main distinguishing structural element of this peptide family is the GASA domain in the C-terminal region of the peptide. Twelve conserved cysteine residues compose this GASA domain, which are known to contribute to the overall stability and activity of the peptide (Nahirñak *et al.*, 2012). Their inhibitory properties have been reported in a number of studies. Segura *et al.*, 1999 discovered that the AMP from potato tubers (Snakin-1, also known as StSN1) has antimicrobial and antifungal abilities against the potato pathogens *C. michiganensis* subsp. *Sepedonicus* and

Botrytis cinerea. Alfalfa Snakin-1 (*Ms*SN1) from the Alfalfa plant *Medicago sativa* L. was identified and its activity against *Agrobacterium tumefaciens* and *Phoma medicaginis* var. *medicaginis* characterised (García *et al.*, 2014). Both strains were sensitive to the *Ms*SN1 extract which resulted in the inhibition of fungal spore germination in the case of *Phoma medicaginis* var. *medicaginis* (an alfalfa pathogen), and high inhibitory activity against *Agrobacterium tumefaciens* growth (walnuts, grapevines, sugar beets, horseradish and rhubarb pathogen). In addition, the Snakin peptide, Snakin-2 (StSN2) has also been characterised from potato tubers (Bártová, Bárta and Jarošová, 2019). It was found to be very active against the bacterial strain *C. michiganensis,* and even more so against the fungal pathogens *Botrytis cinerea* (wine grapes pathogen), *Fusarium* spp., *Plectosphaerella cucumerina* (potato pathogens), *Aspergillus flavus* (cereal grains and legume pathogen) and *Bipolaris maydis* (maize pathogen) (Berrocal-Lobo *et al.*, 2002).

Hevein- and knottin-type peptides are another group of AMPs, which, like most plant AMPs, are cysteine rich and found as part of their defense system. Heveins are chitin-binding proteins that were first discovered in rubber tree latex *(Hevea brasiliensis)* (Soedjanaatmadja, Subroto and Beintema, 1995). They have not been found to be potent antimicrobial or antifungal agents; therefore, this group has not been as extensively researched as the rest of the AMPs. However, peptides homologous to heveins, (known as hevein-like peptides) have been discovered in some plants, specifically in amaranth *(Amaranthus caudatus)* seeds and sweet peppers (*Capsicum annuum*) – inhibiting microbial growth in vitro (Broekaert *et al.*, 1997).

Knottin peptides were discovered from *Ecballium elaterium* seeds as strong trypsin inhibitors. Knottin-type peptides on the other hand, were extracted from the plant *Mirubilis julupu*. These peptides get their name from the knotted structure that they

form due to the presence of the disulphide bridges within the peptide sequence (Le Nguyen *et al.*, 1990). Hevein- and knottin-type peptides are usually grouped together because of similarities in their cysteine motifs (Chagolla-Lopez *et al.*, 1994). Hevein-type peptides extracted from *A. caudatus* seeds, named Ac-AMP1 and Ac-AMP2, were studied by Broekaert *et al.*, 1992 and it was found that a variety of plant pathogenic fungi were sensitive to the two hevein-like AMPs. The fungal species *Alternaria brassicicola* (cabbage, cauliflower, broccoli pathogen), *Ascochyta pisi* and *Fusarium culmorum* (pea pathogens), *B. cinerea*, *Colletotrichum lindemuthianum* (bean pathogen), and *Verticillium dahlia* (causing Verticillium wilt in plants such as potatoes, peppers, potatoes, cotton) were all negatively affected and their growth reduced. Knottin-type peptides *Mj*-AMP1 and *Mj*-AMP2, isolated from the seeds of the plant *Mirabilis jalapa L.* were tested for their antifungal activity towards a series of fungal and bacterial pathogens (Cammue *et al.*, 1992). The fungal pathogens *Cercospora beticola* (sugar beets and spinach pathogen), *Pyricularia oryzae* (rice pathogen), *Phoma betae* (beet pathogen) and *Venturia inaequalis* (apple pathogen) were amongst some of the species that were affected by the presence of the peptides, with *Mj*-AMP2 having a greater effect. Growth of the bacterial plant pathogens *E. coli* and *Erwinia carotovora* (carrot, tomato, potato pathogen) were also inhibited to various extents. Shao *et al.*, 1999 examined the purification and antifungal properties of the AMP PAFP-s, a peptide very structurally similar to the AMPs purified by Cammue *et al.*, 1992, derived from the seeds of the plant *Phytolacca americana*. The study found PAFP-s to be active against *Fusarium oxysporum* (banana, sweet potato, tomato pathogen), *Fusarium graminearum* (wheat, corn and barley), and *Alternaria tenuis* (now known as *Alternaria alternata*, causing spoilage in tomatoes). To a lesser degree, *P. oryzae* was also affected.

Cyclotides are relatively new members to the family of plant AMPs. They are small proteins that contain around 30 amino acids in their sequence and are distinguished by their head to tail cyclic backbone and disulfide bonds that are arranged in a knotted structure. Plant families *Violaceae, Rubiaceae* and *Cucurbitaceae* contain abundant cyclotide peptides (Craik *et al.*, 2006). In a study by Pränting *et al.*, 2010, the food pathogens *Klebsiella pneumoniae* (raw meat, vegetables, ready to eat meals), *Salmonella enterica* serovar *Typhimurium* and *E. coli* were sensitive to the cyclotide peptide called CyO2, isolated from the plant *Viola odorata*. A separate study by Zarrabi *et al.*, 2013 looked at the same cyclotide peptide and its activity against the plant pathogens *Xanthomonas Oryzae* (rice) and *Rhizobium cicil* (legumes) (based on radial diffusion assay results). Food pathogens *S. aureus* (MIC of 1.6 µg/mL^{-1}), *E. coli* (MIC of 3.2 µg/mL^{-1}) and *Pseudomonas aeruginosa* (dairy products, meat, fish, fresh vegetables) (MIC of 6.4 µg/mL^{-1}) were also inhibited.

Lipid transfer proteins (LTP) are another family of AMPs that can be found in plants. They are small (7-10 kDa), basic proteins present in high amounts in higher plants. Their role is involved in the enhancement of the *in vitro* transfer of phospholipids between membranes, and thus play a key role in the physiological processes of a plant (Kader, 1996). This family of peptides retain eight cysteine residues in a conserved pattern, as well as a hydrophobic cavity consisting of the lipid binding site (Carvalho and Gomes, 2007). Many LTP have been observed to cause antimicrobial and antifungal effects against a variety of microorganisms. The LTP isolated from the seeds of a chilli pepper (*Capsicum annuum*), named Ca-LTP(1), displayed antifungal activity against the common bean plant fungal pathogen *Colletotrichum lindemuthianum* (MIC of 400 µg/mL) (Diz *et al.*, 2006). A separate study isolated LTPs from the leaves of *Arabidopsis thaliana* and spinach that resulted in the

inhibitory activity against bacterial tomato and potato pathogen *C. michiganensis* and *P. solanacearum* (EC-50 in the range of 0.1-1 µM). While peptide concentrations of 10 µM caused inhibition against the fungal potato pathogen *F. solani* (Segura, Moreno and García-Olmedo, 1993).

2.6 Defensins – origin, structure and functionality as potential preservatives

Defensins are a major group of AMPs found in nearly all living organisms. Mammals (Lehrer, 2004), birds (van Dijk, Veldhuizen and Haagsman, 2008), insects (Hoffmann and Hetru, 1992) and plants (Thomma, Cammue and Thevissen, 2002) have all developed the ability to produce defensins as a defense mechanism against microbial, viral and fungal infections. Plant defensins are structurally similar to those found in other lifeforms and are a component of the innate immune system. They are small (45-54 amino acids), cationic and contain a large number of cysteine residues that form multiple disulfide bridges (Tam *et al.*, 2015). Plant defensins were first discovered in the early 1990s in wheat and barley grains (Colilla, Rocher and Mendez, 1990; Mendez *et al.*, 1990), however at the time, these were known as γ-thionins due to the similarity in the size and cysteine content found in thionins. The term "plant defensins" was coined after the discovery of two peptides that were purified from the seeds of the radish plant (*Raphanus sativus*) (Terras *et al.*, 1992) that revealed similarities between them and insect and mammalian defensins (Stotz, Thomson and Wang, 2009).

Plant defensins are structurally stable peptides. The presence of a cysteine stabilised alpha/beta motif (CSαβ-motif) forms one alpha helix and three antiparallel beta sheets, making them well conserved in structure. Four to five disulfide bridges can be formed from the cysteine residues present. Some defensins differ in the number of disulfide bridges present; those with 4 disulfide bridges are known as 8C, while those with 5 are termed 10C. Examples of peptides with 4 disulfide bridges are those found in *Nicotiana alata* (NaD1), *Vigna radiate* (VrD1), *Medicago sativa* (Ms-Def1), *Pisum sativum* (Psd1) and *Raphanus sativus* (Rs-AFP1, Rs-AFP2). Peptides containing 5 sulfide bridges are found in the plant *Petunia hybrida* (PhDs).

Defensins can be produced in the leaves, fruit, bark, pods, seeds, tubers and floral organs of a plant as a defense mechanism against pathogens, but also as a method to cope with environmental stresses (Carvalho and Gomes, 2009; Ishaq, Bilal and Iqbal, 2019). The largest proportion of defensins are thought to be produced in the seeds of a plant. This is due to the extra stress that seedlings must cope with in order to survive. An example of this mechanism is found in the radish plant. The seeds of this plant can contain up to 0.5% defensins of the total protein present; during germination, the seed coat is disrupted, resulting in an increase of these proteins to 30%. This process occurs as a means to protect the seedling (Stotz, Thomson and Wang, 2009). Defensins function to protect the plant against microbial or fungal pathogens in a variety of ways. However, fungal pathogens are more susceptible than microbial pathogens (Cools *et al.*, 2017). A number of mechanisms have proposed for this inhibition, one of which is through the interaction with the cell membrane of the pathogen. Membrane permeabilisation has been most studied. Permeabilisation can occur through the action of the positively charged peptide accumulating on the negatively charged membrane, compromising its integrity and resulting in cell death. This mechanism has been observed with fungal cells (Thevissen, Terras and Broekaert, 1999) but also in yeast cells by the action of the synthetic analogue of the radish defensin, Rs-AFP2 (Shwaiki, Arendt and Lynch, 2020). Permeabilisation can lead to cell leakage and death, for example through the efflux of potassium ions. When this happens, the action of potassium transporters such as Tok1 and Nha1 aid in restoring proper membrane potential that would have been disrupted by the permeabilising action of AMPs (Yenush, 2016). This consequently leads to the efflux of potassium ions from the cell membrane, triggering membrane depolarisation. The damage caused to the cell membrane and the release of potassium ions can prompt an imbalance to the osmotic

pressure within and around the cell, leading to cell death (Liu *et al*. 2020). Another hypothesised mechanism of action of defensins is through the stimulation of the target cells to over-produce reactive oxygen species (ROS) (Hayes *et al.*, 2013). Excessive production of these ROS ultimately leads to cell death. In yeast cells, ROS is generated during normal cell function in the mitochondria, however the overproduction of these reactive species can lead to cell apoptosis (Wang *et al*. 2015). These proposed mechanisms of actions have been observed to be linked to each other, where the permeabilisation of the cell membrane leads to a cascade of reactions, including the overproduction of ROS (Struyfs *et al.*, 2020). Figure 1 illustrates these proposed mechanisms of action on a yeast cell.

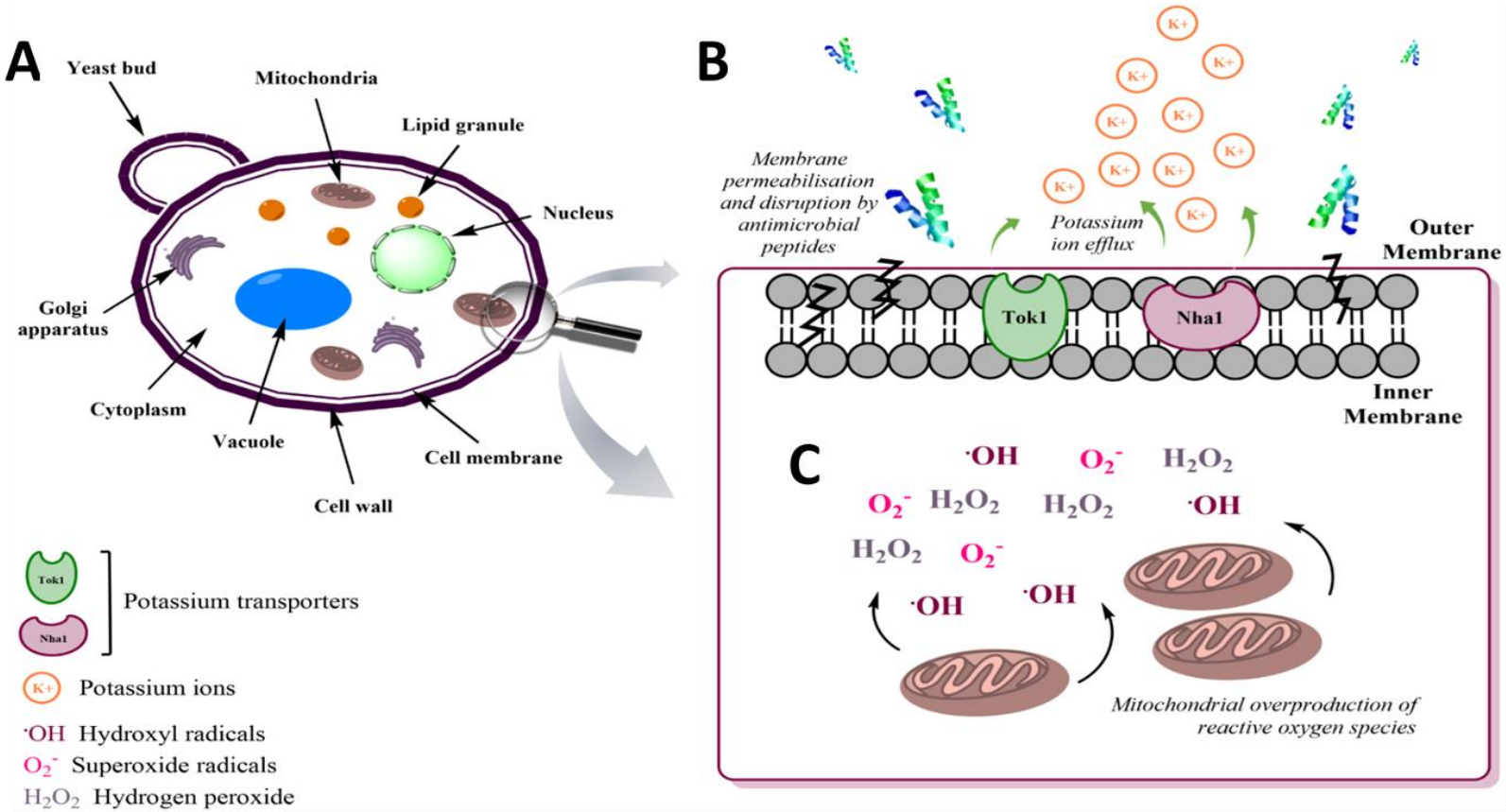

Figure 1: Figure illustrates a potential mechanism of action of AMPs on a yeast cell (A). Permeabilisation of the negatively charged yeast membrane is caused by the presence of positively charged peptides. This leads to the disruption of the membrane and subsequently, to the efflux of potassium ions. Tok1 and Nha1 are potassium transporters representing the efflux of potassium ions from the cell (B). As a result of the membrane permeabilising activity of the AMP, mitochondrial overproduction of reactive oxygen species (ROS) can also occur, which can lead to cell death (C).

There are various factors that determine the potency of an AMP; the size (number of amino acids), sequence and structure, overall charge, and the net hydrophobicity and amphipathicity (a peptide presenting both hydrophobic and hydrophilic regions, allowing it to interact with both the hydrophobic-hydrophilic structures of membrane lipids). As previously discussed, a more cationic peptide results in better interaction with microbial cell membrane, therefore a sequence with positively charged residues is preferred. A more amphipathic and hydrophobic structure also enables a peptide to insert itself more readily into microbial membranes. The amino acid composition of an AMP determines its amphipathic and hydrophobic properties, thus influencing its ability to bind membranes (Stark, Liu and Deber, 2002; Edwards *et al.*, 2016).

Hydrophobic residues such as leucine, valine and alanine, can establish the hydrophobic content of a peptide. Some of these residues, such as tryptophan and phenylalanine can impart increased hydrophobicity but also enhance antimicrobial activity and resistance to salts (Muñoz *et al.*, 2007). A study by Thery *et al.*, 2019 found that a tryptophan substitution with an unnatural β-diphenylalanine residue resulted in stronger antifungal activity due to the increased hydrophobicity caused by this substitution. However, increasing the hydrophobic content beyond a certain value can also enforce intramolecular binding, which may lead to reduced antimicrobial activity (Chen *et al*. 2007). Furthermore, a high hydrophobic content can also be linked to increased haemolytic activity (Jiang *et al.*, 2008). Charged aromatic residues dictate the amphipathicity of a peptide by aiding in their location in the microbial bilayer. Studies have found that increasing the amphipathic properties of a peptide may result in increased haemolytic activity (Jin *et al*. 2016; Kondejewski *et al*. 1999), however, others have examined the opposite effect (Zhang *et al*. 2016).

Defensins are produced in different areas of a plant and can be isolated and purified using various extraction methods (Figure 2).

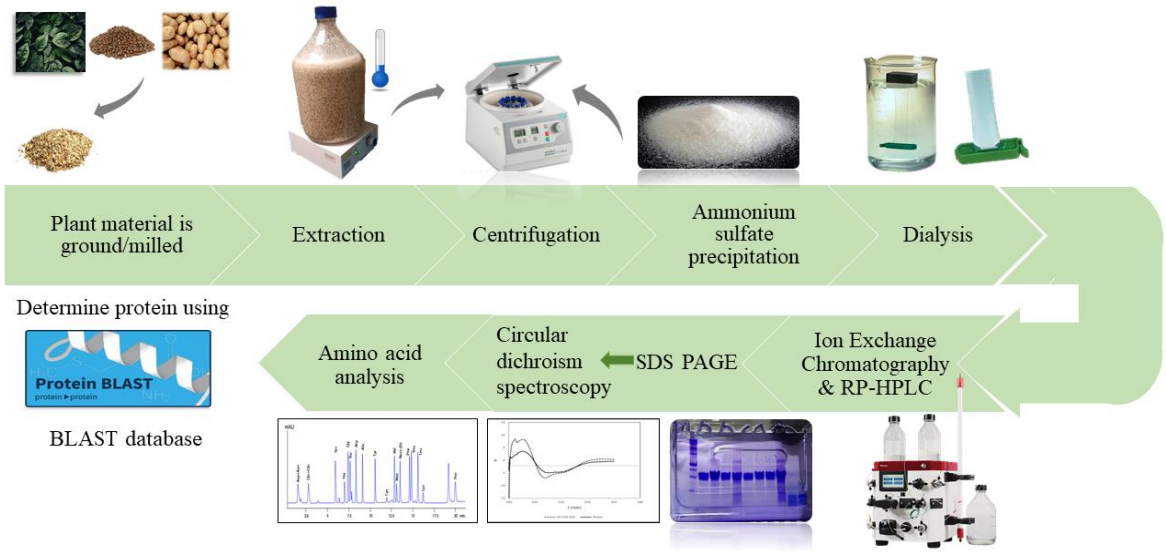

Figure 2: A workflow illustration of a common peptide extraction method from plants. Plant materials (seeds, tubers, leaves) are ground for their extraction in a buffer solution at low temperatures. This is followed by centrifugation, ammonium sulfate precipitation and the removal of any remaining salts (dialysis) after a final centrifugation step. Ion exchange chromatography and RP-HPLC are carried out on this crude extract to further purify it. SDS PAGE and circular dichroism better analyse the peptide present by studying its molecular weight and secondary structure, respectively. Finally, an amino acid analysis is performed to determine the sequence of this peptide and through the Protein BLAST database, its origin and similarities to other AMPs can be determined.

The plant material is milled or ground to fine particles and extracted in a buffer (formulated based on the type of defensin being extracted) for a number of hours at low temperature. The supernatant is collected, and the proteins are precipitated with 70-80% ammonium sulfate (salting-out method) followed by extensive dialysed to remove any remaining salt. The crude extract is then put through ion exchange chromatography (IEXC) and reverse-phase high performance liquid chromatography (RP-HPLC) for further purification of the proteins. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) can be used to verify the proteins

present in the fractions collected by chromatography. Analysis or characterisation of the proteins can be performed, for example circular dichroism spectroscopy or amino acid analysis from the data obtained from the SDS PAGE gels (Tang *et al.*, 2018). These methods respectively analyse the secondary structure of the protein present and identify the potential amino acid sequence. Various studies on plant antimicrobial peptides have looked at the inhibitory effects of defensins after extraction and purification processes. A good example of this is the purification of the defensins Rs-AFP1 and Rs-AFP2 from the seeds of the radish plant *R. sativus* (Terras *et al.*, 1992). The study found the defensins to be inhibitory against common plant pathogens such as *C. lindemuthianum*, *C. beticola*, *Pyrenophora tritici-repentis* (wheat, barley, rye pathogen), *Septoria nodorum* (wheat pathogen), and *V. dahlia*, to name a few. Table 3 lists some known defensins and their activity against plant pathogens.

Table 3: Names of plant defensins, their origin and their inhibitory effect against plant pathogens. Common plant/foods typically spoiled by these pathogens are also listed.

Type of AMP	AMPs	Pathogen	Food/Plant	Reference
Defensins	*Raphanus sativus* (Radish) Rs-AFP1 & Rs-AFP2	*Colletotrichum lindemuthianum*	Beans	(Terras *et al*. 1992)
		Cercospora beticola	Sugar beets, spinach	
		Pyrenophora tritici-repentis	Wheat, barley, rye	
		Septoria nodorum	Wheat	
		Verticillium dahliae	Potato, tomato, cucumber	
	Expression of *Raphanus sativus* (radish) defensins	*Alternaria solani*	Increased resistance in transgenic tomato plants	(Parashina *et al*. 2000)

	Expression of *Medicago sativa* (alfalfa) defensin alfAFP	*V. dahliae*	Study found increased resistance in transgenic potato plants	(Gao *et al*. 2000)
	Expression of *Pisum sativum* defensin gene (pea) DRR206	*Leptosphaeria maculans*	Study found increased resistance in transgenic canola plants	(Wang *et al*. 1999)
	Medicago sativa (Alfalfa) MsDef1 & MtDef4	*Fusarium graminearum*	Wheat, corn, barley	(Sagaram *et al*. 2011)
	Trigonella foenum-graecum L. (Fenugreek) Tfgd1	*Rhizoctonia solani*	Potatoes, cucumber, cereals, rice	(Olli and Kirti 2006)
		Phaeoisariopsis personata	Peanut	
	Nicotiana alata (Jasmine tobacco) NaD1	*Fusarium oxysporum f. sp. Vasinfectum*	Cotton	(Van Der Weerden, Lay, and Anderson 2008)
		V. dahliae	Potato, tomato, cucumber	
		Thielaviopsis basicola	Root plants, e.g., carrots	
		Leptospheria maculans	Brassica crops; broccoli, cabbage, turnip	
	Aesculus hippocastanum (Horse-chestnut) Ah-AMP1	*Botrytis cinerea*	Wine grapes	(Osborn *et al*. 1995)
	Clitoria ternatea (Asian pigeonwings) Ct-AMP1	*Cladosporium sphaerospermum*	Citrus fruit	
	Dahlia merckii (Hardy Dahlia) Dm-AMP1	*Penicillium digitatum*		
	Heuchera sanguinea (Coral bells) Hs-AMP1	*Fusarium culmorum*	Wheat	
		Leptosphaeria maculans	Brassica plants	
		Septoria tritici	Wheat	

	Verticillium albo-atrum	Hops, alfalfa, cotton	
Pisum sativum (Pea) Psd1 & Psd2	*Aspergillus niger*	Fruit and vegetables, peanuts	(Almeida *et al*. 2000)
	Aspergillus versicolor	Wheat and barley crops, cereals and oilseeds	
	Fusarium moniliforme	Corn	
	Fusarium solani	Peas, beans, potatoes, tomatoes	
	Neurospora crassa	Bakery products; bread	
Petunia hybrida (Petunia flower) PhD1 & PhD2	*Fusarium oxysporum*	Cotton, bananas	(Lay, Brugliera, and Anderson 2003)
	B. cinerea	Wine grapes	

Studies have also explored the potential for the exploitation of defensins through genetic engineering, for the development of plants with enhanced resistance to fungal pathogens. In a study by Parashina *et al.*, 2000, the expression of a radish defensin caused increased resistance of a tomato plant to *Alternaria solani,* while the expression of a pea and alfalfa defensin resulted in enhanced fungal resistance in *Brassica napus* (Rapeseed) and potato plants, respectively (Gao *et al*. 2000; Wang *et al*. 1999). An alternative genetic engineering approach to combat food spoilage has also been proposed. The study by James *et al*., 2014 looks at genetically engineering a human β-defensin into a common lager yeast, accomplishing the secretion of the defensin by the yeast during fermentation and consequently reducing microbial spoilage in the beer.

The ability of defensins to influence plant pathogens can lead to/result in a change in the way that plants respond to disease. Increasing a plant's resistance to fungal and

microbial pathogens genetically can greatly benefit the food industry by reducing incidence of infection and therefore lessen the impact of agricultural and food loss. Not only this, but it can play an important role to help reduce dependency on chemical pesticides.

Naturally extracting defensins for food preservation and developing more resistant transgenic plants come with challenges. With any large extraction process, the raw materials required for the extraction of AMPs can be expensive, and the procedure to fully purify the final peptides can be time consuming. The expression of certain defensin genes in transgenic plants can also be an issue. Studies have shown that the continuous expression of some defensins can have toxic effects on a plant on their growth and development including altered organ growth or a reduction in pollen and seed production (Allen *et al.,* 2008; Stotz, Spence and Wang, 2009). The antifungal peptides MsDef1 and MtDef1 (*Medicago sativa)*, Rs-AFP2 (*Raphanus sativus*) and fungal toxin KP4 (*Ustilago maydis*) have been found to block root development and consequently stop the growth of root hair in germinating *Medicago truncatula* seeds. This altered development was observed at concentrations similar to those required to cause antifungal activity (Allen *et al.*, 2008). Furthermore, genetically modifying plants to be resistant to infections is unlikely to gain wide acceptance currently due to societal concerns around genetically modified organisms. The fear of genetically modified plants that represent a perceived health risk, or their association industrialised farming, are some of the factors that need to be addressed and discussed in modern society. Fortunately, there is another potential route for the exploitation of AMPs.

2.7 Chemical synthesis of AMPs

The importance of chemical synthesis as an alternative method for the study and potential future exploitation of AMPs has received increasing attention in recent years. Different methods exist for the chemical synthesis of peptides. One of these strategies is through solid-phase peptide synthesis (SPPS). This mechanism can result in a peptide of high purity through the effective removal of by-products that may already be present in the sample (Münzker, Oddo and Hansen, 2017). It is one of the most common and preferred methods of synthesising AMPs. Compared to liquid-phase peptide synthesis (LPPS), SPPS is selected for the synthesis of long peptides and for its efficiency, speed and simplicity (Bayer and Mutter, 1972). Methodologies for SPPS have been reviewed by Amblard *et al.*, 2006. Chemically synthesising antimicrobial peptides and testing for their antimicrobial/antifungal activities has become commonplace in research due to increased accessibility and affordability to SPPS methods. As for naturally extracted AMPs from plants, chemical synthesis of plant AMPs based on the sequence of wild-type peptides, and their application for reducing plant pathogens has become a focus for research. A study by Thevissen *et al.*, 2005 revealed the antifungal effects of a synthetic peptide and its synthetic analogues derived from the seeds of the plant *Impatiens balsamina.* The study showed the different extents of inhibition of the synthetic peptide and the derivatives against plant pathogens *Neurospora crassa* (bread mould), *B. cinerea,* and *F. culmorum*, and pathogenic strain *S. cerevisiae.* Harris *et al.*, 2014 chemically synthesised Snakin-1 and Snakin-2 originating from potato tubers. These were active against *Plectosphaerella cucumerina* (melon and potato pathogen) and *Fusarium oxysporum* f. sp. *conglutinans* (cabbage pathogen). Table 4 provides a list of plant-derived synthetic peptides.

Table 4: Table containing different examples of synthetic plant AMPs and their use as inhibitors of some common food/plant pathogens. Synthetic AMPs, their derivatives and analogues, and their use in transgenic plants are named. The food/plant that each pathogen is found to spoil are also identified.

Type of synthetic peptide	AMPs	Pathogen	Food/Plant	Reference
Synthetic plant AMPs	*Impatiens balsamina* (Balsam plant) Ib-AMP1 and Ib-AMP4	*Neurospora crassa*	Bread	(Thevissen *et al*. 2005)
		Botrytis cinerea	Wine grapes	
		Fusarium culmorum	Wheat	
		Saccharomyces cerevisiae	Alcoholic beverages	
	Solanum tuberosum (Potato tubers) Snakin-1 & Snakin-2	*Plectosphaerella cucumerina*	Melon, potato	(Harris *et al*. 2014b)
		Fusarium oxysporum f. sp. conglutinans	Cabbage	
	Vigna unguiculate (Cowpea plant) KT43C	*F. culmorum*	Wheat	(Thery and Arendt 2018)
		Penicillium expansum	Apples	
		Aspergillus niger	Fruit and vegetables, peanuts	
	Raphanus sativus (Radish) Rs-AFP1 & Rs-AFP2	*S. cerevisiae*	Alcoholic beverages	(Shwaiki, Arendt, and Lynch 2020)
		Zygosaccharomyces bailii	Fruit juices, jams, preserves	
		Zygosaccharomyces rouxii	Salad dressings, sugar syrups	
		Debaryomyces hansenii	Meats and cheeses	
		Kluyveromyces lactis	Dairy products	
Synthetic AMP derivatives	Novel cationic peptides KK14 & its analogues	*F. culmorum,*	Wheat	(Thery *et al*. 2019) (Shwaiki *et al*. 2019)
		P. expansum	Apples	
		A. niger	Fruit and vegetables, peanuts	
		Z. bailii	Fruit juices, jams, preserves	
		D. hansenii	Meats and cheeses	
		Z. rouxii	Salad dressings, sugar syrups	
		K. lactis	Dairy products	

		S. cerevisiae	Alcoholic beverages	
	Cationic hexapeptide PAF26 derivatives	*Penicillium digitatum*	Citrus fruit	(Muñoz *et al*. 2007)
		S. cerevisiae	Alcoholic beverages	
		Escherichia coli	Raw/uncooked meat, dairy products, contaminated vegetables	
	Ultra-short cationic peptide (O3TR)	*F. culmorum*	Wheat	(Thery *et al*. 2018)
		P. expansum	Apples	
		A. niger	Fruit and vegetables, peanuts	
		Z. rouxii	Salad dressings, sugar syrups	
		S. cerevisiae	Alcoholic beverages	
		K. lactis	Dairy products	
		Z. bailii	Fruit juices, jams, preserves	
		D. hansenii	Meats and cheeses	
	Cationic polypeptide *Flo* derivatives	*Pseudomonas aeruginosa*	Dairy, meat, fruit, vegetables	(Suarez *et al*. 2005)
		Salmonella enterica	Raw meat, poultry, seafood	
		Staphylococcus aureus	Meat, dairy products	
Synthetic AMPs in transgenic plants	Transgenic *Brassica juncea* (Black Mustard Seed) expressing synthetic AMP MsrA1	*Alternaria brassicae*	Brassica (cabbage) family	(Rustagi *et al*. 2014)
	Biopeptide BP100 expressed in *Oryza sativa* (rice)	*Dickeya chrysanthemi*	Pea	(Nadal *et al*. 2012)
		Fusarium verticillioides	Maize	
	Lycopersicon esculentum Mill (tomato) plant defense gene, PAL5 transformed into *Solanum tuberosum L.* (Potato)	*Erwinia carotovora*	Carrot, tomatoes, potatoes	(Yi *et al*. 2004)

The ability to chemically synthesis AMPs based on natural peptides has also meant that changes can be easily made to peptide sequences with the aim of examining the effect of changes or substitutions and ultimately increase the activity or potency of these peptides.

Such derivates of plant AMPs have been examined in studies against food spoilage fungi and yeast. De novo synthetic, modified plant AMPs were synthesised and designed on basis of natural cationic plant AMPs: these peptides were observed to show antifungal and antiyeast activity against common food pathogens (Shwaiki *et al.*, 2019; Thery *et al.*, 2019). *F. culmorum*, *Penicillium expansum* (apple pathogen) and *Aspergillus niger* (grapes, onions, apricots, peanut pathogen and common contaminant of food) were the fungal species sensitive to the peptides, while the yeast species *Z. bailii, Z. rouxii, D. hansenii, S. cerevisiae* and *K. lactis* were similarly inhibited by the peptides. Both studies also demonstrated the application of the synthetic peptides in different beverage products commonly spoiled by these yeast and fungal species. In some cases, a complete reduction in fungal and yeast growth were observed in the beverages. Another examined synthetic antimicrobial peptides derived from the radish plant that successfully inhibited the spoilage of different food and beverage products by the yeasts *Z. bailli, Z. rouxii, D. hansenii, K. lactis* and *S. cerevisiae* (Shwaiki, Arendt and Lynch, 2020). Fruit juices and a soft drink inoculated with *Z. bailii* and peptides were found to completely inhibit its growth and the inoculation of salad dressing with peptides and yeast resulted in its partially inhibition. The inhibition of fungal spoilage in dough was examined by Thery and Arendt, 2018 which incorporated a synthetic peptide based on a natural AMP derived from the cowpea plant and reduced spoilage in the dough matrix (Schmidt, Arendt and Thery,

2019). Table 4 lists some of these and more studies that explore synthetic AMPs derivatives and transgenic plants containing synthetic peptides.

Chemically synthesising peptides can be advantageous over natural extractions as it reduces the time, labour and equipment cost of extracting from plant material. This can have the potential to reduce the necessity to divert the value of seeds from food supplies, an issue that could arise due to the amount of seed material that would be necessary to extract a significant economically viable quantity of AMP.

The synthesis of very pure peptides can be accomplished, reducing contaminant compounds in a sample. Without the advent of chemical peptide synthesis methods generation of (potentially enhanced) AMP derivatives through amino acid sequence modification would not have been possible. A number of challenges, however, remain that will hinder their application as part of the solution for reducing food losses (application for crops; reduction of losses at farm and producer levels) and food waste (application in foods; reduction at retail and consumer level). The state of the current synthesis technology means that production of a large scale is generally cost prohibitive; a 40 amino acid peptide can cost as high as $1600 per gram (Latham, 1999). Furthermore, the more structurally complex the peptide, the more expensive the synthesis will be; for example, it is more expensive to produce cyclic and crosslinked peptides, than it is to produce those that are short and linear. However, cyclic peptides are known to be more resistant to degradation, making it the preferred choice for peptide production (Wimley, 2019). Therefore, the current high costs make chemically synthesised peptides less appealing for their incorporation as food preservatives at this time. However, over time as the technology develops further and the cost of synthesis begins to drop, chemical synthesis of peptides could represent part of a much-needed solution for the control of food waste. The design of enhanced

synthetic peptides with increased potency could enable the application of lower quantities of peptide (thus offsetting the current high costs associated with chemical synthesis on a large scale). In addition to the cost-associated challenges facing chemically synthesised AMPs, consumer perception of these peptides as chemical preservatives, also poses a potential challenge that would need to be addressed. Therefore, fundamental studies performed now, like those described above are important in demonstrating proof of principle and laying the foundation for future research and development.

2.8 Safety of AMPs

Consumers increasingly demand more natural forms of food preservatives. Naturally extracted and chemically synthesised plant AMPs can have the potential to meet this demand. However, as with destined for consumption and incorporated into foods, safety is of paramount importance. As previously discussed, AMPs are cationic and interact with the negatively charged membrane of microorganisms. Most human cells are neutral or slightly positively charged, making AMPs selective for microbial/fungal cells (Chen *et al*. 2016). Although AMPs are selective, weak hydrophobic interactions between AMPs and mammalian cell membranes can occur. Even though these interactions are weak compared to the electrostatic interactions that microbial and fungal membranes have with AMPs, cytotoxicity is still a potential issue for mammalian cells (Mahlapuu *et al.*, 2016).

An agent's cytotoxic effect refers to its ability to cause reduced cell viability or proliferation; in this case AMP cytotoxicity to human cells is a potential and undesirable effect (Aslantürk, 2018). This can occur via a number of mechanisms. The cell membrane's integrity can be compromised and can result in cell lysis. Leakage of the cell's content follows, resulting in a rapid mechanism called necrosis (Paredes-Gamero *et al.*, 2012). Apoptosis can also occur; a process of programmed cell destruction (Hengartner 2000; Silva, Do Vale, and Dos Santos 2008). In addition to this, some AMPs may cause cell proliferation, i.e., an increase in the number of cells. This mechanism is associated with and seen to increase in tumour development (López-Sáez, 1998). Cell proliferation is amongst one of the activities associated with AMPs, including wound repair, cytokine release and chemotaxis of leukocyte cells. Although beneficial in the case for wound repair or chemotaxis of leukocytes, cell proliferation can be a marker for the development of tumours in mammalian cells

(Ackermann, 2016). The method by which AMPs can cause cell proliferation has been proposed to be through the promotion of DNA synthesis through the increased expression of cell differentiation-relating factors, accelerating cell proliferation (Liu *et al*. 2018).

Various studies have found AMPs to be cytotoxic. This aspect could be beneficial for example, in the case of inhibiting the viability in cancerous cells (Rozek *et al.*, 2000; Anaya-López *et al.*, 2006; Galdino da Rocha Pitta, Galdino da Rocha Pitta and Lins Galdino, 2010). However, for AMPs destined for application in food products and on plant crops, the cytotoxicity level needs to be minimal. The majority of defensins, typically, are known for their low toxicity (Thevissen *et al.*, 2004). Other AMPs like thionins (Stec 2006; Loeza-Ángeles *et al*. 2008; Li *et al*. 2002) and cyclotides (Lindholm *et al.*, 2002; Svangård *et al.*, 2007) can be significantly cytotoxic. A snakin peptide called PG-2, derived from the potato tuber *Solanum tuberosum* L cv. Gogu Valley, however, was not found to be non-toxic to human cells (Kim *et al.*, 2013). This demonstrates the variability among AMPs and indicates that some peptides may be better suited than others for use in food preservation/crop protection. Thus, besides the level of activity or potency, an equally important factor is peptide safety.

This cytotoxic property can be avoided or reduced through the design of synthetic peptides. AMP derivatives or analogues can be generated that have reduced cytotoxic effects. Linear cationic AMPs destabilise mammalian cell membranes to a lesser extent than fungal membranes (Mason, Marquette and Bechinger, 2007; Thery *et al.*, 2018). The overall hydrophobicity, a major factor contributing to cytotoxicity, is reduced as a result of the removal of disulfide bridges from a non-linear peptide, forming a linear one. Studies that examine synthetic peptides have shown this (Ferre *et al.*, 2006; Porto *et al.*, 2018). Increasing the hydrophobicity and amphipathicity

generally leads to an increase in cytotoxicity to mammalian cells, therefore peptides with such properties are less desirable.

The haemolytic activity of an AMP refers to its ability to rupture red blood cells (Oddo and Hansen, 2017). The ability to cause haemolysis is used as a general indicator of peptide cytotoxicity (Ruiz *et al.*, 2014). There are numerous studies that have treated erythrocytes with different plant AMPs and found the haemolytic activity of these peptides to be insignificant (Phansri *et al.*, 2011; Thery and Arendt, 2018; Thery *et al.*, 2019; Shwaiki, Arendt and Lynch, 2020).

For AMPs to be considered safe, a peptide's sensitivity to proteolytic degradation needs to be taken in account. For the use in therapeutics, they need to be able to withstand degradation. Various studies have examined this and found different AMPs to be suitable (Starr and Wimley 2017; Strömstedt *et al*. 2009; Meng and Kumar 2007; Shao *et al*. 2019). In food applications, however, a peptide may be readily broken down when ingested. For application in food preservation, where the activity is only necessary up until the time of consumption, proteolytic degradation of the peptide during digestion is not an issue; indeed, it may be desirable to minimise any potential cytotoxic or allergenic effects. However, depending on the food application and type of product, it would be important that the peptide is resistant to any potential endogenous proteolytic activity that may exist in a food product, which could potentially lessen or destroy the activity of the peptide during the very time that the peptide's antimicrobial (preservative) activity is desired. Therefore, a peptide's susceptibility to proteolytic activity in certain products would determine its application in different food matrices.

Studies that have analysed how certain synthetic AMPs react to proteolytic enzymes discovered that they can in fact be prone to proteolytic digestion (Thery *et al.*, 2019; Shwaiki, Arendt and Lynch, 2020). Synthetic peptides and their derivatives/analogues can be generated to have increased or reduced resistance to proteolytic degradation, depending on the proposed application or food product. Studies have revealed that cyclisation and fatty acid modifications result in a stable and more resistant peptide, therefore non-cyclic, linear peptides could be more appropriate for food application (Montesinos and Bardají, 2008). Peptides that contain L-amino acids are also more prone to degradation, compared to D-amino acids. Structural modification of a peptide sequence may lead to an improvement of one property; however, it is important to consider the changes these modifications can have on other functional properties or the activity of the peptide. For example, reducing the cytotoxicity or proteolytic resistance may have a negative effect on the antimicrobial activity of the peptide (Zeitler *et al.*, 2013).

The safety of AMPs for human consumption is therefore a very important factor to consider. Much of the literature on AMPs focuses on increasing cytotoxic effects and proteolytic resistance for application in therapeutics and drug development. More research needs to be conducted on the safety of AMPs with respect to food applications. In addition, a better understanding of how these peptides interact with the food matrix will aid in improving the field of AMPs as potential food preservatives (Borch, Kant-Muermans and Blixt, 1996).

2.9 Other plant compounds, secondary metabolites and hormones as antifungal agents

Plants are complex organisms, and as previously discussed, they have numerous adaption mechanisms and compounds to help survive harsh environments in nature. The section on *'Natural plant compounds as food preservatives'* reviewed some of the more well-known plant compounds and their potential for food and crop protection. In this section, other plant compounds, hormones and secondary metabolites that have not been as extensively researched for crop protection will be reviewed.

Methyl jasmonate, an ester belonging to the jasmonate family, is involved in the regulation of plant growth. This plant hormone provides defense against biotic and abiotic factors by mediating communication within and outside a plant system, making them critical as secondary metabolites (Reyes-Díaz *et al.*, 2016). Studies focused on the application of methyl jasmonate pre- and post-harvest to food crops have reported significant findings in favour of this compound. The treatment of tomato plants with methyl jasmonate in their early vegetative stages of development found that the plant was less susceptible to the plant pathogen *Monilinia fructicola* (causative agent of brown rot in stone fruits) (Li *et al*. 2010). Post-harvest treatments of fruit have also benefited. In a study by González-Aguilar, Buta, & Wang, 2003, the exposure of the papaya fruit to methyl jasmonate vapours inhibited fungal decay, with and without the combinational treatment of MAP.

Another secondary metabolite involved in triggering resistance to plant pathogens are phytoalexins. Most of these compounds are less potent than the fungicides found on the market, however there is strength in numbers when it comes to phytoalexins as they are able to accumulate to concentrations beyond what is needed to cause fungal

inhibition (Jeandet *et al.*, 2002). There are many types of phytoalexins that have antifungal activity. An example of one of these compounds is resveratrol, a natural compound also researched for its potential effect as an anticarcinogenic and antioxidant agent (Adrian and Jeandet, 2012). The plant pathogen *B. cinerea* was tested for its inhibition in the presence of resveratrol and extensive conidial disruption was found (Adrian & Jeandet, 2012). This demonstrates the potential of phytoalexins for plant protection. A review by Vestergaard & Ingmer, 2019 also highlights the capability of resveratrol as antifungal and antimicrobial agents.

Ureases differ from the secondary metabolites described above as they are enzymes that are responsible for nitrogen bioavailability. However, like methyl jasmonate, salicylic acid and phytoalexins, they are also responsible for defending a plant against pathogens. Studies that have focused on plant derived ureases found that they can have inhibitory effects against various plant pathogens. Becker-Ritt *et al.*, 2007 demonstrated that ureases extracted from soy and jack beans (*Glycine max* and *Canavalia ensiformis*, respectively) cause cell wall damage to fungal pathogens including *Trichoderma* spp..

Saponins are a group of plant glycosides well-known for their soap-like properties. Their use as a soap component makes their antifungal properties somewhat overlooked. Saponins extracted from *Aesculus hippocastanum* (aescin), *Glycine max* (soyasaponin) and *Hedera helix* (hederagenin) were inhibitory against important fungal pathogens, with aescin being the most effective. The pathogens *Microdochium nivale* and *Pyrenophora teres* were amongst the fungal species effected, major pathogens of wheat and barley, respectively (Trdá *et al.*, 2019).

2.10 Summary and conclusion

The aim of this review was to provide an update on our understanding of plants' defense mechanisms and how these can be exploited as part of a solution for the global food waste challenge that exists today. A large percentage of food waste today is due to microbial or fungal spoilage. Although the established preservation techniques have tackled this issue, consumers increasingly desire more natural forms of food preservatives.

Plants are a natural reservoir of antimicrobial/antifungal compounds and can be exploited to reduce food spoilage in a variety of ways. Even with the current knowledge on plant defense mechanisms and biology, there is still a lot that can be learned from plant systems and how they can further be applied in the field of food science and food preservation. There is a large body of knowledge on the activity of plant compounds, specifically AMPs, against microbial and fungal plant pathogens. Their application in food can be advantageous as their natural origin may be seen as a more conservational and healthier solution to current preservatives. The introduction of these plant-based preservatives can also encourage a more sustainable food production system as it would reduce the resources needed to produce and generate the existing forms of preservatives. Furthermore, the potential application of such AMPs presents an opportunity for reducing the use of chemical agricultural pesticides, which can negatively affect consumer perception of crop productions. Using plant compounds as preservatives has a number of potential advantages to traditionally used preservation methods. In the case of AMPs, plants are readily available and can be easily cultivated. This also applies for the other plant compounds discussed, such as EOs, phenolic compounds and propolis (Antolak and Kregiel 2017).

The application of AMPs, whether natural, synthetic or in transgenic plants, have their challenges. The expense, labour, and time necessary to produce plant AMPs at quantities where they could be beneficial are challenges that must be overcome before they can be exploited to their fullest and promising potential in the food industry. As the technologies improve and develop, plant AMPs could be exploited to a fuller extent and the research being conducted at present and reviewed here represents an excellent foundation for future development.

2.11 Acknowledgements

This work was supported by the Department of Agriculture, Food and the Marine (F.I.R.M.), Project Reference 15/F/731.

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Chapter 3

Future of antimicrobial peptides derived from plants in food application – a focus on synthetic peptides

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Published in Trends in Food Science and Technology:

Shwaiki, L. N., Lynch, K. M. & Arendt, E. K. (2021) 'Future of antimicrobial peptides derived from plants in food application – a focus on synthetic peptides', Trends in Food Science and Technology. Elsevier. doi: 10.1016/j.tifs.2021.04.010.

3.1 Abstract

Background

Food spoilage is caused by the undesirable growth of spoilage microorganisms in food products. This spoilage can lead to the global loss and waste of food. It is estimated that 1.3 billion tons of edible food produced for human consumption is either lost or wasted each year. Different preservation techniques have been developed to prevent this spoilage; however, the problem still occurs. Plants have the ability to produce compounds to protect themselves from the harsh environment. This has led to the exploitation of plant antimicrobial peptides (AMPs) which have been naturally extracted to study their activity against plant, food, and human pathogens. The chemical synthesis of such peptides has also grown in popularity over the years.

Scope and Approach

This review will focus on the different techniques that can be applied to generate peptide sequences, produce them through various chemical and biological methods, and predict their activity. These approaches are reviewed for their ability to develop synthetic peptides (based on plant AMPs) with greater antimicrobial activity and characteristics essential for their application as potential food preservatives. The development of these synthetic peptides with reduced toxicity to human cells, improved activity and stability can be a possible solution to the continuous fight against food spoilage and food waste.

Key Findings and Conclusions

Synthetic antimicrobial peptides are being developed and modified in such a way to encompass potent and safe characteristics. This knowledge can be exploited for the potential application of such peptides in foods as preservative agents. A deeper

understanding of their structure, function and mechanisms of action can be used to integrate them into food for the reduction of food spoilage, and consequently, of food waste. Although their development and production through the methods reviewed can generate peptides with suitable characteristics for reducing food spoilage, the cost of synthesis can be a drawback to such methods. Nevertheless, as the technologies improve and develop over time, the development of these synthetic AMPs can be fully exploited for their potential role in the food sector.

3.2 Introduction

Food waste is increasingly prevalent in modern society and can be attributed both to consumer behaviour and the undesirable growth of spoilage microorganisms (bacteria, fungi (moulds) and yeast that cause food spoilage). Food waste and food loss are terms that the Food and Agriculture Organization (FAO) define as the "decrease in quantity or quality of food along the food supply chain" at different stages (FAO, 2019). In addition, a clear distinction between them can be made by identifying food waste as occurring "at the retail and consumption level", while food loss is predicated to occur "along the food supply chain from harvest/slaughter/catch, up to, but not including, the retail level". These distinctions are important to further understand the role of spoilage microorganisms in the food production chain. An estimated 1.3 billion tons of edible food produced for human consumption is either lost or wasted each year. In Europe and North America, 280-300 kg of food is lost or wasted per capita every year – a high figure compared to the 120-170 kg/year of food lost in sub-Saharan Africa and South/Southeast Asia. Due to consumer behaviour, per capita food waste can be as high as 115 kg/year in developed countries, compared to 6-11 kg/year in developing countries (FAO, 2019).

Spoilage microorganisms play a large role in determining the degree of food waste and food loss worldwide. Different food groups can be affected by microorganisms such as bacteria, fungi (mould) and yeast. The contributing factors for their growth in food are dependent on the type of substate; with particular focus on the water, sugar, salt, oxygen and nutrient content (Petruzzi *et al.*, 2017). Just like pathogenic spoilage bacteria (that can cause disease and the spoilage of food), fungal spoilage pathogens (yeast and moulds) are also likely to grow and cause outbreaks. The production of mycotoxins by some fungal species can result in the contamination of major crops

such as maize (aflatoxins, fumonisins and zearalenone mycotoxins (Tarazona *et al.*, 2020)), rice (aflatoxin, citrinin and deoxynivalenol mycotoxins (Tanaka *et al.*, 2007)) and wheat (deoxynivalenol and zearalenone mycotoxins (Tibola *et al.*, 2015)). These toxins can go on to cause food-borne illnesses (Benedict, Chiller and Mody, 2016). Food spoilage by fungal pathogens is nevertheless a factor that contributes to the global food waste and food loss concern and should be regarded as part of the problem.

Although foodborne illnesses are a devastating result of pathogenic spoilage microorganisms, the loss of food at different stages of production through to consumption due to spoilage can be economically destructive. The exact figure for the total economic loss caused by food spoilage is unknown, however it is estimated that 25% of food produced globally is lost due to spoilage microorganisms (Bondi *et al.*, 2014).

The use of food preservation techniques, in the form of temperature control, food additives (chemical and natural), modified packaging, thermal and ionisation processes are all applied with the aim to reduce spoilage (Bhat, Alias and Paliyath, 2012). However, the loss of food in this way continues to be a problem. Consumer perceptions of food and their best-by date increases the challenges faced in the battle to reduce food loss. Consumers begin to pass judgment on the consumability of a food product that may be safe for consumption due to the ‘best-by’ or ‘best-before’ dates. In addition, consumer behaviour related to the amount of food purchased and the sale of increasing large serving or pack sizes for products, has led to an ever-increasing amount of food being discarded, either because it was not consumed before it deteriorated or because it was simply forgotten about; such behaviour further contributes to food loss. In the EU, it was reported that household waste was one of

the highest contributor to food waste with approximately 47 million tonnes ± 4 million tonnes reported in 2012 (Stenmarck *et al.*, 2016).

Increasing preservative levels in foods may, in theory, be a way to tackle this problem. However, additives like chemical preservatives that are linked to increasing shelf-life are perceived as negative by consumers; increasingly there is a demand for different and more natural forms of preservatives (Bedale, Sindelar and Milkowski, 2016).

Antimicrobial peptides (AMPs) are compounds found in animals, plants and even microorganisms, that function to protect against microbial pathogens. In plants, this mechanism is crucial for survival. (Adem Bahar and Ren, 2013; Nawrot *et al.*, 2014). Plant AMPs have been researched for their potential to inhibit different pathogens, including food spoilage pathogens, from bacterial to mould and yeast species. A new and increasingly recognised avenue for the study of plant AMPs for such applications is their synthesis through various approaches. This review will look at these novel approaches and at the methods employed for the design of plant AMPs and their functional prediction; furthermore, the status of these novel AMPs in the future of food preservation is discussed. The focus of this review is to develop an understanding of plant AMPs and how they can be exploited for their antimicrobial activity for the prevention of food loss and food waste. AMPs from other sources (e.g., animals and humans) will also be considered in areas where their knowledge can be applied for plant AMPs.

3.3 Plant antimicrobial peptides (AMPs)

Plants are stationary entities whose ability to photosynthesise and produce the oxygen that we need to survive make them one of the most remarkable organisms on earth. Nevertheless, they can find themselves vulnerable to environmental factors, herbivores, and like other organisms, to microbial (bacteria and fungi) infections. Plants have evolved and adapt to such stresses by developing physical or chemical defences. Physical traits may include the production of thorns or spines to deter herbivores, or the production of bark or waxy cuticles to deter microorganisms; while chemical defences are within the plant system (Moles *et al.*, 2013). Amongst these is the production of AMPs, whose synthesis occurs in nearly all parts of a plant during any stage of its life (Campos *et al.*, 2018). For example, the seeds of the radish plant continuously produce AMPs belonging to the defensin family. During seed germination, the production of these defensins are significantly increased to aid in protecting the seedlings against pathogens (Stotz, Thomson and Wang, 2009). Just like defensins, there are many other AMPs that plants can produce; the major ones belong to the families of thionins, cyclotides, snakin, hevein (and hevein-type peptides), knottin (and knottin type peptides), lipid transfer proteins (LTP) and 2S albumin proteins (Nawrot *et al.*, 2014). These AMPs have been the focus of essential research to understand their relationship and interaction with plant pathogens (Benko-Iseppon *et al.*, 2010), food spoilage pathogens (Hintz, Matthews and Di, 2015) and even human health (De Souza Cândido *et al.*, 2014).

AMPs from different families have been studied for their effect against plant pathogens and food spoilage pathogens with the aim to reduce spoilage (and ultimately loss) throughout the food chain. Since the discovery of these AMPs, most research has been performed to investigate the microbial inhibition potential of many of these

peptides. Plant pathogens such as *Pythium graminicola*, *Botrytis cinerea*, *Verticillium dahlia* and *Aspergillus niger* are significant crop spoilage organisms, which have been successfully inhibited by AMPs extracted from rice (thionin) (Ji *et al.*, 2015), potato tubers (snakins) (Segura *et al.*, 1999), amaranth (hevein-type peptide) (Broekaert *et al.*, 1992) and peas (defensins) (Almeida *et al.*, 2000), respectively. A thionin peptide produced by *Arabidopsis thaliana* was found to be inhibitory against common food spoilage pathogens *Staphylococcus aureus* and *Escherichia coli* (Loeza-Ángeles *et al.*, 2008), while *Klebsiella pneumoniae* was inhibited by the presence of a cyclotide peptide from the plant *Viola odorata* (Pränting *et al.*, 2010). Table 5 gives a list of peptides from various families of plant AMPs. Their structure, source, and their antimicrobial properties against common food/crop spoilage pathogens are included in this table.

Table 5: Peptides in the different families of plant AMPs that can have an application in food as potential preservative agents.

Type of AMP	Name of AMP	Source	Chemical and structural Properties	Antimicrobial Properties	Potential in food applications	Reference
Defensin	Rs-AFP1 Rs-AFP2	*Raphanus sativus*	Highly basic peptides, rich in cysteine residues; molecular weights of 5 kDa; Contain net charge of +4 and +6, respectively. Cysteine-stabilized αβ-motif typical of plant defensins	Antifungal and antiyeast activity	Synthetic Rs-AFP1 and Rs-AFP2 contain antiyeast activity against food spoilage yeast (*Zygosaccharomyces bailii)* in various beverages	(Terras *et al.*, 1992; Shwaiki, Arendt and Lynch, 2020a)
	IbAMP1	*Impatiens balsamina*	Highly basic peptide. Contains four cysteine residues which form two intramolecular disulfide bonds. Contains a net charge of +5	Antifungal and antibacterial activity	Antimicrobial activity was found against common enteric foodborne pathogens (*Escherichia coli, Salmonella enteric, Pseudomonas aeruginosa)*	(Tailor *et al.*, 1997; Wu, Di and Matthews, 2013)
	Cp-thionin II	*Vigna unguiculata*	Conformation is stabilized by four disulfide bonds between cysteine residues. Contains a net charge of +8	Antifungal and antibacterial activity	Prevention of the contamination of wheat grains during storage (against the fungal spoilage pathogens *Fusarium culmorum, Aspergillus niger, Penecillium expansum)*	(Schmidt, Arendt and Thery, 2019)
	MsDef1 MtDef4	*Medicago sativa*	A conserved region of two antiparallel beta strands with an interposed loop containing a cationic charge that participates in one to four disulfide bonds. The net charge for MsDef1 is +3 and +6 for MtDef4	Antifungal activity	Antifungal activity against a common wheat and barley pathogen (*Fusarium graminearum*)	(Sagaram *et al.*, 2011)

Thionins	Tu-AMP 1 Tu-AMP 2	*Tulipa gesneriana*	Both peptides are rich in cysteine and basic amino acids (arginine and lysine). Tu-AMP 1 is a single-chain peptide which contains four disulfide bonds. Tu-AMP 2 had a unique structure; it was a heterodimer consisting of α-chain and β-chain	Antifungal and antibacterial activity	Antifungal and antimicrobial activity against plant pathogens (e.g., *Fusarium oxysporum* and *Curtobacterium flaccumfacien*) known to cause spoilage of important food crops (potatoes, tomatoes, legumes)	(Fujimura *et al.*, 2004)
	Wheat β-Purothionins	*Triticum aestivum*	8 cysteine and 10 basic residues are distributed throughout the peptide. 4 disulfide bonds are present	Antibacterial activity	Antibacterial activity against common food pathogens that cause major spoilage in commercially significant plants such as tomatoes and peppers (*Corynebacterium michiganense*, *Xanthomonas campestris*)	(Fernandez de Caleya *et al.*, 1972; Mak and Jones, 1976)
	Thionin 2.4	*Arabidopsis thaliana*	A 5kDa peptide with 3 disulfide bonds present within its sequence. It contains a net charge of +1	Antifungal activity	Activity against the destructive crop fungal pathogen *F. graminearum* (targeting barley, wheat and corn crops)	(Asano *et al.*, 2013)
Snakin/GASA	St-SN1	*Solanum tuberosum*	St-SN1 is highly basic and has a short, central hydrophobic stretch. It contains a net charge of +9	Antifungal, antiyeast and antibacterial activity	Antipathogenic properties of the Snakin peptide revealed the potential to inhibit growth of potato and wine grape pathogens. The synthetic form of St-SN1 reduced yeast spoilage in various beverages.	(Segura *et al.*, 1999; López-Solanilla *et al.*, 2003; Shwaiki, Arendt and Lynch, 2020c)

					Listeria monocytogenes, a major pathogen in ready-to-eat foods was also found sensitive to this peptide	
	MsSN1	*Medicago sativa*	MsSN1 contains 12 cysteine residues within a conserved C-terminal region	Antifungal and antibacterial activity	Antifungal activity of this peptide reduced the growth of pathogenic fungal species effecting the alfalfa crop (*Phytophthora medicaginis*). The same fungal specie is also a common pathogen of the chickpea and lentil plant	(García *et al.*, 2014)
	Snakin-Z	*Ziziphus jujuba*	A 31 amino acid peptide with a molecular weight of 3.318 kDa	Antifungal and antibacterial activity	Strong antibacterial action against the pathogen *Staphylococcus aureus* that can be transmitted through contaminated/spoiled food	(Daneshmand, Zare-Zardini and Ebrahimi, 2013)
Cyclotides	Cycloviola cin O2	*Viola odorata*	Peptides consists of a circular backbone and 3 disulfide bridges which are arranged in a knotted fashion. Peptides contains net charge of +2	Antifungal and antibacterial activity	Antipathogenic properties against common food spoilers (*Salmonella, E. coli)* and antifungal activity against fungal pathogens known to target rice and legume crops	(Pränting *et al.*, 2010; Zarrabi *et al.*, 2013)
	Cycloviola cin O8			Antifungal activity	Antifungal activity against a common wheat and barley pathogen (*F. graminearum*)	(Parsley *et al.*, 2018)
Knottin-type	PAFP-S	*Phytolacca americana*	Cationic and highly Basic peptide containing three disulfide bridges in its structure (6 cysteine	Antifungal activity	Common crop pathogens of legumes, rice and barley (*F. oxysporum* and *Pyricularia oryzae*) are susceptible to	(Shao *et al.*, 1999)

			residues responsible for disulfide bridges). Contains a net charge of +4		the peptide's strong antifungal activity	
	Mj-AMP1 Mj-AMP2	*Mirabilis jalapa*	Peptides are highly basic and contain 3 disulfide bridges within their structures. Both peptides contain a net charge of +3	Antifungal activity	Major pathogenic fungal species (*F. oxysporum)* known to target agriculturally significant crop such as rice were sensitive to the antifungal properties of these knottin-type peptides	(Cammue *et al.*, 1992)
Hevein-type	Ee-CBP	*Euonymus europaeus*	Peptide contains 5 disulfide bridges (linked by 10 cysteine residues present in its structure) and a net charge of +5	Antifungal activity	Strong antifungal properties against *F. culmorum* and *Alternaria brassicicola*, pathogens of wheat and cabbage crops	(Van Den Bergh *et al.*, 2002)
	SmAMP3	*Stellaria media*	A basic and cysteine-rich peptide containing a net charge of +2 and 3 disulphide bridges in its structure	Antifungal activity	The growth of fungal fruit (grapes and apricot) pathogen *Aspergillus niger*, and legume pathogen *Fusarium solani* were inhibited by the antifungal activity of the havein-type peptide	(Rogozhin *et al.*, 2015)
	EAFP1 EAFP2	*Eucommia ulmoides*	Both sequences contain 41 amino acid residues. Both contain 10 cysteines, cross-linked to form five disulfide bridges	Antifungal activity	Pathogenic fungal species that cause spoilage to tomato, wheat and potato crops (A*culops lycopersici)* are susceptible to the antifungal activity of both peptides	(Huang *et al.*, 2002)

Lipid Transfer Protein (LTP)	Ha-AP10	*Helianthus annuus*	A basic 10 kDa peptide containing 4 disulfide bridges	Antifungal activity	Fungal pathogen *Fusarium solani* that effects major economically significant food crops was susceptible to the antifungal properties of the peptide	(Regente and De La Canal, 2000)
	Ca-LTP1	*Capsicum annuum*	A 9 kDa peptide with 4 α-helices within its structure	Antifungal activity	The growth of the common bean plant pathogen *Colletotrichum lindemunthianum* was inhibited by the peptide's antifungal activity	(Diz *et al.*, 2011)
	Mung bean nsLTP	*Phaseolus mungo*	Peptide consists of four α-helices stabilized by four disulfide bonds and a net charge of +7	Antifungal and antibacterial activity	Peptide's antifungal activity resulted in strong inhibitory effects against major crop fungal pathogens (*F. solani, Fusarium oxysporum).* Antibacterial action against *S. aureus,* a major spoiler and contaminant of foods such as meat and dairy products	(Wang *et al.*, 2004; Lin *et al.*, 2005)
2S albumin proteins	Pe-AFP1	*Passiflora edulis*	The peptide consists of 2 disulfide bonds in its sequence formed by 2 conserved cysteine residues	Antifungal and antiyeast activity	The peptide was successful in causing inhibition to the fungal pathogen *F. oxysporum*, a spoiler of agriculturally significant crops	(Pelegrini *et al.*, 2006)
	CW-1	*Malva parviflora*	A 15 amino acid peptide with a molecular weight of 1.783 kDa	Antifungal activity	Antifungal activity against a common grain crop pathogen *Fusarium graminearum*	(Wang and Bunkers, 2000)

These AMPs can be extracted from plants using different extraction and purification methods and variations of these methods have also proven successful. One approach for the purification of a plant peptide is to perform an extraction procedure using the plant of interest and a buffer of a specific pH. This solution can undergo several centrifugation and precipitation (ammonium sulfate) stages. Ion exchange chromatography is used to separate the target peptide by charge (anion and cation exchange chromatography) and an extra chromatography step can be applied (reverse-phase high performance liquid chromatography (RP-HPLC)) for its further purification. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is performed to approximate its molecular weight and an amino acid analysis can determine a portion or the majority amino acid sequence of the isolated peptide. Its potential name and origin can be further assessed using a protein BLAST search on an online database (Tang *et al.*, 2018). This approach of natural extraction can be time consuming and the yield concentration of peptide is typically low, therefore a large-scale extraction procedure is required. In addition to this, AMPs extracted and purified from a plant host can display antimicrobial activity at higher concentrations compared to their synthetic form (i.e., they can contain lower antimicrobial properties than the synthetic form). Some studies have shown higher antimicrobial activity by the synthetic version of an AMP that has been originally purified from the host plant. A prime example of this is the synthetic linear analogue of the native Cp-thionin II peptide from the seeds of the cowpea plant. Minimum inhibitory concentrations (MIC) of 20 and 200 µg/mL were observed against the food spoilage fungi *Fusarium culmorum* and *Penicillium expansum*, respectively (Thery and Arendt, 2018). This synthetic AMP resulted in more potent antimicrobial activity compared to the AMP

from which its sequence was synthesised from. The extracted and purified peptide from the seeds of the cowpea plant displayed higher MIC values against the same fungal spoilers in the study by Schmidt *et al*., 2019. MICs of 50 µg/mL against *F. culmorum* and > 500 µg/mL against *Penicillium expansum* were observed, both levels higher than what the synthetic cowpea peptide required to cause inhibition. These studies demonstrate the lower antimicrobial properties that naturally extracted AMPs can have compared to their synthetic counterparts.

The synthesis of AMPs via various chemical processes are being employed as an alternative method to produce AMPs such as those mentioned above, but also more potent derivatives, or even totally novel peptides, based on best 'design' and structural and functional characteristics of the most potent natural plant AMPs. These methods can be exploited to increase the safety of food as well as reduce food waste, as their directed design could provide a potency and stability surpassing that of naturally extracted AMPs from plants.

3.4 Synthetic AMPs from plants

3.4.1 Approaches to develop synthetic peptides

Plant AMPs have been successfully extracted and purified from different sources and their antimicrobial activity has been characterised. The chemical synthesis of AMPs is being recognised as an alternative to natural extraction for its potential in reducing production time. An entire plant peptide sequence may be analysed for the synthetic development of novel peptides with even more potent antimicrobial activities. This has been explored through the natural cleavage or *in-vitro* proteolysis of AMPs to acquire short natural fragments with improved characteristics (Thery, Lynch and Arendt, 2019). Although not well studied in plant AMPs, this method of peptide production has been performed on the human milk protein, Lactoferrin, producing analogs (Lf (1–11) and Lfcin H17–31) with positive antifungal activity against the human fungal pathogens *Aspergillus fumigatus* and *Candida albicans,* respectively (Håversen *et al.*, 2010; Fernandes and Carter, 2017). Another study that explored this approach looked at the analogs developed from the amphibian cationic α-helical peptide Temporin-SHf. These peptide analogs showed antimicrobial and antifungal activity against various Gram positive and Gram negative bacteria, and yeasts, including *Staphylococcus aureus, Escherichia coli,* and *Candida albicans.* In addition to this, at antimicrobial concentrations, cytotoxic effects were not observed (André *et al.*, 2015). Peptides obtained via this approach not only display improved activity, but also reduced cytotoxicity to human cells, increasing their value as they are both potent and safe.

A different approach for the generation of synthetic peptides is through *de novo* synthesis using combinatorial libraries. This method bases the assembly of new peptides on already established AMPs that may have strong antimicrobial properties but encompass negative characteristics such as cytotoxicity to human cells. Such peptides can be modified to fit desired characteristics for safety and improve their potential for future applications. The Antimicrobial Peptide Database (APD_3) (Wang, Li and Wang, 2015) is amongst one of the numerous databases available. The input of sequences into these databases calculates the hydrophobicity, structure, total net charge – amongst other properties – of a peptide, as well as correlate its sequence to known AMPs already on the database. This method has proved successful in generating potent peptides and will be discussed in more detail in the section that explores predicting peptide activity. In one particular study, three *de novo* cationic peptides were designed and found to exhibit strong activity against several pathogenic spoilage yeast and fungal species, including *C. albicans,* and *Aspergillus* and *Fusarium* species (Maurya *et al.,* 2013). This ability to modify peptide sequences and alter their properties is valuable for the production of more stable and active peptides. A study by Chu et al., 2013 found the substitution of tryptophan or histidine residues with D-β-naphthylalanines resulted in a peptide that was less sensitive to high salt concentrations. This resistance to high salt concentrations signifies the peptide's stability. Its ability to retain its antimicrobial activity in a high salt solution denotes the peptide's potent antimicrobial properties. A peptide's ability to resist high salt concentrations is a good indicator of its potential as a food preservative. The salt concentrations encountered in various food products can cause the deactivation of

AMPs, therefore a higher resistance to such salt concentrations is optimal for their potential application as food preservatives.

These studies highlight what can be achieved through the modification of known peptide sequences. Whether through natural cleavage, *in vitro* proteolysis, or the generation of a novel peptide based on already established peptides, plant AMPs can be used to generate more potent, stable peptides that can be further exploited for their use in food preservation.

3.4.2 Production of synthetic peptides

Following the design of a peptide where its functional properties will be maximised through targeted design and consideration of its sequence, it will be synthesised (figure 3 summarises the various methods of peptide synthesis).

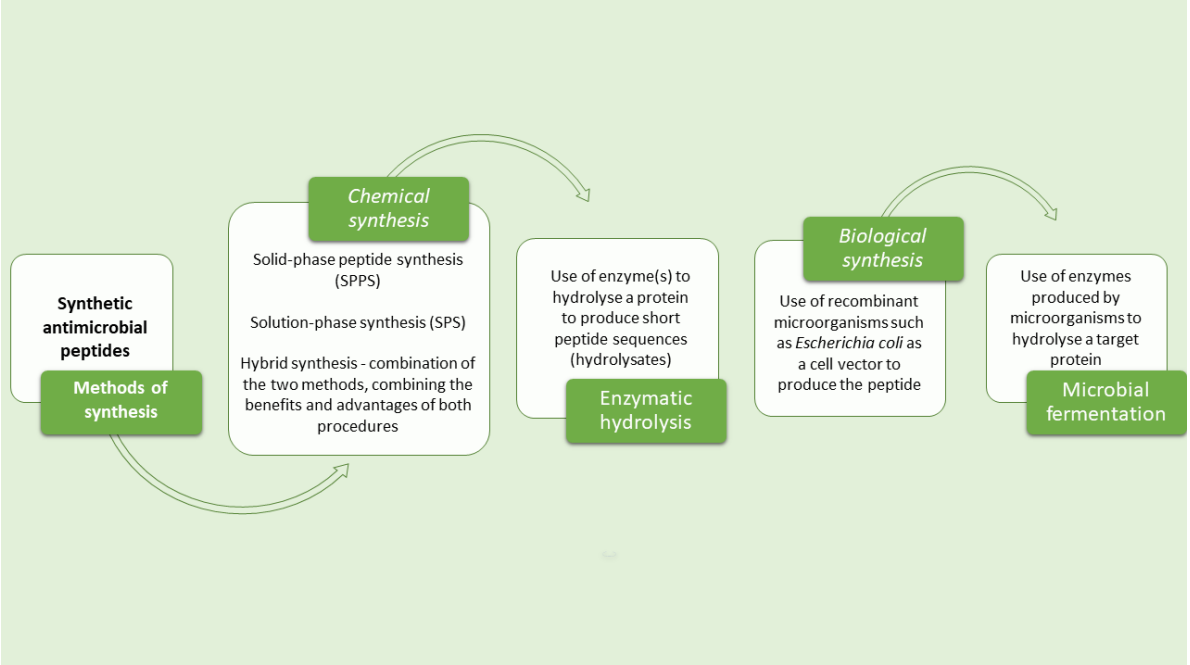

Figure 3: A summary of the different approaches that peptides can be synthesised through chemical and biological synthesis.

Chemical synthesis is preferred over the biological synthesis of peptides due to the possibility of incorporating non-natural components into a sequence (for example alpha, beta-didehydrophenylalanine) (Maurya *et al.*, 2013; da Costa *et al.*, 2015). Solid-phase peptide synthesis (SPPS) and solution-phase synthesis (SPS) are commonly practised forms of chemical synthesis employed in the production of peptides. SPPS was introduced after the development of SPS for its simplicity, efficacy, and compatibility with automated synthesis. This made it possible to produce and develop peptides on a much larger scale than SPS, whose long and laborious production process was a disadvantage for clinical use. Hybrid synthesis is a combination of the two methods, combining the benefits and advantages of both procedures (Goodwin, Simerska and Toth, 2012). To better understand these methods and the processes involved, the articles by Raibaut et al., 2015 and Tsuda & Okada,

2011 explore, in more depth, the steps required for peptide production using these techniques.

Biological synthesis refers to the use of recombinant organisms for the production of peptides or proteins. The early 1980s brought about the production of insulin using this type of recombinant technology. Two small genes containing nucleotide sequences coding for human insulin was inserted into *E. coli* cells, allowing for the production of large quantities of the peptide (Riggs, 1981). This discovery made it possible for human insulin to be manufactured for large scale therapeutic use. The production of AMPs by recombinant methods has since been recognised as an efficient system to generate larger peptides – such as defensins, that typically contain 30-50 amino acid residues (in comparison to the peptides discussed already, which may have as little as 11 residues in their sequences). Although proven effective, some disadvantages exist such as low production yield and the necessity to overcome the potential toxic effect of the peptide on the host microorganism. In addition, the introduction of non-biological components cannot be accomplished using this method. To overcome this, these AMPs may be fused with other proteins such as thioredoxin, which not only prevents cell lysis but also aids in the formation of disulfide bonds. Various cell vectors have been used for this function, including the most well-known, *E. coli*, the fungal species *Aspergillus*, yeast cells belonging to *Saccharomyces cerevisiae*, and even chloroplast expression systems (Matejuk *et al.*, 2010). Potato AMPs, Snakin-1 (sn1) and Defensin-1 (pth1) were successfully generated through biological synthesis via expression in *E. coli* cells. The peptides were found to be inhibitory against bacterial and fungal potato pathogens including *Clavibacter*

michiganensis subsp. *sepedonicus* and *Colletotrichum coccoides,* and the wine grape pathogen *Botrytis cinerea* (Kovalskaya and Hammond, 2009). A separate study found similar results when expressing genes for the AMP Pg-AMP1, found in the seeds of the guava (*Psidium guajava*) plant, into *E. coli* cells. This AMP inhibited various gram-negative and gram-positive bacterial food spoilage pathogens (Tavares *et al.*, 2012). The production of synthetic AMPs by this cell vector method further demonstrates the potential of synthesising such peptides to reduce the growth of food spoilage pathogens in order to develop them further for their use as potential food preservatives.

A different method for obtaining peptides from plants is through the process of enzymatic hydrolysis. During this process, the use of one or more enzymes are applied to hydrolyse a specific protein (in this case, a plant protein) to produce short peptide sequences in the form of hydrolysates (Daliri, Oh and Lee, 2017). A study by Aguilar-Toalá et al., 2020 gives insights into this method to produce hydrolysates derived from the seeds of the *Salvia hispanica* plant. The study successfully generated peptides with antimicrobial activity against food spoilage microorganisms *Escherichia coli* and *Listeria monocytogenes*. They accomplished this through the hydrolysing actions of enzymes alcalase and flavourzyme on the proteins present in the seeds of the plant. A separate study by Sornwatana et al., 2013 used a similar approach to generate antibacterial peptides from the fruit protein of *Fructus Bruceae* by peptin hydrolysis. This method is often preferred over the process of employing microorganisms to perform this hydrolysing mechanism. This approach (referred to as microbial fermentation) involves hydrolysing a target protein through the action of enzymes that

a microorganism releases as it grows. This aids the proteins to break down into peptides that can be screened for their antimicrobial activities. In addition to the short reaction time, the ease of scalability and predictability, the enzymatic hydrolysis approach renders it the preferred method over microbial fermentation (Daliri, Oh and Lee, 2017).

These approaches (summarised in figure 4) have been proven effective for the synthetic production of AMPs. Although some of these methods may have drawbacks, the design of potent peptides with optimal characteristics can be taken one step further. Modifying a peptide sequence can lead to the improvement of its antimicrobial activity, stability, and cytotoxic properties (safety).

3.4.3 Designing synthetic AMPs

For AMPs to be considered for food applications, it is crucial to identify any negative effect that they may have on human cells. A peptide's toxic effect, as well as its haemolytic activity and susceptibility to proteolysis are important factors that need to be considered when applying AMPs in foods that will be consumed. In AMPs from natural plant sources, these factors can pose a challenge. However, in synthetic assembly, a peptide may be designed in such a way that can exclude these negative effects as much as possible. Designing AMPs is a sensitive process as one modification, while positive from the point of reducing cytotoxicity and increasing safety, may have a detrimental effect on the overall antimicrobial characteristics and potency of the peptide. Factors including cationicity, hydrophobicity, amphipathicity, secondary structure formation (for example α-helical and β-sheet) and sequence length

all contribute to the formation of a stable peptide with potent activity (Thery, Lynch and Arendt, 2019). Table 6 summarises these different factors and their significance for the generation of a synthetic AMP.

Table 6: Factors to consider when designing synthetic AMPs.

Designing synthetic antimicrobial peptides			
Factors to consider	**Summary**	**Examples**	**Reference**
Cationity & distribution of residues	Positive charged AMPs facilitate the interaction with microbial membrane. Lys, Arg, and His strongly interact with membrane lipid layer, enabling strong antimicrobial activity. An AMP incorporating too many Arg residues can cause the haemolytic activity to rise. Insertion of Arg or Lys in the hydrophobic face of a helix peptide can reduce haemolysis.	Addition of Arg residues in the temporin-Shf analogs increased activity against *Saccharomyces cerevisiae* – MIC as low as 12.5 µM was observed for some analogs.	(André *et al.*, 2015)
Hydrophobicity	Hydrophobic residues can facilitate insertion into microbial membrane. Increasing hydrophobicity above a certain point can result in loss of activity and increase in toxicity.	Single amino acid substitution increased hydrophobicity of HPRP-A1 analogs. Good antimicrobial and antifungal activity were observed, however accompanied by strong haemolytic activity (>120% difference in haemolysis between HPRP-A1 and some of its analogs with similar activity).	(Zhao *et al.*, 2013)
Amphipathicity	A peptide that contains both hydrophobic and hydrophilic residues in its sequence. An amphipathic peptide can have strong antimicrobial activity, however above a certain threshold, it can have haemolytic/toxic effects.	A significant increase in the affinity to red blood cells was observed in a modified peptide (with the same hydrophobicity to the parent peptide) consisting of Tyr and Leu instead of two Lys residues. Haemolysis at 55 µM	(Hollmann *et al.*, 2016)

		increased from 8 ± 2 (parent peptide) to 85 ± 12 (modified peptide).	
Length	A peptide must be of at least 7-8 amino acid residues in length for good amphipathic structure to develop. Shorter AMPs are known for their strong antimicrobial activity and reduced toxicity/haemolysis and salt sensitivity.	The 18-mer peptide (RI18) derived from the 36-residue parent peptide (PMAP-36) showed strong inhibitory activity – 99.9% clearance – against *E. coli* in the presence of 25 and 50% serum.	(Lyu *et al.*, 2016)

3.4.3.1 Cationicity and distribution of residues

The activity of AMPs against microorganisms is a consequence of a peptide's overall positive net charge. The cationicity of a peptide refers to its positive charge that allows it to interact with negatively charged microbial membranes. The potency of a peptide can be explained by its interaction with negatively charged biological membranes. Basic amino acid residues arginine (Arg) and lysine (Lys) cause initial interaction with the anionic surface membrane, facilitating the residues' insertion into the membrane lipid bilayer (Thery, Lynch and Arendt, 2019). Furthermore, these positively charged residues can participate in covalent and non-covalent interactions with other residues of the membrane bilayer (such as valine (Val), tryptophan (Trp) leucine (Leu), alanine (Ala), tyrosine (Tyr) and phenylalanine (Phe)) (Li, Vorobyov and Allen, 2013). The incorporation of Arg residues has been found to generate stronger antimicrobial activity than Lys; however, a downside to this is the high haemolytic activity that accompanies it. The stable interactions observed between the side chains in an Arg residue and those found in the membrane bilayer can make it easier for it to navigate past the hydrophobic membrane core of microorganisms (Schibli *et al.*, 2006). This was observed in the study by André et al., 2015, that found that the addition of Arg residues in the temporin-Shf analogs increased their activity against yeast spoilage strain of *Saccharomyces cerevisiae* (spoiler of alcoholic beverages).

The distribution or location of these residues in a sequence can determine activity or potency. Lowering the haemolytic activity of an α-helical peptide was found to be promoted via the insertion of Arg or Lys residues in the hydrophobic face of the helix – causing a decrease in the hydrophobic moment (measure of amphipathicity) and

ultimately in the haemolytic activity of the peptide (Jin *et al.*, 2016). Furthermore, the amino acid residue histidine (His) can be used as an alternative to Arg and Lys, due to the lower isoelectric point and its net charge increasing under acidic conditions. If the amphipathicity of a peptide is negatively affected by the presence of an Arg or Lys residue, the more neutral His residue can be used instead (Sharma *et al.*, 2010).

3.4.3.2 Hydrophobicity

Hydrophobic residues are just as important in a peptide sequence. The hydrophobicity of a peptide defines the overall charge distribution as non-polar and refers to the presence of hydrophobic residues in a sequence. The nature of the side chains found on amino acids determine their hydrophobicity (glycine (Gly), valine (Val), and proline (Pro) are examples of such residues). The binding of peptides to the membranes of microorganisms is facilitated by these residues, making them crucial for activity. Increasing the hydrophobicity of peptides can reduce their sensitivity to high salt conditions which can result in the loss of membrane-disrupting effects (Saravanan *et al.*, 2014). However, the hydrophobicity of a peptide can only be moderately increased before losing selectivity and potency and increasing its cytotoxic effects (Ong, Wiradharma and Yang, 2014). This is due to the capacity of peptides with high hydrophobicity to readily enter the inner mammalian bilayer and cause haemolysis. Zhao et al., 2013 demonstrated this in their study that observed the effect of single amino acid substitution on the hydrophobicity of the bacterial (*Helicobacter pylori*) peptide HPRP-A1. The study found these analogs to exert good antimicrobial and antifungal activity, but also developed strong haemolytic activity. This

corroborates the assumption that hydrophobic characters in a peptide sequence may be linked to high haemolytic activity due to the high hydrophobicity of a peptide enabling interaction with various types of lipid bilayers. It is therefore important to understand the balance of hydrophobic residues in a peptide sequence to reduce the likelihood of toxic effects towards mammalian cells.

3.4.3.3 Amphipathicity

Amphipathicity is the state in which a peptide contains both hydrophilic and hydrophobic residues. This attribute permits the hydrophobic elements of a peptide to further permeate microbial membranes after initial attachment to the phospholipid head groups. It does this through its insertion and interaction with the phospholipids present (Takahashi *et al.*, 2010). The hydrophobic moment is used to measure the amphipathicity of a peptide in an alpha-helical conformation. The amphipathicity of a peptide is typically associated with helix motifs; as such hydrophobic and hydrophilic residues are located between the opposite faces of a helix, resulting in a fitting distribution for membrane binding (Drin and Antonny, 2010). Alpha helical peptides are one of the most encountered peptides in research, due to their reputation as highly potent AMPs. Alpha-purothionin from wheat (Han *et al.*, 1996) and Ah-AMP1 from horse chestnut (Fant, Vranken and Borremans, 1999) both contain helical structure in their overall form.

Similar to the property of hydrophobicity, studies have demonstrated that modulating the amphipathicity can result in different outcomes with respect to antimicrobial activity and cytotoxic effects of a peptide. Hollmann et al., 2016 demonstrated this

when cationic peptides based on a previously studied AMP were designed and modified to have the same hydrophobicity and different hydrophobic moments. A significant increase in the affinity to red blood cells was observed in the modified peptide consisting of Tyr and Leu instead of two Lys residues, due to the presence of a higher hydrophobic moment. This higher hydrophobic moment caused the peptide to become highly amphipathic and develop a secure helical structure, thus explaining the increase in the haemolytic activity.

A similar outcome was observed by Zhang et al., 2016, where a dramatic decrease in the amphipathicity of their peptide resulted in lower haemolytic effects for some of the analogs originated, while also preserving good antimicrobial activity. The minimum inhibitory concentration (MIC) for the majority of bacterial species tested was double that of the original, native peptide; 12.5 μM vs 6.25 μM. Although the activity was not fully preserved, the resulting activity is still deemed effective. The slight increase in the MIC was a resulting consequence for the lower haemolytic activity. This was accomplished through the substitution of residues on the non-polar face of the peptide, resulting in the lower haemolytic effect. This effect has been suggested to be due to the disruption of the alpha-helical amphipathic structure that is linked to enhanced haemolytic activity.

3.4.3.4 Length

As observed with the cationicity, hydrophobicity and amphipathicity, the length of a peptide bares great importance on its activity. The connection between an amphipathic peptide and its length is ensured through the presence of at least 7-8 amino acids

residues in the sequence. This is also true when considering the structure of a peptide. For example, an effective alpha-helical peptide requires at least 22 amino acids to traverse the lipid bilayer, while a beta-sheet requires at least 8 amino acids (Adem Bahar and Ren, 2013). Shortening a peptide sequence can, however, result in the increase in activity and reduction in toxicity, haemolysis and in some cases, salt/serum sensitivity. In a study by Mohanram & Bhattacharjya, 2016, various 12-residue cationic, amphipathic *de novo* peptides were designed and tested for their bactericidal activity in both a serum and salt solution, with their activity being retained. Likewise, shorter analogs of the porcine cathelicidin peptide (PMAP-36) were designed into an 18-mer peptide (RI18) with better fungicidal activity and salt resistance (Lyu *et al.*, 2016). This further supports the concept of employing shorter antimicrobial peptides for increased activity. In addition, shortening the length of a peptide reduces production costs, making it more economically viable to manufacture.

The effect of each of the discussed parameters or properties on their own is not well established, as the activity and selectivity of peptides are not dependant on one factor alone; instead, they are all dependant on one another. The studies mentioned highlight the necessity for a better understanding of the structure-function relationship of peptides, and how modifying a sequence for enhanced activity may be accompanied by increased negative effects. A balance between good antimicrobial activity and reduced cytotoxicity is therefore critical for the development of these peptides. However, the more that is understood about relationship and interdependency of the different parameters in a peptide, the more efficient it can be to the development and synthesis of effective, enhanced and safe peptides. Adopting an integrated thinking on

peptide design could be revolutionary in designing synthetic AMPs that are potent, safe and economical to combat the effects of crop and food spoilage pathogens right across the food chain.

3.5 Mechanism of Action

3.5.1 Membrane interactions

A cationic AMP can exert its antimicrobial activity through membrane interactions that result in the disruption of the membrane and eventual death of a cell. Unlike conventional antibiotics, AMPs act rapidly. What AMPs can do in a matter of minutes, antibiotics may take hours to accomplish (Fantner *et al.*, 2010). This fast response is not only a result of membrane interaction with peptides, but it is also the effect of their interference with metabolic activities and cytoplasmic components through the interference of various signalling processes such as wound repair, chemotaxis or cell migration (Splith and Neundorf, 2011).

Plant AMPs have been extensively studied for their mechanism of action that targets the cell membranes of microorganisms (Shwaiki, Arendt and Lynch, 2020c). This has led to different hypothetical model systems being proposed for their interaction with and effects on cells. The amphipathic and cationic structure of AMPs allow for electrostatic attraction to the negatively charged phospholipids, teichoic acids or lipopolysaccharides found in the membranes of microorganisms. This leads to the accumulation of the peptide on the membrane surface, after which, having reached a threshold concentration, the peptides begin to reorient in the lipid bilayer and the collapse of the membrane follows. The manner by which this happens has been proposed through different mechanisms which can further be classified into different groups based on their primary mode of action; 1) the formation of pores or 2) through membrane disruption. The pore forming mechanisms include the “barrel-stave” and the “toroid pore” (wormhole) models. In the barrel-stave model, alignment of the

hydrophobic regions of the peptide with the lipid tail occurs, leading to the formation of the inner surface of the pore with hydrophilic residues. The toroidal pore model is a result of the charged residues in the AMP causing an electrostatic shift in the hydrophilic heads of the lipids. This causes the membrane to bend back on itself, causing the top and bottom monolayers to be continuous (Nawrot *et al.*, 2014). Some AMPs act via non-specific membrane permeabilisation (membrane disruption), one of which is called the carpet model. It gets its name from the way that the peptide begins to cover the surface of the membrane once it has reached a critical concentration, in a "carpet-like" fashion. Eventually, there is a destabilisation and collapse of the membrane structure into micelles. This final step is caused by the peptides saturating the surface of the membrane and, in a detergent-like manner, breaking the lipid bilayer into separate micelles (Lee, N. Hall and Aguilar, 2015). Figure 4 illustrates these 3 mechanisms.

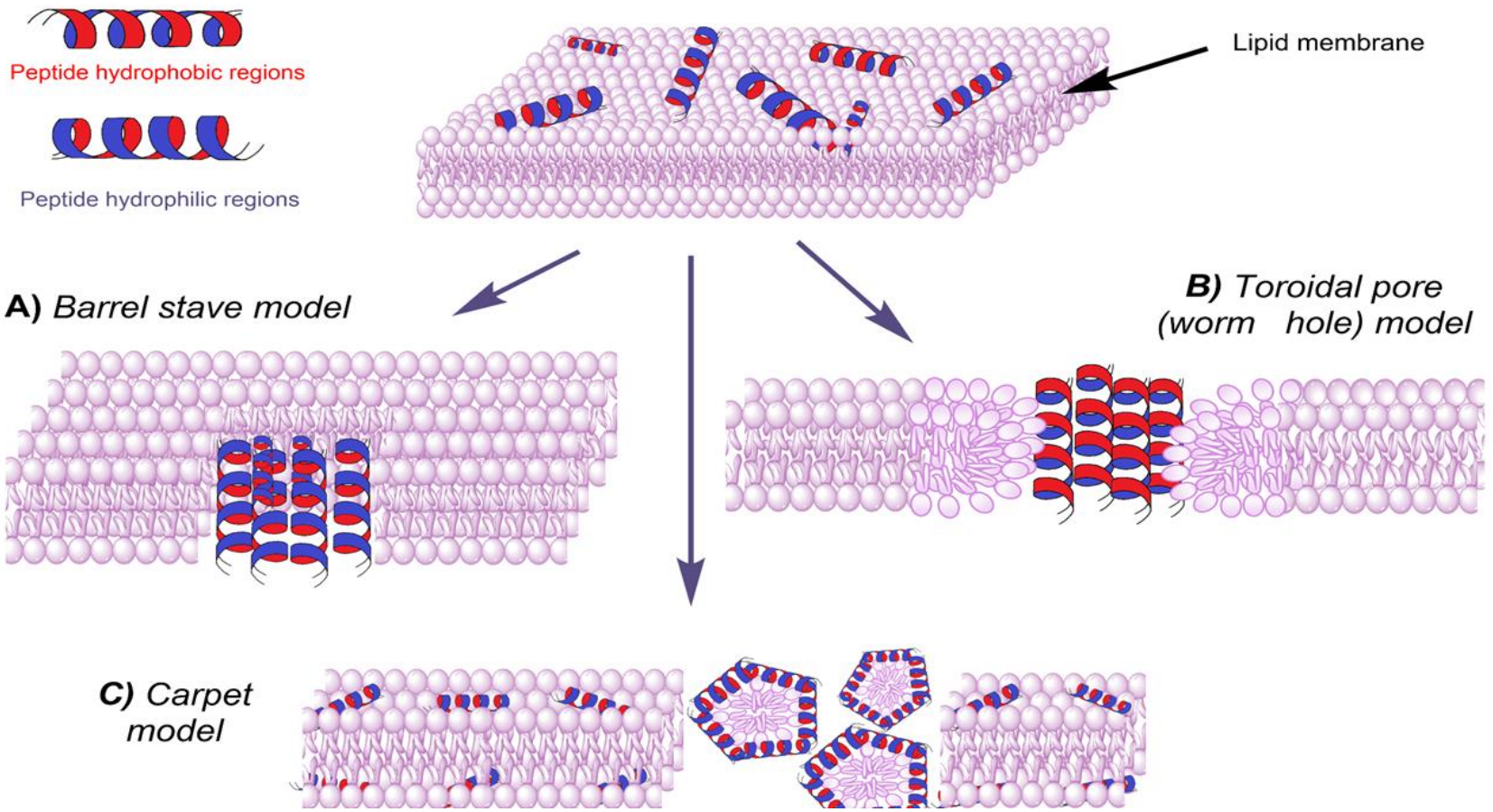

Figure 4: Illustration of the mechanism of action of AMPs through the formation of pores and the disruption of the membrane. The barrel-stave model (A) and the toroidal pore (worm hole) model (B) signify a peptide's pore forming abilities that can result in the further disruption of a cell's normal function. The carpet model (C) is illustrative of the disruptive mechanism that AMPs can act on to form micelles from the membrane structure.

3.5.2 Intracellular mechanisms

As discussed above, at high concentrations plant AMPs can permeate the cell membrane. It is important to note that when this happens, intracellular mechanisms may be affected too for example, through the rapid efflux of ions and cytoplasmic membrane depolarization. In the case of the efflux of ions, it has been shown that membrane permeabilisation can lead to the leakage of potassium ions from the cell, leading to cell death (Kim and Lee, 2019; Shwaiki, Arendt and Lynch, 2020b).

Reactive oxygen species (ROS) are potent oxygen-containing molecules that can lead to the oxidative stress of a cell if present in excess. Plant AMPs have been found to induce apoptosis via the accumulation of these ROS (Oyinloye, Adenowo and Kappo, 2015). Microorganisms are reliant on these species for various processes including their metabolism and stress response. However, the overproduction and accumulation of ROS such as hydroxyl radicals, superoxide anions, hydrogen peroxide and peroxide radicals can ultimately lead to cell death (Ciociola *et al.*, 2016). This generation of ROS can result in oxidative damage to macromolecules and ultimately lead to protein oxidation or fragmentation, or DNA damage. Superoxide anions can be converted to hydrogen peroxide via the mitochondrial respiratory chain of a cell. This ROS can then react to form the reactive and more potent hydroxyl radical, whose presence will subsequently lead to the induction of apoptosis (Cho and Lee, 2011). AMPs can promote the generation of these ROS, leading to such events (Oyinloye, Adenowo and Kappo, 2015).

3.6 Safety of AMPs

The safety of plant AMPs is an important factor to consider for their application in food. Just like the therapeutic application of drugs, it is essential that AMPs in food applications are deemed safe for human consumption. The cationic nature of AMPs makes them attracted to the negatively charged membranes of microorganisms, making them the ideal antimicrobial agents. Due to this selectivity, AMPs are less likely to target human cells that are neutral or slightly positively charged. In addition, the component of a mammalian membrane bares differences to the membrane of microorganisms. An example of this is the higher content of cholesterol in human cell membrane, thus providing protection against AMPs. Nevertheless, weak hydrophobic interactions can occur, making cytotoxicity a potential risk (Chen *et al.*, 2016).

In the synthetic production of peptides, the cytotoxic effects described can be minimised, through targeted design. Some ways that this can be achieved is through the disruption of alpha-helical structures or modification of the peptide backbone. The ability of a peptide to bind and form a helical configuration upon interaction with neutral membranes has been found to cause enhanced toxicity (Mihajlovic and Lazaridis, 2012; Zhang *et al.*, 2016). Modification of the peptide backbone has resulted in improved biological properties of peptides, including reduced toxic effects. This has been accomplished through the substitution of residues with un-natural amino acids, for example, the replacement of the naturally occurring L-amino acid for the unnatural D-form (Shah *et al.*, 2020). Furthermore, this modification has been shown to improve sensitivity to proteases of such modified peptides (Manabe and Kawasaki, 2017). However, it is speculated that improved stability in the presence of proteases

can also occur from such modifications. This could be due to the longer *in vivo* half-lives that peptides containing D-amino acids possess, in comparison to their natural counterparts (Wade *et al.*, 1990). Using the full D-enantiomeric structure of a peptide by substituting all L-amino acids with their D-counterparts is known to create more resistant peptides to proteolytic digestion (Melchionna, E. Styan and Marchesan, 2016). Resistance to proteolytic digestion is important when considering plant AMPs for their application in foods, as a peptide must also be capable of being broken down by enzymatic activity in the gut, where its action is no longer desired.

For the systemic application of these AMPs, a low haemolytic activity against red blood cells must also be considered. Plant AMPs are known to rupture human erythrocytes, an indicator sign of cytotoxicity, making them unsuitable for food applications. As discussed in the previous section, the length, hydrophobicity and amphipathicity are all factors that can be considered when designing synthetic AMPs, for reducing the haemolytic (cytotoxic) activity of the peptide and increasing overall safety.

3.7 Predicting and modulating activity of novel AMPs

There are various methods that can facilitate the synthetic design of potent, stable, and safe AMPs. An effective peptide may be designed on the basis of a natural peptide or number of peptides that can be used as a template. By selecting the characteristics and properties of desirable peptides (as described in section 3.3), such derivative, chimeric peptides can be designed, incorporating the desirable characteristics of the original natural peptide(s), for greater antimicrobial activity, better stability, and reduced cytotoxicity. In addition, this can be achieved through single amino acid substitutions to help identify the significance of certain residues and understand their role in the peptide sequence and the link to the peptide properties (Fjell *et al.*, 2012). This form of modification is known as post-translational modification (PTM), and can also involve disulfide bonds, C-terminal amidation, bromination, chlorination, or the presence of D-amino acids (Falanga *et al.*, 2016). In the study by Carroll et al., 2010, an AMP derived from bacteria was used as the template for three enhanced peptides called nisin S, T and C. These derivatives were based on the parent peptide and through the incorporation of newly introduced residues, resulted in the enhancement of antimicrobial activity over the native peptide. Panteleev et al., 2015 used the template-based approach to reduce the cytotoxic activities of analogs from the AMP, arenicin. This was done by identifying the amino acid residues responsible for this toxicity and performing a single amino acid substitution with hydrophilic residues on the non-polar face of the peptide. This method of prediction relies heavily on the clear understanding of the structure and function of each residue present in the template peptide (as mentioned in section 3.3).

A different method to the template-design approach is the use of biophysical techniques to examine the structural features of AMPs as they interact with a target membrane. This is of importance as some peptides can adopt a different structure upon interaction or binding with a membrane and understanding their structure and mode of interaction can be of benefit when developing AMPs (Avci, Akbulut and Ozkirimli, 2018).

Virtual screening is a more complete but also more complex approach for the prediction of peptide activity. Quantitative structure-activity relationships (QSAR) is a common technique based on molecular modelling of small AMPs. QSAR takes into account the numerous descriptors of a peptide, such as size, charge, hydrophobicity, etc., and generates an overview of the predicted biological activity of the peptide (Barley, Turner and Goodacre, 2018). Machine learning algorithms and statistical analysis techniques can also be used for this aim. Techniques such as logistic regression (Veltri, Kamath and Shehu, 2017), artificial neural networks (ANN) (Torrent *et al.,* 2011), support vector machines (SVM) (Meher *et al.,* 2017) and random forests (RF) (Bhadra *et al.*, 2018) have all been exploited for the prediction of AMP activities. These approaches are being increasingly exploited for their high speed, low cost, and high efficiency. The relationship between the antimicrobial activity and the biochemical attributes of a peptide can be interpreted using these machine learning systems. Several AMP databases that adopt these techniques are available and contain an extensive list of AMPs. Some databases may focus on AMPs derived from specific sources, such as PhytAMP and PlantPepDB that focus on plant AMPs, or ones that deal with AMPs derived from all sources of organisms (CAMPR$_{3,}$

APD3, DBAASP). These databases can be used as prediction tools and in the case of many of these, peptide sequence assembly and functionality are frequently based on machine learning algorithms. The CAMP database, for example, employs SVM, ANN and RF (Su *et al.*, 2019).

This method of prediction is a powerful and efficient way of understanding and developing a potent, stable and safe peptide. Not only is it economically viable and time-efficient, but virtual screening of AMPs or potential AMPs can serve as a guiding tool for the time-consuming work that wet-researchers perform (in the laboratory, but also to the *in-silico* research involved). Nevertheless, the complexity of these computational tools for the development of novel AMPs may be intimidating and sometimes difficult to interpret. This downside of virtual screening can result in researchers avoiding this approach and relying more on template-based or biophysical approaches.

3.8 Benefits and challenges of synthesising AMPs in the aim to reduce food waste

The virtual screening and chemical synthesis of AMPs are promising methods that can aid in the production of peptides with potent antimicrobial properties. The ability to modify a peptide to achieve better antimicrobial properties and improved stability makes them prospective candidates that can be applied to food to reduce spoilage. Exploiting these methods can therefore generate peptides with enhanced properties. A peptide with a greater tolerance to high salt concentrations generally found in foods, with greater sensitivity to proteolytic degradation to allow for their breakdown in the human gut, and reduced toxicity against human cells, can be developed. The knowledge required to develop an AMP with suitable characteristics for its application in food to reduce the growth of spoilage microorganisms can be acquired through the understanding of how AMPs can be generated and synthesised. The basis of these synthetic peptide sequences is derived from plants, a source from which AMPs are constantly being discovered. The vast number of peptides already known and the potential for more to be discovered is a benefit for the development of synthetic peptide sequences derived from this source.

Consumer perception of current preservation techniques, especially chemical preservatives, has increased the desire for more natural forms of preservatives in foods. The approach of synthesising peptides based on plant peptide sequences can be seen as a possible solution to this. Various studies have been successful in providing evidence for the inhibition of spoilage microorganisms with AMPs and, with future developments in peptide understanding, production and synthesis, the further reduction of food spoilage can be envisaged. All of the techniques and approaches

presented in this review can be further developed and built upon for the production of effective peptides whose sequences, configuration and properties are based on natural plant AMPs.

The challenge that synthetic AMPs face in the application of food waste reduction is its cost of synthesis. The low market cost of current preservative agents (chemical preservatives such as sodium benzoate for example) gives these forms of preservatives the advantage. The cost of chemical synthesis processes is currently expensive, thus, the production of large quantities of peptide required for application could be currently cost prohibitive. Regardless of these challenges, as the technologies improve and develop over time, the development of synthetic AMPs could be more fully exploited. The research reviewed here exemplifies their potential to be considered as a real solution to the growing problem of food waste. Figure 5 illustrates a **SWOT** analysis demonstrating the **S**trengths, **W**eaknesses, **O**pportunities and **T**hreats of utilising synthetic AMPs derived from plants to reduce food spoilage and food waste.

Figure 5: A SWOT analysis demonstrating the Strengths, Weaknesses, Opportunities and Threats of utilising synthetic AMPs derived from plants to reduce food spoilage and food waste.

3.9 Summary and conclusion

Food loss and waste caused by pathogenic spoilage microorganisms has led to an increasing amount of food being lost globally. Although food preservation using traditional techniques has assisted in reducing the burden of food waste due to the growth of spoilage microorganisms, it is still an issue that presents a challenge. This review examined the use of plant AMPs as a possible solution to food waste, and more specifically their synthetic counterparts and derivatives. To fully exploit synthetic peptides, there needs to be an awareness on how to develop, design and predict such peptides and their antimicrobial activity. Developing an understanding of the structure-function relationship of a peptide sequence can serve as a starting point for the improvement of plant AMPs, whose inhibitory activity may be ideal but whose safety towards human cells may not be optimal. Generating synthetic AMPs has proven successful via natural and in-vitro proteolysis or even *de novo* synthesis using combinational libraries. Various methods have been adopted to predict the activity and functionality of novel AMPs. Virtual screening provides the most beneficial technique thus far for this aim, as it combines the use of machine learning with already well-established databases available. Both chemical and biological methods of peptide synthesis can be used; however, chemical production via SPPS is currently the more common method due to its time efficient, flexible and direct production processes.

As the purpose of these synthetic AMPs is for the prevention of the growth of spoilage microorganisms in food systems, their safety is of utmost importance. The probability of developing a peptide with cytotoxic effects can be avoided by generating a peptide with certain desirable sequence characteristics, as described in this review. It is

therefore important to find a balance between the level of antimicrobial activity and the level of cytotoxicity that a peptide may demonstrate. However, this negative trait cannot be disregarded when discussing the drawbacks of the incorporation of, either natural or synthetic, AMPs into foods.

The research presented in this review illustrates the potential for the application of synthetic AMPs derived from plants to reduce food/crops spoilage. Synthetic AMPs are reviewed for their consideration as possible solutions to the continuous existence of food waste.

3.10 Acknowledgements

This work was supported by the Department of Agriculture, Food and the Marine (F.I.R.M.), Project Reference 15/F/731.

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Chapter 4

Anti-yeast activity and characterisation of synthetic radish peptides Rs-AFP1 and Rs-AFP2 against food spoilage yeast

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Published in Food Control as:

Shwaiki, L. N., Arendt, E. K. and Lynch, K. M. (2020) 'Anti-yeast activity and characterisation of synthetic radish peptides Rs-AFP1 and Rs-AFP2 against food spoilage yeast', *Food Control*, 113, p. 107178. doi: 10.1016/j.foodcont.2020.107178.

4.1 Abstract

Food spoilage resulting from the presence of yeast is a common problem in the food industry. The development of natural food preservatives is a growing area of interest for the food industry. The application of antimicrobial peptides derived from plants can be a simple and natural method of preserving food. This study looked at the antiyeast activity of two chemically synthesised radish antimicrobial peptides, Rs-AFP1 and Rs-AFP2, for their inhibitory effect against different yeast species. The minimum inhibitory concentration (MIC) of both peptides was generated. Two mechanisms of action were studied (membrane permeabilisation and the overproduction of reactive oxygen species (ROS)) and both were found to occur with Rs-AFP2, while only the overproduction of ROS was detected for Rs-AFP1. The effect of the peptides on the yeast cells was also visualised by scanning electron microscopy. Their safety in terms of human consumption was studied and no adverse effects were found. Lastly, the stability of the peptides in different conditions, such as high salt, heat and a range of pH were studied in addition to their antiyeast activity in different food matrices such as soft drink, fruit juices and salad dressing, further supporting the peptides' potential for use in food preservation.

4.2 Introduction

Antimicrobial peptides (AMPs) are a large group of host defence proteins of short amino acid sequence and positive charge (Hancock & Diamond, 2000). They are found in different life forms ranging from microorganisms to animals and humans (Adem Bahar & Ren, 2013; Jenssen, Hamill, & Hancock, 2006; Mahlapuu, Håkansson, Ringstad, & Björn, 2016). They are part of the host defence system and are antimicrobial towards a wide range of pathogens (Brown & Hancock, 2006; Hancock, 1999). AMPs isolated from plants amount to a large group of these proteins and are found in various parts of the plant, constituting part of their host defence system. Many of these peptides have been isolated from areas such as the roots, seeds, flowers, leaves and stems of plants (Goyal & Mattoo, 2016; Tang, Prodhan, Biswas, Le, & Sekaran, 2018). Defensins are one major group of AMPs in the plant kingdom. They are small proteins with 45-54 amino acids (approximately 5 kDa) and are rich in cysteine residues (De Samblanx et al., 1997a; Lay & Anderson, 2005; Neuhaus, 1999). They provide protection against fungal and bacterial pathogens during the plants' life cycle (Garvey et al., 2013).

AMPs have recently garnered increased interest in different areas of scientific research, from their integration as potential sources of novel antibiotics (Seo, Won, Kim, Mishig-Ochir, & Lee, 2012; Zaiou, 2007), to their use in food preservation (da Silva Malheiros, Daroit, & Brandelli, 2010; De Vuyst & Leroy, 2007; Schmidt, Arendt, & Thery, 2019; Shwaiki, Arendt, Lynch, & Thery, 2019). In the food industry, food spoilage can occur during stages of production (Fung, 2009), packaging (Korkeala & Johanna Björkroth, 1997) or consequentially during the storage of the food product. This contamination may be caused by the unfavourable growth of bacteria, yeast or fungal species (de W. Blackburn, 2010). Yeast species

Zygosaccharomyces, *Saccharomyces*, *Debaryomyces* and *Kluyveromyces* are notorious for their manifestation in products such as soft drinks and salad dressing (Thomas & Davenport, 1985), wine (Kalathenos, Sutherland, & Roberts, 1995), meats and cheeses (Houtsma, de Wit, & Rombouts, 1993; Westall & Filtenborg, 1998), and dairy products (Fleet & Mian, 1987; Mayoral et al., 2005), respectively.

The use of AMPs in the prevention of food spoilage is becoming a topic of interest in the field of bio-preservation (Ahmad et al., 2017; Cleveland, Montville, Nes, & Chikindas, 2001; Fry, 2018) as the natural aspects of using plant AMPs is appealing. Numerous plant species have been used for the extraction and purification of such AMPs (Carvalho, Machado, Da Cunha, Santos, & Gomes, 2001; Okamoto, Mitsuhara, Ohshima, Natori, & Ohashi, 1998; Tailor et al., 1997; Zhang & Lewis, 1997). The radish plant, *Ruphanus savitus*, is recognized as the source to the two defensins, Rs-AFP1 and Rs-AFP2. These two proteins are highly basic and rich in cysteine residues with a molecular weight of approximately 5 kDa (Terras et al., 1992). They have been previously extracted and purified from the seeds of the radish plant. The production of these defensins can be accomplished either from natural extraction processes using the seeds of the plant (Osborn et al., 1995; Terras et al., 1992), but can also be chemically synthesised (Koczulla & Bals, 2003). Chemically synthesising peptides can be expensive; however, a very high purity can be achieved, leading to the production of a peptide free of any unwanted compounds.

For the purpose of this study, the chemical synthesis of Rs-AFP1 and Rs-AFP2 was carried out and their activity against 5 food spoilage yeast, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis* was investigated. Their mechanism of antiyeast action was

studied alongside their stability under different conditions. The safety and incorporation of the peptides into different food matrices was also explored.

4.3 Material and Methods

4.3.1 Rs-AFP1 and Rs-AFP2 synthesis

Rs-AFP1 and Rs-AFP2, two homologous defensin peptides originating from the seeds of the radish plant *Raphanus sativus*, were chemically synthesised by GL Biochem (Shanghai) Ldt. Both peptides contain 51 amino acid and differ by 2 residues (Table 7). They were synthesised to a purity of 80% as indicated by the supplier. Both peptides were resuspended in water at a concentration of 2 mg/mL.

Table 7: One-letter amino acid sequences of Rs-AFP1 and Rs-AFP2

Peptide	Amino Acid Sequence
Rs-AFP1	QKLC**E**RPSGTWSGVCGNNNACKNQCI**N**LEKARHGSCNYVFPAHKCI CYFPC
Rs-AFP2	QKLC**Q**RPSGTWSGVCGNNNACKNQCI**R**LEKARHGSCNYVFPAHKCI CYFPC

4.3.2 Yeast strains

Zygosaccharomyces bailli Sa 1403, *Zygosaccharomyces rouxii* ATCC 14679, *Kluyveromyces lactis* ATCC 56498, *Debaryomyces hansenii* CBS 2334 (DMSZ (Germany)), and *Saccharomyces cerevisiae* Baker's yeast (Puratos, Belgium) were used throughout this study. Each yeast was grown aerobically in Sabouraud dextrose (SD; Sigma-Aldrich) agar at 25°C. Overnight incubation of the yeast was performed in SD broth at the same temperature under gentle agitation. All media and reagents used were obtained from Sigma-Aldrich (MO, USA), unless otherwise stated.

4.3.3 Antiyeast Assays

The minimum inhibitory concentration (MIC) of peptides Rs-AFP1 and Rs-AFP2 was determined using a microbroth dilution method as outlined by the National Committee

for Clinical Laboratory Standards (NCCLS M-27A, NCCLS 2002). The yeast suspensions were prepared in SD broth from overnight cultures which were adjusted to 10^4 cfu/mL. Two hundred and ninety microliters was transferred to a flat-bottom 96-well microtitre plate (Sarsdedt, Nümbrecht, Germany) followed by 10 µl of peptide ranging in concentration from 12.5 to 400 µg/mL. In order to serially dilute the peptides, 100 µL of the content in this first well was transferred in the subsequent wells containing 200 µL of yeast suspension. The positive control contained water, instead of peptide. The plates were incubated for 48 h at 25 °C in a microtitre plate reader (Multiskan FC Microplate Photometer, Thermo Scientific, MA, USA) under gentle agitation. The optical density was measured at 2 hr intervals at a wavelength of 620 nm. This assay was repeated in triplicate on 2 different plates.

In order to better visualise the peptides' antiyeast activity against *Z. bailii* over a longer period of time, an antiyeast assay was carried out and incubated for 6 days under the same conditions as above.

The fungistatic and fungicidal activity of the peptides were determined by spotting 100 µL of the yeast suspension from an antiyeast assay onto SD agar plates and incubating at 25 °C for 48-72 hr, depending on the optimal incubation time of the yeast. *D. hansenii, S. cerevisiae* and *Z. bailii* were incubated for 48 hr while *Z. rouxii* and *K. lactis* were incubated for 72 hr. The fungistatic/fungicidal activity of the peptides was determined by their ability to cause complete inhibition of the yeast.

4.3.4 Colony Count Assay

A colony count assay was performed to determine the peptides' ability to kill the yeast at different concentrations and observe the time course of this killing, as described by

Jang *et al.*, 2006. This allowed for the confirmation of the peptides' activity over time. *Z. bailii* was chosen for this assay as it was the most sensitive yeast to the peptides. One millilitre of a 10^4 cfu/mL yeast suspension was prepared in SD from an overnight culture and incubated with 100 µL of peptide, ranging in concentration from 50 to 200 µg/mL. This suspension was incubated at 25 °C. One hundred microliter of the suspensions was spotted onto SD agar plates every hour over a period of 6 hr and incubated for 2 days at the same temperature. The plates were counted, and a time course of the peptides' activity was determined.

4.3.5 Heat, pH and Salt stability of peptides

The peptide's stability in high heat, high salt and a range of pH was tested to determine their antiyeast activity when exposed to different environmental conditions. Peptide concentrations of 25, 50 and 100 µg/mL were tested against *Z. bailii* as the indicator yeast.

To study the effect of heat on the peptide's activity, Rs-AFP1 and Rs-AFP2 were heated for 15 min at 100°C and left to cool for 30 min before testing. An antiyeast assay was carried out as described in section 2.3.

Different ranges of pH were tested by carrying out an antiyeast assay using SD broth of which the pH was modified to 3, 5, 7, 9 and 11. The pH of the broth was modified using 1 M sodium hydroxide and 0.1 M hydrochloric acid. SD broth modified to the different pH ranges were used as a control, but without the addition of the peptides.

The stability of the peptides in different salt solutions were tested using 1- and 5-mM magnesium chloride ($MgCl_2$) and 50 and 150 mM potassium chloride (KCl). The

antiyeast assay was carried out on *Z. bailii* in the presence of the four salt concentrations. Control consisted of media containing the salts without peptide.

4.3.6 Membrane Permeabilisation

The peptide's membrane permeabilisation potential was examined to determine if this was a mechanism of antiyeast action. The peptides' permeabilising activity against the cell membrane of the yeast could be detected using the dye propidium iodide, as this dye binds and stains the nucleic acids of the yeast, but this is only made possible in the case of the yeast's permeabilised membrane. A cell suspension of 10^6 cfu/mL was prepared from an overnight culture. Concentrations of peptides (10 µL) ranging from 50 to 400 µg/mL were added to 90 µL of yeast and incubated at 25°C for 2 hr. Subsequently, a final concentration of 5 µM propidium iodide (SIGMA) was added and the suspension was left to incubate at room temperature for 20 min in dark conditions, before being washed with SD broth by centrifugation at 3,000 *g* for 5 min, to remove unbound dye. Fifty microliters of these suspensions were loaded onto slides and viewed under a Confocal Laser Scanning Microsocope (CLSM) (Olympus FV1000, incorporating an IX81 inverted microscope, Germany). The negative and positive control consisted of 0.1% Triton X-100 and water, respectively. A maximal excitation (λEx) and maximum emission (λEm) wavelengths of 535nm and 617nm, respectively were used.

4.3.7 Overproduction of Reactive Oxygen species (ROS)

The overproduction of ROS by yeast in the presence of the peptides was determined using a similar assay to the membrane permeabilisation. The method established by

Hayes *et al.*, 2013 was followed. A *Z. bailii* yeast suspension of 10^6 cfu/mL was incubated with 5 µg/mL of Dihydrorhodamine 123 (Sigma-Aldrich) at 25°C for 2 hr. Dihydrorhodamine 123 is an indicator dye that, in the presence of ROS, oxidises to rhodamine 123 after being taken up by the cell (Djiadeu *et al.*, 2017). After incubation, the cells were washed with SD broth by centrifugation at 3500 *g* for 5 min. Ten µL of Rs-AFP1 and Rs-AFP2 were added at different concentrations (50 to 400 µg/mL) and then incubated for 1 hr at 25°C. The cells were washed with 0.6 M potassium chloride and viewed under the CLSM by measuring the fluorescence at the maximal excitation (λEx) and maximum emission (λEm) wavelengths of 488nm and 538nm, respectively. The positive and negative control consisted of 2 mM hydrogen peroxide (H_2O_2) and water, respectively.

4.3.8 Peptides' haemolytic activity

The haemolytic activity of the peptides refers to the peptides' ability to cause the release of haemoglobin from defibrinated sheep erythrocytes, due to the cell lysis. This assay was carried out according to the method described by Thery and Arendt, 2018. Equal volumes of phosphate-buffered saline (PBS) solution was used to wash the fresh sheep's blood (Oxoid™) three times by centrifugation at 900 *g* for 15 min. This solution was made up to 4% using PBS and 80 µL was added into Eppendorf tubes in conjunction with 20 µL of peptides at different concentrations (6.25 to 400 µg/mL). The samples were incubated for 1 hr at 37°C before centrifuging them again for 10 min at 1,000 *g*. The supernatant was added into a 96 well microtiter plate and the absorption was measured at a wavelength of 405 nm. A positive control consisting of erythrocytes treated with 0.1% Triton X-100 and a negative control of PBS alone were

used. The percentage of haemolysis was calculated using the absorbance measured and inputting it in the formula below.

$$\% \text{ Haemolysis} = \frac{(A405\ peptide\ treatment) - (A405\ PBS)}{(A405\ 0.1\%\ Triton\ X{-}100) - (A405\ PBS)}$$

4.3.9 Peptides' cytotoxic activity

The peptides' cytotoxic activity against Caco-2 cells, a colonic cell line, was performed as described by Thery *et al.*, 2019. Caco-2 cells (ECACC) were passaged in Dulbecco's Modified Eagle Media (DMEM) supplemented with 1% non-essential amino acids and 10% fetal bovine serum (FBS) and diluted to $1x10^5$ cells/mL. Two hundred microliters of this cell solution was added into wells of a flat-bottom 96 well microtitre plate and incubated for 24 hr at 37 °C with 5% CO_2. This media was removed, and the two peptides were added at different concentrations in conjunction with DMEM with 2.5% FBS, bringing the volume in each well to 200 µL. The peptides were tested at concentrations of 100 to 600 µg/mL. A control consisting of sterile water and DMEM with no peptide was also tested. The plate was incubated for 24 hr at 37 °C. Subsequently, the media was removed and 100 µL of DMEM and 10 µL of MTT labelling reagent (Cell proliferation Kit I MTT; Sigma, Ireland) were added to each well and incubated for 4 hr. This was followed by the addition of 100 µL solubilisation buffer and overnight incubation. The viability of the cells was measured using a fluorometric spectrophotometer at 570 nm with a background reading of 690 nm. The assay was carried out on triplicate samples for each peptide at each concentration.

4.3.10 Peptides' resistance to proteolytic digestion

The peptides' resistance to proteolytic digestion was tested with α-chymotrypsin (Sigma, St Louis, MO, USA), a common digestive enzyme found in the human gut. The assay was carried out as described by Thery *et al.*, 2019, in order to try and mimic the environment that the peptides may encounter if used as preservatives in food and subsequently digested. The peptides were incubated at concentrations in conjunction with α-chymotrypsin at different peptide: enzyme molar ratios of 60:1, 250:1, 2500:1, for 4 hr at 37 °C. The α-chymotrypsin was then inactivated by heat at 80 °C for 10 min before an antiyeast assay testing the peptide at concentrations of 50, 100, 200 and 400 µg/mL was performed against *Z. bailii.* The α-chymotrypsin was stored in solution in a digestion buffer consisting of 50 mM Tris–HCl (pH 7.4) and 5 mM $CaCl_2$.

4.3.11 Scanning Electron Microscopy

In order to visualise the peptides' effect on the growth of *Z. bailii* cells, samples were prepared by following the protocol of Murtey and Ramasamy, 2016. A yeast cell suspension of $1x10^6$ cfu/mL was prepared from overnight cultures. The peptides were added to individual 1.5 mL micro-centrifuge tubes containing a total of 1 mL yeast suspension (to peptide concentrations of 400 µg/mL) and incubated for 0 and 4 hr before centrifuging at 900 *g* for 2 min for fixation. The pellet was resuspended in 5% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2). After 30 min, the glutaraldehyde solution was removed by centrifuged and the pellet was washed twice with 0.1 M phosphate buffer (pH 7.2). A series of ethanol washes comprising of 35%, 50%, 75%, 95% and absolute ethanol were carried out in order to dehydrate the samples. For each step, the samples were left for 30 min before centrifuging and

resuspending the pellet. The last two ethanol washes comprising of 95% and absolute ethanol were repeated twice, before the addition of the first round of hexamethyldisilazane (HDMS) for another 30 min. The supernatant of the second round of HDMS was discarded and the samples were left in a desiccator to dry overnight. These were then fixed onto plain aluminum stubs and coated with a 5 nm gold-palladium layer (80:20) using a Gold Sputter Coater (BIO-RAD Polaron Division, SEM coating system, England), and viewed under a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan), under constant accelerating voltage of 5 kV. Samples containing yeast with no peptide were used as controls.

4.3.12 Peptides' application in different food matrices

The application of Rs-AFP1 and Rs-AFP2 were assessed in different food matrices. *Z. bailii* is a yeast commonly known to spoil foods of high sugar and salt content such as salad dressings, soft drinks, syrups and wines (Blackburn, 2006; Kuanyshev, Adamo, Porro, & Branduardi, 2017). The antiyeast effect of Rs-AFP1 and Rs-AFP2 were investigated in some of these different foods.

The soft drink tested was Fanta Orange (Coca-Cola, Ireland). This was done via the microtiter plate method using filter sterilised Fanta orange inoculated with 10^2 cfu/mL yeast from an overnight culture of SD broth. This concentration of yeast was used in order to represent the number of cells found to spoil such beverages. The peptide concentrations tested ranged from 50 to 400 µg/mL. The antiyeast activity of the peptides in the Fanta Orange was measured by observing growth of the yeast over 48 hr and measuring the optical density at 620 nm. Controls consisted of Fanta Orange inoculated with 10^2 cfu/mL of yeast without peptide and Fanta Orange with no yeast added. The pH of the Fanta Orange was recorded as 3.1, a pH lower than what was

found for the SD broth (pH 5.3). This protocol was also used to test the antiyeast activity of the peptides in orange juice (*SuperValu* Chilled Orange Juice) (pH 3.86), apple juice (*CYPRINA*, Apple Juice) (pH 3.54), and cranberry juice (*SuperValu*, Chilled Cranberry Juice) (pH 2.7). The controls of each beverage consisted of the corresponding beverage inoculated with 10^2 cfu/mL of yeast and no peptide.

Salad dressing (*MILANO* House Light Dressing) (pH 3.1) was used to test the peptides' activity in a more viscous food matrix. A sample of the salad dressing was inoculated with 10^2 cfu/mL of *Z. bailii* in conjunction with 400 µg/mL of each peptide. This solution was thoroughly mixed before spreading 100 µL of each sample onto SD agar plates and incubating for 3 days at 25 °C. This allowed for the determination of the peptides' effectiveness in such a viscous matrix. In order to observe the long-term effect of the peptides in this food, the assay was repeated with the exception of incubating the peptides in the salad dressing for 48 hr before spreading the solutions onto SD agar plates. For both assays, salad dressing with no peptide was used as the control. The cranberry juice was subjected to the same treatment and spread onto SD agar plates in order to better visualise the peptides' antiyeast effect over 48 hr and to further confirm the results of the micro broth dilution assays in this beverage.

4.4 Results

4.4.1 Minimum inhibitory concentrations

The results of the antiyeast assay revealed Rs-AFP1 to be less potent than Rs-AFP2 (Table 8). *Z. bailii* was the most sensitive yeast with MICs ranging between 25 and 50 µg/mL for both peptides. *Z. rouxii* and *D. hansenii* were only inhibited by Rs-AFP2 at concentrations ranging between 50 and 100 µg/mL, and *S. cerevisiae* and *K. lactis* were not affected by either peptide, even at the highest concentration of 400 µg/mL.

The fungistatic/fungicidal activity of the peptides was determined. The only fungicidal activity observed was with Rs-AFP2 against *Z. bailii* at the highest concentration of 400 µg/mL. Rs-AFP1 was found to only be fungistatic against *Z. bailii*, the only yeast sensitive to the peptide. Rs-AFP2's fungistatic activities was also observed against both *Z. rouxii* and *D. hansenii.*

Performing an antiyeast assay for 6 days resulted in the same inhibitory effect of the peptides against *Z. bailii*. At the concentration range of the peptides' MIC (25 to 50 µg/mL), they were able to cause inhibition and continue to do so over the 6-day incubation period.

Table 8: Ranges of minimum inhibitory concentrations of Rs-AFP1 and Rs-AFP2 against *Zygosaccharomyces bailli* Sa1403, *Zygosaccharomyces rouxii* ATCC14679, *Saccharomyces cerevisiae* Baker's yeast, *Kluyveromyces lactis* ATCC56498 and *Debaryomyces hansenii* CBS2334.

	***Zygosaccharomyces bailli* Sa1403**	***Zygosaccharomyces rouxii* ATCC14679**	***Saccharomyces cerevisiae* Baker's yeast**	***Kluyveromyces lactis* ATCC56498**	***Debaryomyces hansenii* CBS2334**
Rs-AFP1	MIC range of 25 to 50 µg/mL – Fungistatic	No inhibition	No inhibition	No inhibition	No inhibition
Rs-AFP2	MIC range of 25 to 50 µg/mL - Fungicidal	MIC range of 50 to 100 µg/mL - Fungistatic	No inhibition	No inhibition	MIC range of 50 to 100 µg/mL - Fungistatic

4.4.2 Colony Count Assay

The rate of *Z. bailii* inhibition affected by the peptides over 6 hr was observed (Figure 6A and 6B). Rs-AFP2 fully inhibited *Z. bailii* at all three concentrations (50, 100, and 200 µg/L) after 2 hr of incubation. The same effect can be seen at 100 and 200 µg/mL of Rs-AFP1, with the exception of 50 µg/mL only causing a reduction in *Z. bailii* after 4 to 5 hr. Increase growth of the yeast was seen for the first 3 hr, before the decrease was observed.

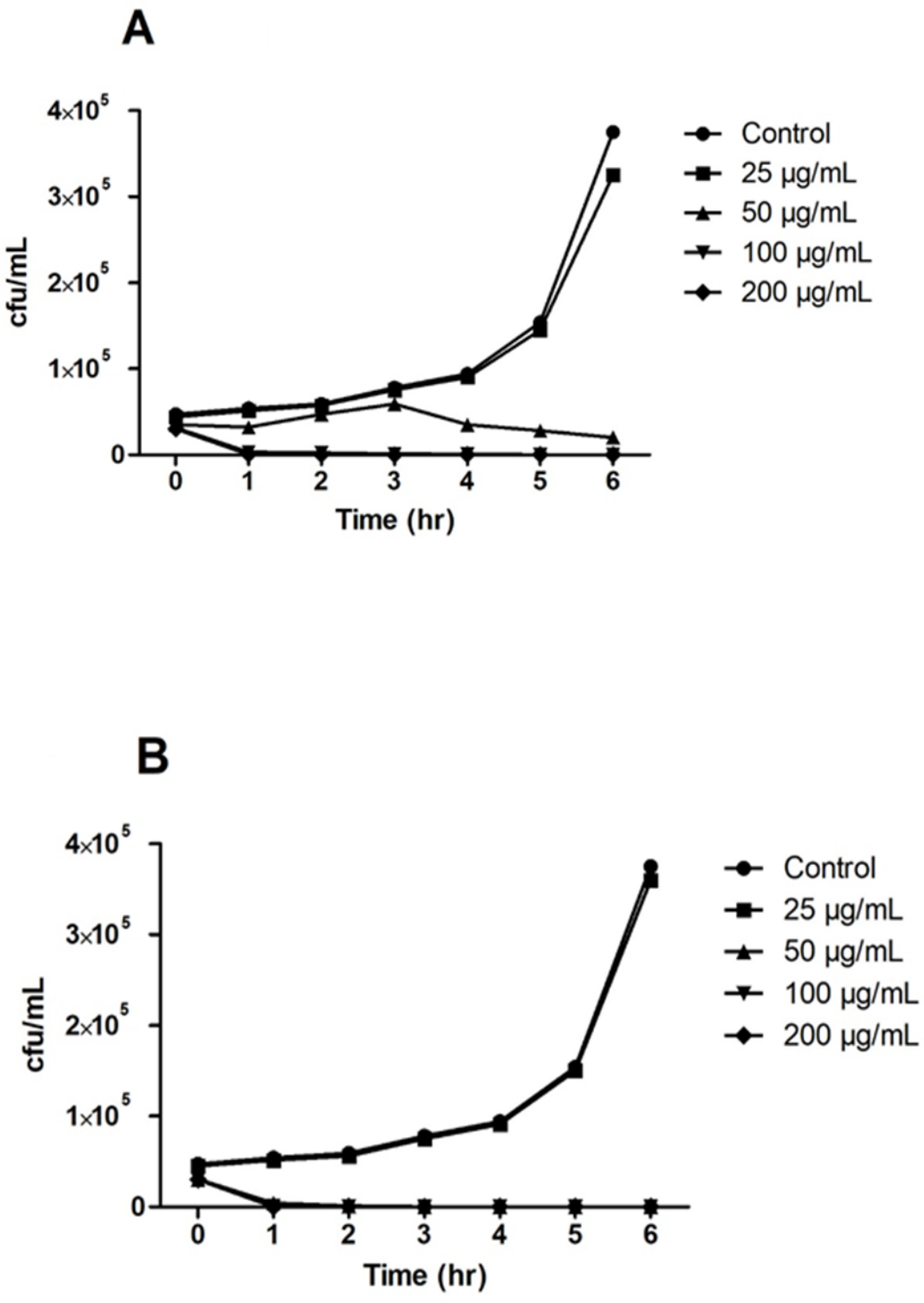

Figure 6: Colony count assay demonstrating the rate of *Z. bailii* inhibition caused by Rs-AFP1 (A) and Rs-AFP2 (B). Yeast growth reduced after only 1 hr of incubation in the presence of both peptides at the highest concentrations, in comparison to the control, which showed a steady increase in growth over the 6 hrs.

4.4.3 Peptides' stability

After being subjected to thermal treatment of 100 °C for 15 min, no change in their antiyeast activity was observed. At the concentration of 50 and 100 µg/mL, the peptides were able to inhibit *Z. bailii*, while at 25 g/mL there was no inhibition observed, as predicted from the antiyeast MIC assays.

The modification of the media's pH resulted in changes to the peptide's antiyeast activity to various degrees (Table 9). At pH 3, a decrease in their activity was observed from the complete growth at all concentrations tested (25, 50 and 100 µg/mL). At pH 5, both Rs-AFP1 and Rs-AFP2 were unaffected. At pH 7, a complete loss in the peptides' activity was observed. pH 9 and 11 showed no yeast growth in the controls of unadjusted SD broth, indicating that the yeast was unable to grow in such basic conditions.

Table 9: The effect of the medium pH change on Rs-AFP1 and Rs-AFP2 and their antiyeast activity.

	pH 3	pH 5	pH 7	pH 9	pH 11
Rs-AFP1	No inhibition	Full inhibition due to peptide activity	No inhibition	No yeast growth	No yeast growth*
Rs-AFP2	No inhibition	Full inhibition due to peptide activity	No inhibition	No yeast growth	No yeast growth*

* No yeast growth was observed due to the yeast's inability to grow at such high pH.

Rs-AFP2's activity in the salt-containing media showed a decrease in antiyeast activity at the higher concentration of $MgCl_2$ (5mM) and in both concentrations of KCl (50 and 150 mM) (Figure 7A). At the highest peptide concentration of 100 µg/mL in 1 mM $MgCl_2$, there was considerably less yeast growth compared to 100 µg/mL in 5 mM $MgCl_2$ and 50 and 150 mM KCl. Rs-AFP1 was affected more; even 1 mM $MgCl_2$ affecting its antiyeast activity (Figure 7A). At 100 µg/mL, *Z. bailii* growth was not

completely repressed, as the OD is nearly half the salt control but not low enough to consider inhibition to be occurring.

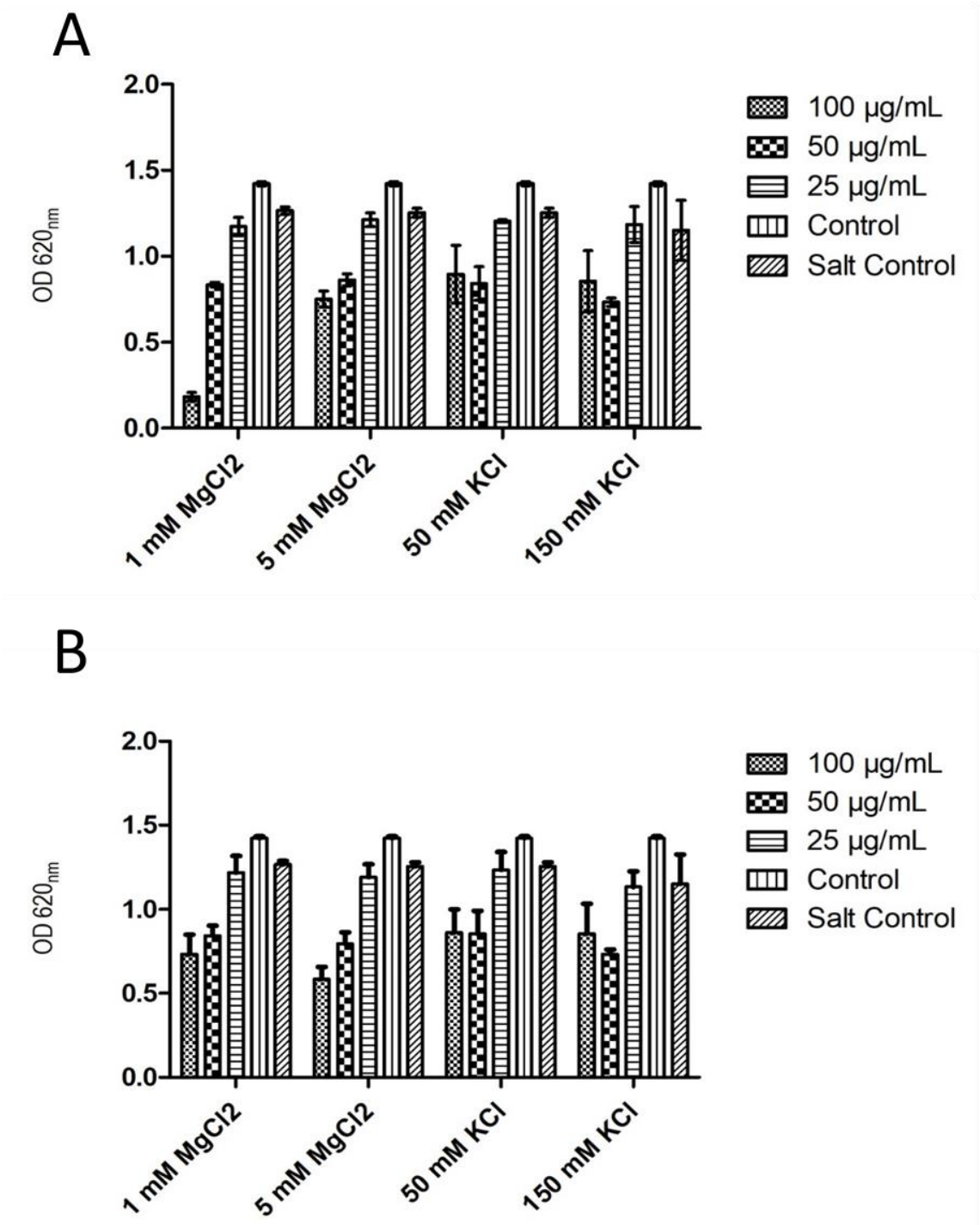

Figure 7: Stability of Rs-AFP2 (A) and Rs-AFP1 (B) in $MgCl_2$ and KCl at different concentrations. The lowest concentration of $MgCl_2$ resulted in no negative effects of Rs-AFP2's antiyeast activity as seen from inhibition of *Z. bailii* at 100 µg/mL.

4.4.4 Peptides' mechanism of action

The permeabilisation of the yeast membranes and the overproduction of Reactive Oxygen Species (ROS) were found to be the peptides' primary mode of action against *Z. bailii.*

At the highest concentration tested (400 µg/mL), Rs-AFP2 was found to completely permeabilise the yeast (8A). The level of permeabilisation decreased as the concentration of peptide was lowered (200 and 100 µg/mL) (8B and 8C). At the minimum concentration tested (50 µg/mL) there was no visible permeabilisation.

Rs-AFP1 did not result in the permeabilisation of the yeast membrane, even at the highest concentration of 400 µg/mL (Result not shown).

The overproduction of ROS in the yeast due the action of the peptides was observed for both Rs-AFP1 and Rs-AFP2. As predicted, the higher concentrations of both peptides (400 µg/mL) produced a higher level of ROS formation in the yeast cells, when viewed by CLSM, in comparison to the yeast subjected to 100 and 200 µg/mL of both peptides (9A, 9B and 9C). At 50 µg/mL, no ROS overproduction was detected (image not shown).

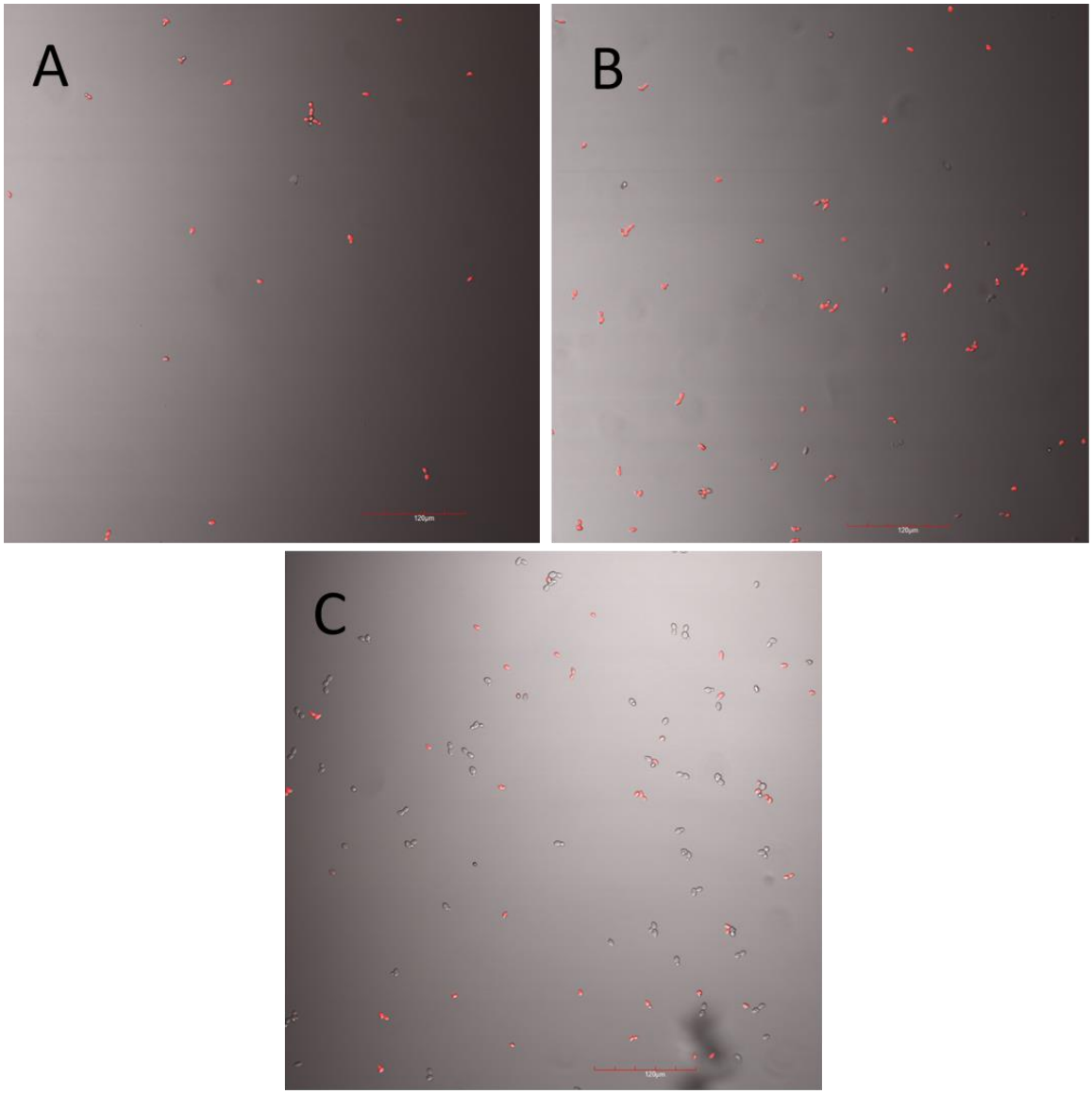

Figure 8: CLSM images showing the impact of Rs-AFP2 on *Z. bailii* cell membrane at 400 µg/mL (A), 200 µg/mL (B) and 100 µg/mL (C). The highest peptide concentration resulted in complete uptake of the dye caused by the permeabilisation of the yeast membrane, suggesting cell death. The level of permeabilisation is seen to reduce with decreasing peptide concentration.

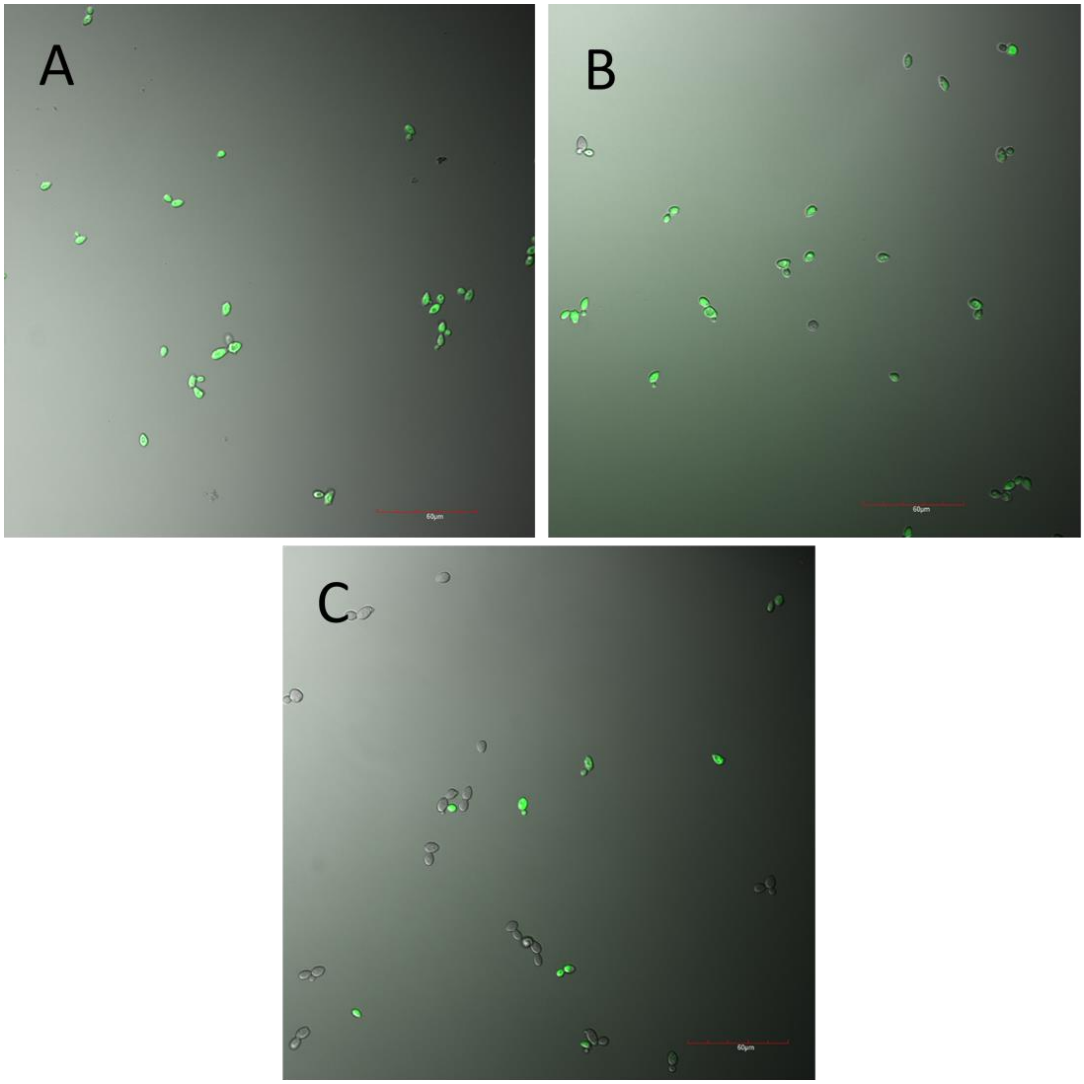

Figure 9: CLSM images showing the overproduction of ROS in *Z. bailii* as a result of Rs-AFP2 at 400 µg/mL (A), 200 µg/mL (B) and 100 µg/mL (C). Fluorescence of the cells indicate oxidation of Dihydrorhodamine 123 dye to rhodamine 123 in the presence of reactive oxygen species.

4.4.5 Scanning Electron Microscopy

The effect of both peptides on the yeast cells morphology showed that the peptide caused the cells to shrink. The control without the presence of the peptides produced visibly healthy and large yeast cells (Figure 10A), in comparison to the yeast inoculated with the peptides, where considerably smaller cells were observed, even at 0 hr (Figure 10B and 10D) and more considerably after 4 hr of incubation (Figure 10C and 10E).

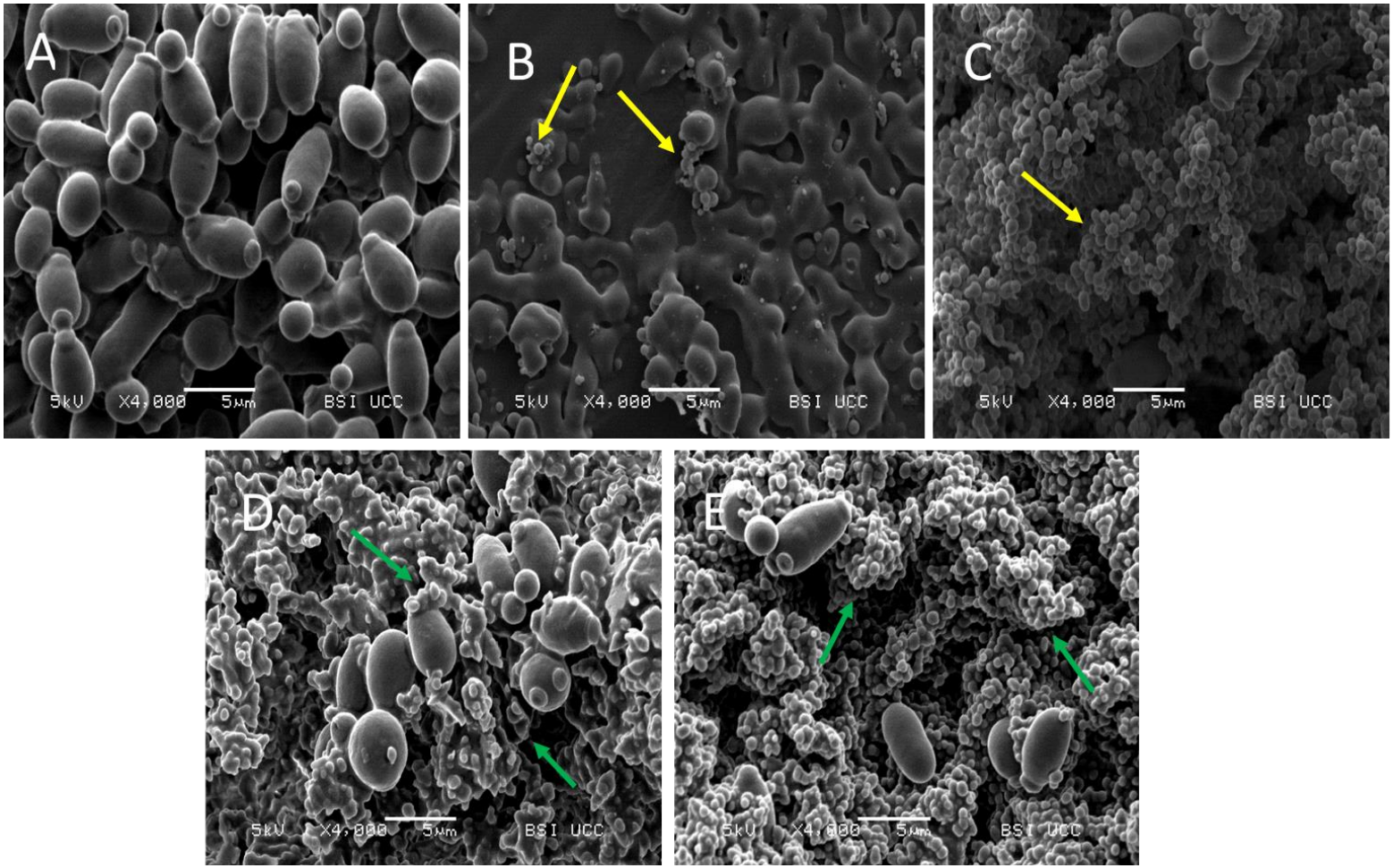

Figure 10: SEM images of *Z. bailii* in the absence of peptide (A), and with Rs-AFP1 after 0 hrs (B) and 4 hrs (C) incubation. Rs-AFP2 after 0 hrs (D) and 4 hrs (E) showed similar images.

4.4.6 Peptides' haemolytic effect

Both peptides were tested for their haemolytic activity against erythrocytes from fresh sheep's blood. At concentrations of 200 and 400 µg/mL, the peptides were found to be significantly haemolytic, with a percentage of haemolysis of more than 50% (Figure 11); 100 µg/mL and lower concentrations resulted in very low haemolysis of erythrocytes, with <10% haemolysis observed.

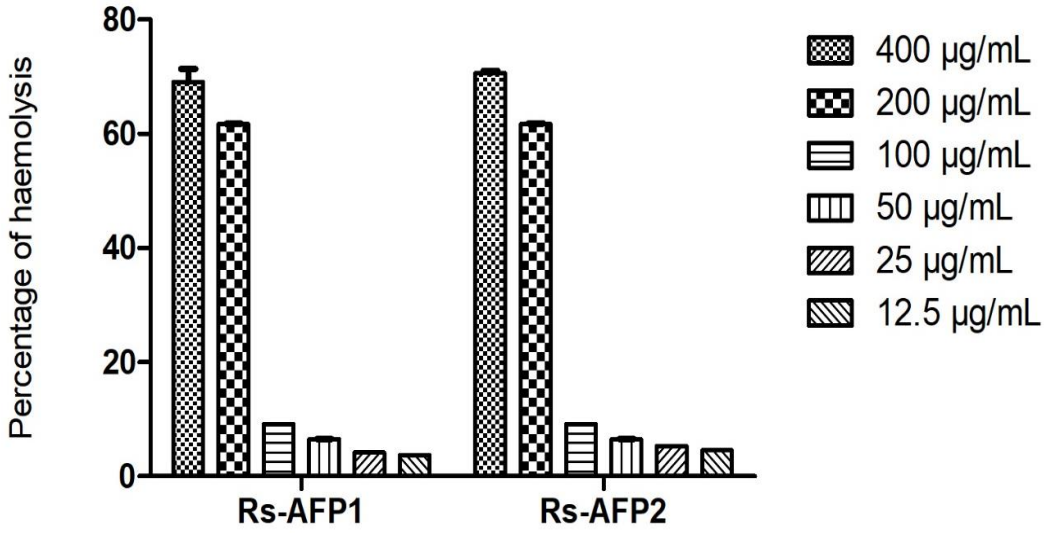

Figure 11: Percentage of haemolysis by Rs-AFP1 and Rs-AFP2 on sheep erythrocytes at concentrations of 12.5 to 400 µg/mL.

4.4.7 Peptides' cytotoxic effect against Caco-2 cells

The cytotoxicity assay indicated that the peptides caused an increase in the cell viability (Figure 12A and 12B), which was proportional to the concentration of the peptide added.

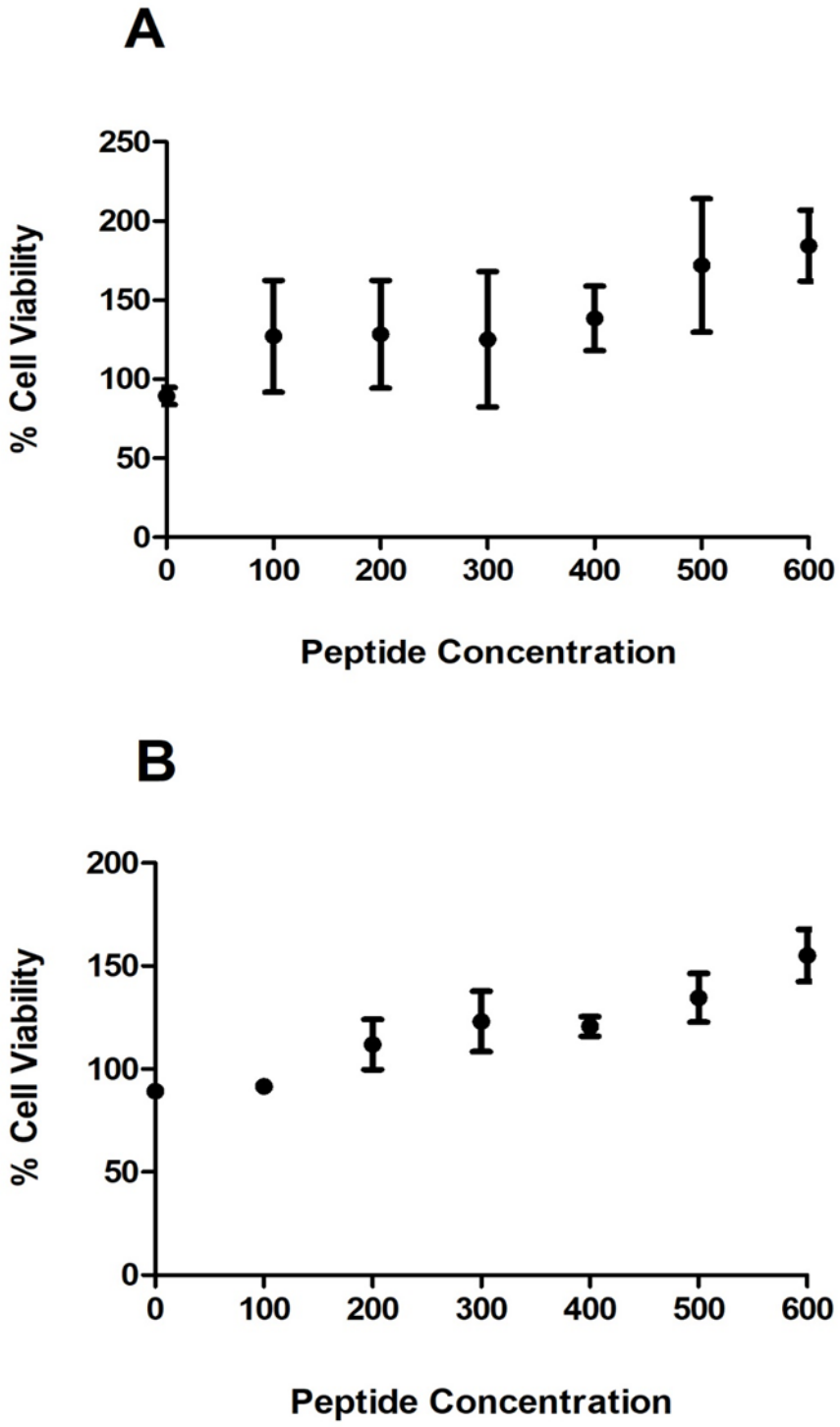

Figure 12: Increase in the viability of Caco-2 cells in the presence of increasing concentrations of Rs-AFP1 (A) and Rs-AFP2 (B) (0-600 µg/mL).

4.4.8 Peptides' resistance to proteolytic digestion

The peptides' resistance to proteolytic digestion by α-chymotrypsin was tested at different molar ratios of peptide: enzyme 60:1, 250:1 and 2500:1. At all molar concentrations, the protease degraded Rs-AFP1 as yeast growth was apparent at all four peptide concentrations (50, 100, 200 and 400 µg/mL). Rs-AFP2 was found to be resistant to the lowest molar ratio of 1:2500 at 400 µg/mL of peptide, as *Z. bailii* inhibition was observed, while the lower concentrations (50, 100 and 200 µg/mL) did not have any effect on the yeast, indicating the degradation of the peptide. At the higher molar ratios of 60:1 and 250:1, the peptide's antiyeast activity was completely eliminated.

4.4.9 Peptides' applications in food

Rs-AFP1 and Rs-AFP2 were tested for their antiyeast activity in different food matrices (Table 10).

Table 10: Antiyeast effect of Rs-AFP1 and Rs-AFP2 on *Z. bailii* in different beverages. Various ranges of inhibition were detected in the different beverages.

	Cranberry Juice	Fanta Orange	Apple Juice	Orange Juice
Rs-AFP1	Full inhibition†	Full inhibition†	Inhibition at 200 and 400 µg/mL	No inhibition
Rs-AFP2	Full inhibition†	Full inhibition†	Inhibition at 200 and 400 µg/mL	No inhibition

†Full inhibition at all concentrations tested.

For the beverages, the peptides were effective in causing full *Z. bailii* inhibition in both the cranberry juice and Fanta Orange. In the apple juice, 200 and 400 µg/mL of peptide caused inhibition, while full yeast growth was observed at 50 and 100 µg/mL. The peptides were ineffective in the orange juice, as they were incapable of preventing

the growth of *Z. bailii* at any of the concentrations tested. The peptide application in the salad dressing caused a reduction if yeast cells after immediate inoculation of the dressing/yeast mixture with the peptides (Figure 13). Incubating the peptides into the salad dressing with the yeast for 48 hr resulted in the yeast's full inhibition of the whole time period. Similar results were obtained for the cranberry juice after incubating for 48 hr; no yeast growth was observed in the samples containing the peptides (at 50, 100, 200 or 400 µg/mL).

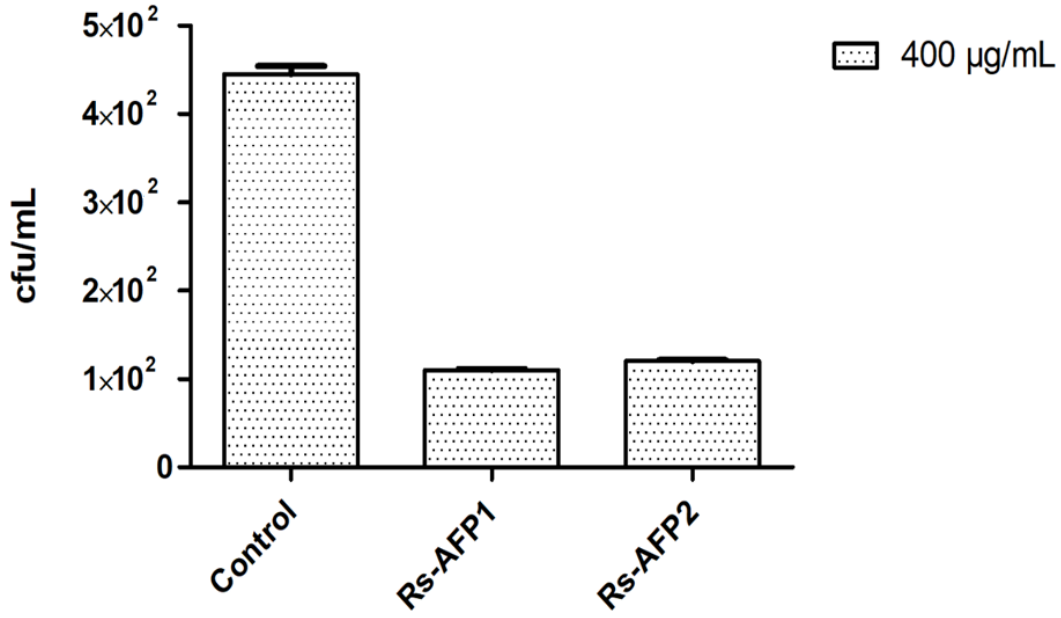

Figure 13: The effect of Rs-AFP1 and Rs-AFP2 (at 400 µg/mL) on *Z. bailii* growth in a sample of salad dressing after the immediate incubation of the peptides with the yeast.

4.5 Discussion

The synthesis of plant antimicrobial peptides through chemical means can be a more direct alternative to the extraction and purification process required to isolate these peptides from plant matrices - a process that can be laborious and time consuming. The chemical synthesis of two radish defensins, Rs-AFP1 and Rs-AFP2, was performed for this study by replicating the peptides' naturally encoded amino acid sequence (Table 1). This paper examines the antiyeast activity of these two defensin peptides, for the inhibition of some common food spoilage yeast and their application in different food matrices.

The peptides were tested for their antiyeast activity against 5 different yeast with inhibition observed against *Z. bailii*, *Z. rouxii* and *D. hansenii*. The ranges of MICs were observed to be between 25 and 50 µg/mL for both peptides against *Z. bailii* and from 50 to 100 µg/mL for Rs-ASP2 against *Z. rouxii* and *D. hansenii.* Rs-AFP1 did not show any inhibitory effect against *Z. rouxii, S. cerevisiae, K. lactis* or *D. hansenii.* The differences in this inhibitory effect against the yeast may be explained by the 2 amino acid residue difference between the peptides. The amino acid residues Asparagine-5 and Glutamate-26 found in Rs-AFP1 are replaced by Glutamine-5 and Argenine-26, respectively, in the sequence of Rs-AFP2. These slight changes result in Rs-AFP1 being less basic and having a lower net positive charge compared to Rs-AFP2. Rs-AFP1 is known to have a net charge of + 4 while that of Rs-AFP2 is + 6. This higher net charge and Rs-AFP2's additional positively charged residues could be the reason for its increased antiyeast activity. It is well known that antimicrobial peptides with a higher net charge and increased number of positively charged amino acid residues result in increased antimicrobial activity (Dathe & Wieprecht, 1999; Hong, Park, & Lee, 2001; Jiang et al., 2008; SD, 2014). The colony count assay

further confirmed this difference in antiyeast activity, where, at the same peptide concentration, Rs-AFP1's inhibitory effect was apparent after a longer incubation period compared to Rs-AFP2.

The peptide sequence alignment of Rs-AFP1 and Rs-AFP2 reveal a pattern of cysteine residues that are involved in the production of 4 disulphide bridges (Fant, Vranken, Broekaert, & Borremans, 1998) and a cysteine stabilised α-β motif (De Samblanx et al., 1997b; Maróti, Downie, & Kondorosi, 2015; Van Der Weerden, Bleackley, & Anderson, 2013). The presence of multiple cysteine residues could explain the peptides' activity against the yeast. It has been reported that peptides rich in cysteine and/or glycine residues have significant antimicrobial properties (Goyal & Mattoo, 2016; Haag et al., 2012; Maróti et al., 2015). AMPs like Rs-AFP1 and Rs-AFP2 are known to effect cell membrane surfaces through the attraction of negatively charged molecules by their cationic residues (Pelegrini & Franco, 2005; Titarenko, López-Solanilla, García-Olmedo, & Rodríguez-Palenzuela, 1997; Whitlow & Teeter, 1985). This attraction ultimately causes the peptides' accumulation on the surface of the cell membrane, resulting in the potential modification of the surface, eventually leading to its death (Goyal & Mattoo, 2016). The presence of lysine (4 in each peptide) and arginine residues (2 in Rs-AFP1 and 3 in Rs-AFP2) in these peptides are the contributing residues for this interaction between the peptides and cell membrane (Sato & Feix, 2008; Yeaman & Yount, 2003), with glutamate (2 in Rs-AFP1 and 1 in Rs-AFP2) also contributing to this attribute (Goyal & Mattoo, 2016). (Illustrative scheme can be seen in Figure 14).

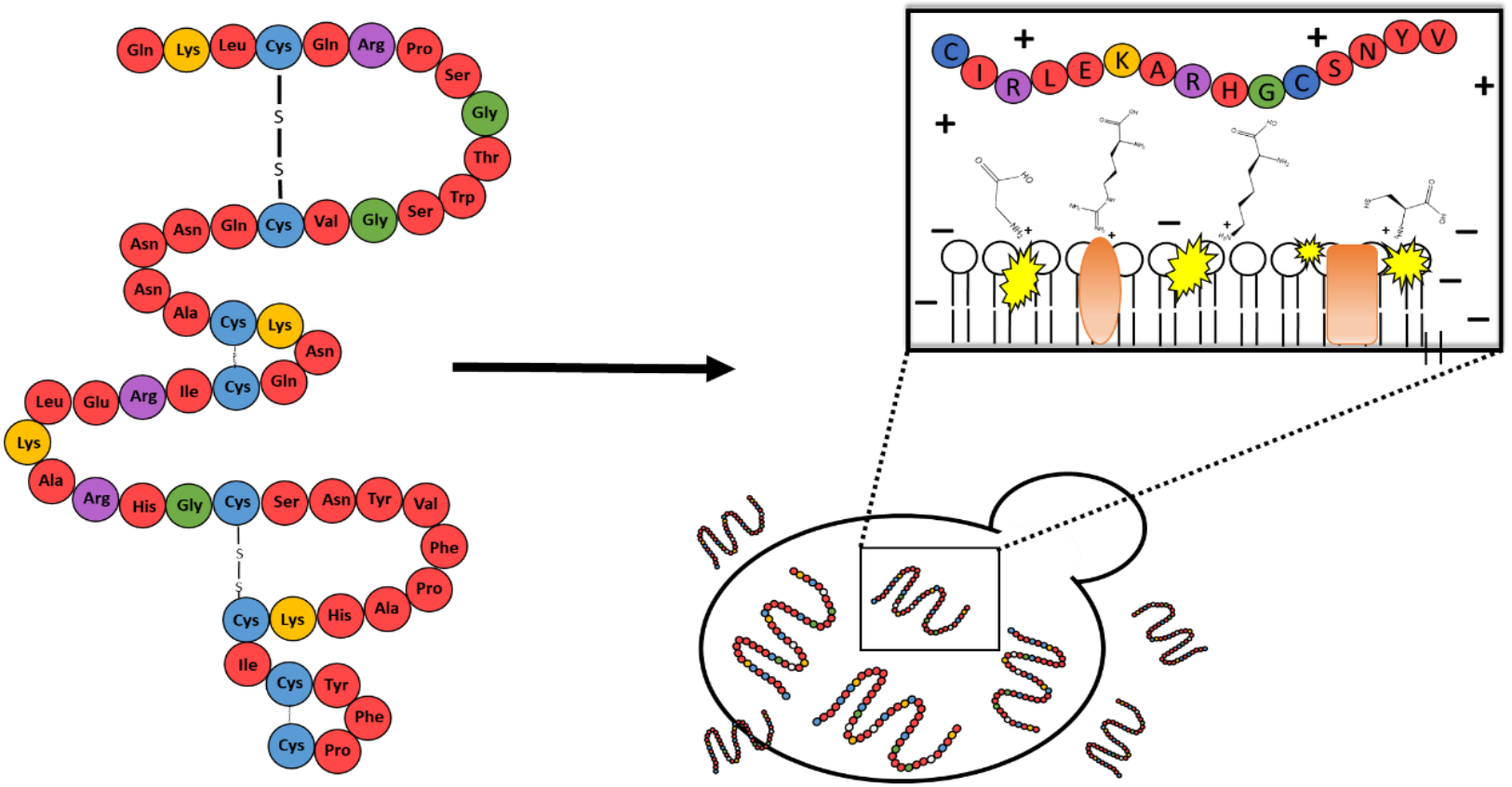

Figure 14: Schematic representation of the mechanism of action of the peptides against yeast. A simple secondary structure of Rs-AFP2 was constructed, revealing 4 disulfide bonds between the 8 cysteine residues present. The 4 main amino acid residues thought to be effectors are highlighted in blue, green, purple and yellow, representing Cys, Gly, Arg and Lys, respectively. The peptide accumulates on the surface of the yeast membrane with interaction of the positively charged amino acid residues with the negatively charged yeast membrane, causing membrane damage.

Due to the intended use of the peptides in food preservation, it is important that they are able to withstand different treatments and conditions to which they may be subjected. At high temperatures, a common process encountered in food preparation, both Rs-AFP1 and Rs-AFP2 were unaffected, causing inhibition even after being subjected to 100 °C. Adjusting the pH of SD media to pH 7 resulted in the greatest change to be the peptides' activity as a loss of antiyeast action was observed. This reduction in antiyeast activity could be due to the change in the peptides' net charge that occurs at neutral and basic pH. The net charge of many cationic AMPs have been studied and observed to be more positively charged at/below pH 7 (Walkenhorst, Klein, Vo, & Wimley, 2013). Table 11 illustrates the effect that different pH have on the net charge of both peptides, with an increase in their net charge observed in more acidic conditions (pH 3 and 5), compared to the basic pH (9 and 11).

Table 11: The net charge of Rs-AFP1 and Rs-AFP2 at the different ranges of pH that were tested during the stability assay. A reduction in the peptides' net charge is observed above neutral pH and in basic conditions. (Data obtained using an online peptide net charge calculator).

	pH 3	**pH 5**	**pH 7**	**pH 9**	**pH 11**
Rs-AFP1 net charge	8.4	6.3	4.1	-3.7	-10.7
Rs-AFP2 net charge	9.5	8.1	6.1	-1.71	-8.8

The salt concentrations tested in the stability assay were based on previous papers that have looked into the stability of peptides in high salt conditions (Betts, Linton, & Betteridge, 1999; Wu et al., 2008). Rs-AFP1 in the presence of salts $MgCl_2$ and KCl resulted in a reduced ability to inhibit *Z. bailii.* This was also observed for Rs-AFP2 at the highest concentrations of the salts. This could be explained by the cations present in the medium interacting with the yeast cell membrane and potentially altering the peptides' overall charge, thus modifying their structure (Baldauf et al., 2013) and

leading to the peptides' reduced antiyeast activity. Relative to Rs-AFP1, Rs-AFP2's unchanged antiyeast activity at the lowest concentration may be due to its more basic nature being a potential factor for its resistance to the salt conditions (Terras et al., 1992).

Both peptides were observed to cause the overproduction of ROS in *Z. bailii*, with the level of overproduction being dose dependant. ROS is known to be generated by yeast cells during normal cell functions, however, under cell stress conditions, the level of ROS increases dramatically, leading to an overproduction in ROS and, ultimately, cell death (Wang et al., 2015). This induction of endogenous ROS has been previously reported to occur in yeast cells and is a recognised mechanism of action of cationic AMPs (Aerts et al., 2007). The more common mechanism of action of AMPs, however, is through the permeabilisation of cell membranes. Rs-AFP2 was shown to permeabilise the cell membrane of *Z. bailii*, while Rs-AFP1 could not. This peptide's increased cationic and amphipathic nature, compared to Rs-AFP1, could explain the level of permeabilisation observed. These attributes enable the peptide to interact with the negatively charged yeast membrane and cause permeabilisation (Kumar, Kizhakkedathu, & Straus, 2018).

Analysing the peptides' inhibitory effect against *Z. bailii* under an electron scanning microscope allowed for the visual observation of their effect on the yeast cells. Cells that were treated with both peptides were observed to have a shrunken nature compared to the untreated yeast. This reduction in the size of the treated cells could potentially be due to the leakage of potassium ions, an important component required for the growth and survival of the yeast cell (Enríquez-Freire, López, & Peña, 1999; Lee & Lee, 2015; Peña, Sánchez, & Calahorra, 2013). Studies have shown that cationic plant antimicrobial peptides, like Rs-AFP1 and Rs-AFP2, can cause this rapid

efflux of potassium ions as a result of membrane damage (De Samblanx et al., 1997b; Enríquez-Freire et al., 1999).

The peptides' safety in terms of their application in foods was also assessed. Both peptides were haemolytic at the highest concentration tested while at the higher end of the MIC range (50 µg/mL) and double the MIC (100 µg/mL), less than 10% haemolysis was observed, a positive attribute if the peptides were to be consumed. Both peptides were sensitive to proteolytic action, an important characteristic for many preservatives. The proteolysis of the peptides supports their gradual degradation after consumption, ensuring that the peptides do not survive the digestion process in the gut.

The evaluation of the peptides' cytotoxicity towards Caco-2 cells found an increase in the cell viability in proportion to the peptide concentration. This behaviour in the presence of the peptides could be linked to their ability to cause cell proliferation. This characteristic has been observed in previous studies in which AMPs were capable of stimulating cell proliferation in dendritic cells, oral epithelial cells and keratinocytes (Ackermann, 2016; Liu et al., 2018; Mi et al., 2018).

Finally, applying the peptides in different beverages matrices revealed that apple juice, cranberry juice and Fanta Orange were suitable food media for their applications. In orange juice, the dense consistency of the liquid could have hindered the peptides' activity against the yeast. In a more viscous matrix like salad dressing, the peptides were able to hinder yeast growth, demonstrating the peptides' potential application in more different food matrices. In order to visualise the effects of the peptides in the preservation of foods with a long shelf life, the peptides incubated for 6 days with the

yeast helped illustrate their inhibitory effect long term, as the peptide were capable of maintaining their antiyeast activity over the 6 days.

4.6 Conclusion

This study helps illuminate the potential use of these synthetic plant peptides for use in food preservation, as illustrated by their applicable function and potentially safe application in different foods. Although the cost of synthesis can be a disadvantage that still needs to be overcome, the cost and time for the extraction and purification of the natural peptide must also be considered. Thus, the chemical synthesis of known antimicrobial peptides derived from natural sources, as outlined in this study, may represent a novel approach to combatting food spoilage. Such an approach, while currently being too expensive for wide-scale adaption, demonstrates a proof of principle which may become more feasible in the future as the cost of process, such as chemical peptide synthesis, reduce.

4.7 Acknowledgements

This work was supported by the Irish Department of Agriculture, Food Institutional Research Measure (F.I.R.M.), Project Reference 15/F/731. A special thank you to Dr Yvonne O'Callaghan for all her help with the cell cultures, Ms. Lilit Ispiryan for her help with the confocal laser scanning microscope and Mr. Jonas Joachim Atzler for the scanning electron microscopy images.

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Chapter 5

Study on the inhibitory activity of a synthetic defensin derived from barley endosperm against common food spoilage yeast

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Published in Molecules as:

Shwaiki, L. N., Sahin, A. W. and Arendt, E. K. (2020) 'Study on the Inhibitory Activity of a Synthetic Defensin Derived from Barley Endosperm against Common Food Spoilage Yeast', *Molecules*. MDPI AG, 26(1), p. 165. doi: 10.3390/molecules26010165.

5.1 Abstract

In the food industry, food spoilage is a real issue that can lead to a significant amount of waste. Although current preservation techniques are being applied to reduce the occurrence of spoilage microorganisms, the problem persists. Food spoilage yeast are part of this dilemma, with common spoilers such as *Zygosaccharomyces*, *Kluyveromyces*, *Debaryomyces* and *Saccharomyces* frequently encountered. Antimicrobial peptides derived from plants have risen in popularity for their ability to reduce spoilage. This study examines the potential application of a synthetic defensin peptide derived from barley endosperm. Its inhibitory effect against common spoilage yeasts, its mechanisms of action (membrane permeabilisation and overproduction of reactive oxygen species) and stability in different conditions was characterised. The safety of the peptide was evaluated through a haemolysis and cytotoxicity assay and no adverse effects were found. Both assays were performed to understand the effect of the peptide if it were to be consumed. Its ability to be degraded by a digestive enzyme was also examined for its safety. Finally, the peptide was successfully applied into different beverages, maintaining the same inhibitory effects in apple juice as was observed in the antiyeast assays, providing further support for the peptide's application in food preservation.

5.2 Introduction

Plants are organisms with distinct mechanisms evolved to protect them from the harsh environments posed by nature. In some plants, the development of physical traits such as thorns and spines are a tool that can deter herbivores (Hanley *et al.*, 2007). Amongst these complex mechanisms is the production of antimicrobial peptides (AMPs) which are known to challenge the presence of microbial and fungal infections. They are part of the innate immune system of a plant and can differ based on their structure and their selective nature (Finkina *et al.*, 2018). Their cationic and cysteine-rich sequences make them effective agents against microbial and fungal cells, but less so towards mammalian cells due to their weaker affinity to neutral/positively charged membranes (Nawrot *et al.*, 2014). Many AMPs are grouped together based on their conformation and sequence. Defensins are one class of plant AMPs that are characterised as cationic, containing 45-54 amino acids and that can form cysteine stabilised alpha/beta motifs (CSαβ-motif) (Carvalho and Gomes, 2009). Thionins, snakins/GASA, hevein- and knottin-type, and cyclotides are other well-recognised plant AMPs known for their impact in plant defence systems (Broekaert *et al.*, 1997).

Plant AMPs can be purified using various extraction methods that ensure their precipitation. These types of natural extractions have been developed for AMPs from a wide range of plants (Zhang and Lewis, 1997; Kim *et al.*, 2013; Almasia *et al.*, 2017; Schmidt, Arendt and Thery, 2019). The chemical synthesis of pure peptides derived from known amino acid sequences is another preferred method of production (Harris *et al.*, 2014). Solid phase peptide synthesis (SPPS) and solution-phase synthesis (SPS) are both forms of chemical synthesis adapted for the production of these peptides.

Microbial spoilage is a challenge faced by the food industry as it can lead to major economic losses and consequently to the waste of a significant amount of food. Spoilage microorganisms can range from bacterial to fungal pathogens. Yeast are sometimes overlooked as spoilers of food products, however a large number of dairy, meat, fruit and vegetable products are effected by yeast species such as *Kluyveromyces, Debaryomyces, Zygosaccharomyces, Pichia, Candida, Yarowia* and *Saccharomyces* (Fleet, 2011). Plant AMPs have been explored for their potential as food preservatives in various studies; most of which deal with reducing infections in plants and crops caused by microbial and fungal pathogens (Fernandez de Caleya *et al.*, 1972; Stotz, Spence and Wang, 2009; Rogozhin *et al.*, 2015). This is significant to combat plant infections that can lead to major losses of agricultural crops. However, more studies need to be conducted on the application of plant AMPs for the preservation of food products. Food preservatives that deal with microbiological spoilage have already been established; the addition of chemicals, pasteurising or freezing foods, irradiation or modified atmospheric packaging are some examples (Harrison, 1906). Regardless of this, spoilage is still a recurring problem in the food industry, making it vital for more effective forms of preservatives to be discovered. Not only this, but consumer perception of the current forms of preservatives on the market has also become an influential driving factor for the development of more natural forms of preservatives (Zink, 1997).

This study will explore the antiyeast activity of a synthetic defensin peptide known as Defensin-like protein 1 (D-lp1) (previously known as gamma-hordothionin). This sulfur-rich defensin is present in barley endosperm and has a known molecular weight mass of 5.25 kDa and a sequence length of 47 amino acids. It was first isolated by Mendez *et al.*, 1990 and designated into the thionin family of plant AMPs. Now it is

known that these peptides correspond to the characteristics of the family of plant defensins. In this study, D-lp1 was tested for its inhibitory effects against common food spoilage yeast. Its minimum inhibitory concentration (defined as the lowest concentration of peptide required to inhibit the visible growth of the yeast) and minimum fungicidal concentrations (the minimum concentration required to prevent yeast growth) (Andrews, 2001) were determined. Its stability in different conditions and application in various beverages were investigated in order to establish its potential as a prospective preservative agent. Its safety and mechanism of action were also examined.

5.3 Methodology

5.3.1 Defensin-like protein 1 (D-lp1) synthesis

D-lp1, a 47 amino acid sequence peptide derived from barley (*Hordeum vulgare*) endosperm was chemically synthesised by GL Biochem (Shangai) Ldt to a purity of 80% as indicated by the supplier. The peptide was resuspended in ultrapure water at a concentration of 2 mg/mL. The amino acid sequence of D-lp1 can be seen in Table 12.

Table 12. D-lp1single letter amino acid sequence.

D-lp1 amino acid sequence
RICRRRSAGFKGPCVSNKNCAQVCMQEGWGGGNCDGPLRRCKCMRRC

5.3.2 D-lp1 secondary structure

The secondary structure of the peptide was developed on the programme RasMol (Valadon, 2009) using the amino acid sequence obtained from the Protein Data Bank (Berman *et al.*, 2000). The structure of the peptide was reviewed using the model built from the programme.

5.3.3 Yeast strains

The different yeast strains used throughout the study were as follows: *Zygosaccharomyces bailli* Sa 1403, *Zygosaccharomyces rouxii* ATCC14679, *Kluyveromyces lactis* ATCC 56498, *Debaryomyces hansenii* CBS 2334 (DMSZ (Germany)), and *Saccharomyces cerevisiae* Baker's yeast (Puratos, Belgium). Each yeast was grown aerobically in Sabouraud dextrose (SD; Sigma Aldrich) agar at 25

°C. Overnight cultures of the yeast were prepared in SD broth (pH 5.3) at the same temperature under gentle agitation at 130 rpm. All media and reagents used were obtained from Sigma-Aldrich (MO, USA), unless otherwise stated.

5.3.4 Determination of D-lp1 antiyeast activity

The MIC of D-lp1 against the 5 yeast strains was also examined as described by Shwaiki, Arendt and Lynch, 2020b. The assay was performed in compliance with the method outlined by the National Committee for Clinical Laboratory Standards (NCCLS M27A- (CLSI, 2008)). Briefly, a 10^4 cfu/mL solution of each yeast strain was prepared from overnight cultures in SD broth and transferred into the wells of a flat-bottom 96-well microtitre plate (Sarsdedt, Nümbrecht, Germany). The peptide was added into the first well and serially diluted to concentrations ranging from 12.5 to 400 µg/mL. A positive control of just distilled water with no peptide was also included. The plates were incubated for 48 h at 25 °C in a microtitre plate reader (Multiskan FC Microplate Photometer, Thermo Scientific, MA, USA) under gentle agitation. The optical density at 620 nm was measured every 2 h against broth as a blank.

The fungistatic and fungicidal activity of the peptide against the susceptible yeast was also examined to determine its MFC as described by Shwaiki, Arendt and Lynch, 2020b. One hundred microlitre of each yeast suspension from an antiyeast assay was spotted onto SD agar plates and incubated for 48 – 72 hrs, depending on the yeast. *D. hansanii, S. cerevisiae* and *Z. bailii* were incubated for 48 h while *Z. rouxii* and *K. lactis* were incubated for 72 h.

5.3.5 Colony Count Assay

A colony count assay was performed as described by Shwaiki, Arendt and Lynch, 2020b. The time required for the peptide to influence yeast growth was observed. Briefly, a yeast suspension of 10^5 cfu/mL was inoculated with different concentrations of D-lp1 (50, 100 and 200 µg/mL) and incubated for a period of 6 h. At 1 h intervals, 100 µL of the suspensions were spread onto SD agar plates and subsequently incubated for 48 h at 25 °C. The control consisted of a yeast suspension with water instead of peptide.

5.3.6 Peptide stability

D-lp1 was tested for its stability so as to determine whether it can retain its antiyeast activity in different environmental conditions which may be encountered in food products. High salt, heat and a range of pH were used to test this. The indicator yeast *Z. bailii* was treated due to its sensitivity towards the peptide. Concentrations of 50, 100 and 200 µg/mL of the peptide were tested.

Salt solutions of 1 and 5 mM $MgCl_2$ and 50 and 150 mM KCl were prepared and added to SD broth. An antiyeast assay was executed using the modified broth. Controls of modified broth, yeast and no peptide were also included.

To determine the effect of thermal treatment, the peptide was heated for 15 min at 100°C and left to cool before an antiyeast assay was performed.

The effect of different pH on the peptide was conducted by changing the pH of the SD media used to perform the antiyeast assay. This was done to examine the peptide's ability to retain its antiyeast activity in various pH conditions that may be encountered

if the peptide was to be applied as a food preservative. pH 3, 5, 7, 9 and 11 were tested by modifying the media with 0.1 M hydrochloric acid and 1 M sodium hydroxide to lower or increase the pH, respectively. Controls of media modified to the different pH containing the yeast and no peptide, were used.

5.3.7 Membrane permeabilisation

One potential mechanism of action of the peptides against the yeast is their ability to permeabilise yeast membrane. This mechanism is detected using propidium iodide, a dye that when inserted through the permeabilised yeast membrane, can bind and stain nucleic acids. The assay was performed as described by Shwaiki et al., 2020. Briefly, a *Z. bailii* cell suspension of 10^6 cfu/mL in SD broth was incubated for 2 h with different peptide concentrations ranging from 100 to 400 µg/mL. A final concentration of 5 µM propidium iodide was added and incubated for 20 min at room temperature in dark conditions. After washing with SD broth, this solution was then viewed under a Confocal Laser Scanning Microsocope (CLSM) (Olympus FV1000, incorporating an IX81 inverted microscope, Germany) using maximal excitation (λEx) and maximum emission (λEm) wavelengths of 535 nm and 617 nm, respectively. Triton X-100 (0.1%) and water were used at the positive and negative controls, respectively.

5.3.8 Overproduction of reactive oxygen species (ROS)

The peptides' ability to cause an overproduction of ROS in the yeast was assessed using the protocol described by Shwaiki, Arendt and Lynch, 2020. Briefly, a yeast suspension of 10^6 cfu/mL was prepared from an overnight solution of *Z. bailii.* Dihydrorhodamine 123 (5 µg/mL) was incubated with this solution at 25°C for 2 h to

facilitate its uptake into the cells and subsequently, its oxidation to rhodamine 123 in the presence of ROS. Following this, the sample was centrifuged at 3500 *g* for 5 min and the cells were washed with SD broth. Different concentrations of peptide were added (100, 200 and 400 µg/mL) and incubated at 25°C for 1 h. After incubation, 0.6 M potassium chloride was used to wash the cells once more before measuring the fluorescence under the CLSM at maximal excitation (λEx) and maximum emission (λEm) wavelengths of 488 nm and 538 nm, respectively. Two mM hydrogen peroxide (H_2O_2) and water were used as positive and negative controls, respectively.

5.3.9 Total nucleotide leakage

The total nucleotide leakage of *Z. bailii* resulting from the peptide's activity was analysed according to the protocol by Li *et al.,* 2016, with some modifications. An overnight culture of *Z. bailli* was used to prepare a 10^6 cfu/mL suspension that was washed twice using phosphate saline buffer (PBS). D-lp1 was added to this yeast suspension at concentrations of 100, 200 and 400 µg/mL and incubated for 4 h at 25 °C, after which the yeast cells were removed via filtration through a 0.22 µm filter. The OD at 260 nm was measured for each concentration. A positive and negative control of 0.1% Triton X-100 and water were used, respectively.

5.3.10 Haemolytic activity

D-lp1's ability to rupture red blood cells and test their safety with regards to their application in food was evaluated. The assay was performed as described by Thery & Arendt, 2018. Fresh sheep's blood (Oxoid™) was washed three times with equal volumes of phosphate-buffered saline (PBS). A 10 mL 4% solution was prepared

using PBS and then transferred into 1.5 mL Eppendorf tubes, in conjunction with 100, 200 and 400 µg/mL of the peptide. After incubation at 37°C for 1 h, the sample was centrifuged, and the supernatant transferred into a 96-well plate to measure its optical density at a wavelength of 405 nm. Triton X-100 (0.1%) and PBS were used as positive and negative controls, respectively. The percentage of haemolysis was calculated using the formula below.

$$\%\ \text{Haemolysis} = \frac{(\text{A405 peptide treatment}) - (A405\text{PBS})}{(A405\ 0.1\%\ \text{Triton X} - 100) - (A405\ \text{PBS})}$$

5.3.11 Cytotoxicity assay

The ability of a peptide to cause a harmful or toxic effect on human cells is referred to as its cytotoxicity activity (Laverty, 2014). A cytotoxicity assay was performed as described by Shwaiki, Arendt and Lynch, 2020b using a colonic cell line. Briefly, Caco-2 cells (ECACC) were prepared in Dulbecco's Modified Eagle Media (DMEM) that had been supplemented with 1% non-essential amino acids and 10% fetal bovine serum (FBS). A cell suspension of 1×10^5 cells/mL was prepared, 200 µL of which was added into the wells of a flat-bottom 96 well microtitre plate and incubated for 24 h at 37 °C with 5% CO_2. Following this incubation, the media was removed and D-lp1 was added into the wells at concentrations ranging from 100 to 700 µg/mL, together with DMEM supplemented with 2.5% FBS. Each well contained a final volume of 200 µL. The controls consisted of sterile water in DMEM. The plate was incubated at 37 °C for 24 h. The media in the wells was discarded and 100 µL of DMEM and 10 µL of the MTT labelling reagents (Cell proliferation Kit I MTT; Sigma, Ireland) were transferred to each well. Following a 4 h incubation period, a solubilisation buffer (100 µL) was added, and the plate was incubated for a further 24 h. A fluorometric

spectrophotometer was used to measure the cell viability in each well at a wavelength of 570 nm using a background reading of 690 nm.

5.3.12 Resistance to proteolytic degradation

This assay was performed following the protocol described by Shwaiki et al., 2020 to characterize the peptide's ability to withstand proteolytic degradation. This was done to simulate what the peptide may encounter if applied as a food preservative. In brief, the digestive enzyme α-chymotrypsin was incubated with the peptide at different concentrations: enzyme molar ratios of 60:1, 250:1, 2500:1, for 4 h at 37 °C. This was followed by inactivation at 80 °C for 10 min. An antiyeast assay was then performed to test the peptide at different concentrations (50, 100, 200 and 400 µg/mL) against *Z. bailii.*

5.3.13 Application of D-lp1 in different food matrices

The application of D-lp1 was assessed in different food matrices against *Z. bailii*, the most susceptible yeast towards the peptide.

The high sugar or salt content found in soft drinks and fruit juices make it a favourable environment for *Z. bailii* to grow (Blackburn, 2006). The antiyeast activity of the peptide was tested in apple juice pH 3.2 (*Tropicana Apple*, pressed apple), Fanta Orange pH 3.1 (*Coca-Cola*, Ireland), and wine (*Faber*, Chardonnay) pH 3.05. D-lp1 was tested via the microtiter plate method using a 10^4 cfu/mL suspension of yeast made up in filter sterilised solutions of the beverages. The peptide was tested at concentrations ranging from 50 to 400 µg/mL and its antiyeast activity was observed

over 48 h by measuring the optical density at 620 nm every 2 h against a sample of each beverage as the blanks. Controls consisting of the beverages inoculated with the same concentration of yeast without peptide was also included.

5.3.14 Statistical analysis

To determine the significant difference between the results obtained for the salt stability assay, analysis of variance with ANOVA (SigmaStat, SPSS Inc., Chicago, USA), was performed. The statistical significance between the inhibitory properties observed in the different salt concentrations (at various peptide concentrations) was analysed. A probability of $p < 0.05$ was considered statistically significant.

5.4 Results

5.4.1 Secondary structure analysis of D-lp1

The secondary structure of the peptide revealed triple-stranded antiparallel beta-sheets, an alpha-helix and corresponding connecting loops (Figure 15).

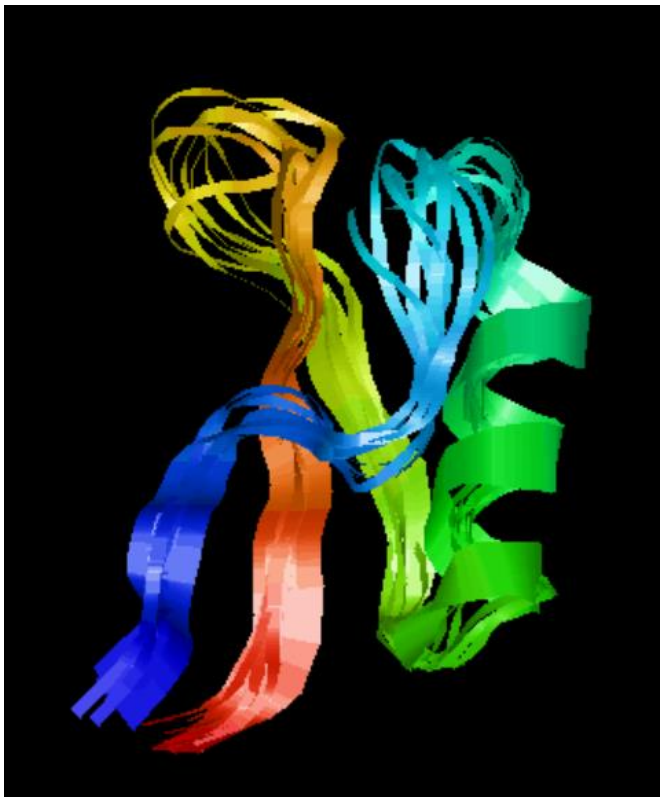

Figure 15: Secondary structure of D-lp1 generated on RasMol demonstrating the triple stranded anti-parallel beta-sheets and alpha helix structure present within the peptide.

5.4.2 D-lp1 Antiyeast activity

The antiyeast activity determined the minimum inhibitory concentration (MIC) or minimum fungicidal concentration (MFC) of the peptide for the 5 yeast. *Zygosaccharomyces bailii* and *Debaryomyces hansenii* were the most sensitive yeasts with MFC ranges of 50-100 µg/mL. *Saccharomyces cerevisiae* and

Zygosaccharomyces rouxii were only inhibited at the highest concentration of 400 μg/mL (MFC and MIC range of 200-400 μg/mL, respectively). The fungicidal activity of the peptide was observed at all inhibitory concentrations against *Z. bailii* and *S. cerevisiae*, while only at 200 and 400 μg/mL against *D. hansenii* (Table 13). The observed fungicidal effects helped to distinguish between the inhibitory mechanism of the peptide (MIC) and its killing mechanism (MFC). No inhibition against *Kluyveromyces lactis* was detected.

Table 13. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of the peptide against the five yeast.

Yeast	**Minimum inhibitory activity (MIC and MFC ranges)**
Zygosaccharomyces bailii	50 - 100 μg/mL (MFC)
Zygosaccharomyces rouxii	200 - 400 μg/mL (MIC)
Kluyveromyces lactis	No inhibition
Saccharomyces cerevisiae	200 - 400 μg/mL (MFC)
Debaryomyces hansenii	50 - 100 μg/mL (MFC at higher concentrations)

5.4.3 Colony count assay

The colony count assay observed the time course of yeast inhibition as a result of the peptide's antiyeast activity. After only 2 h of incubation, 100 (MIC) and 200 μg/mL of peptide resulted in a rapid decrease in cell numbers. The control with no peptide and peptide concentration of 50 μg/mL showed a steady increase in *Z. bailii* growth, whose cell growth after 6 h reached 6.3×10^5 and 5.3×10^5 cfu/mL, respectively (Figure 16).

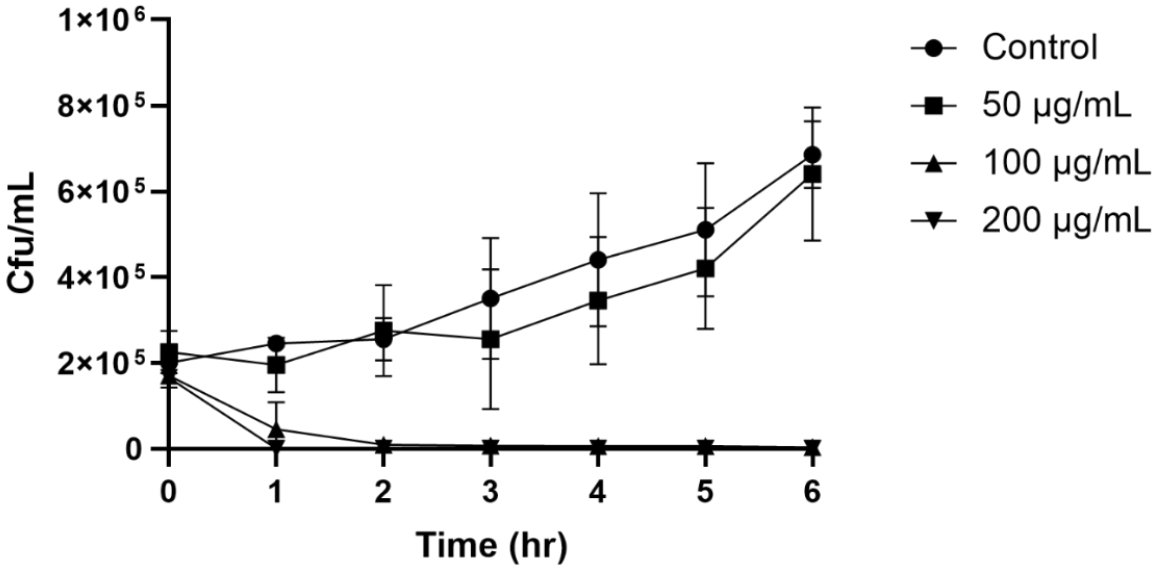

Figure 16: The time-course required for D-lp1 to cause inhibition on *Z. bailii* over a period of 6 hr. Peptide concentrations of 50, 100, and 200 µg/mL were tested alongside a control.

5.4.4 Peptide stability

The peptide's stability in concentrated salt solutions, different pH and high heat was performed to reveal its potential to maintain its antiyeast activity under these conditions.

In 5- and 50-mM magnesium chloride ($MgCl_2$), the peptide's antiyeast activity was only affected at 100 µg/mL, while at the higher concentrations (200 and 400 µg/mL), nearly full inhibition was detected (Figure 17). D-lp1 was affected more in potassium chloride (KCl) as 400 µg/mL was the only concentration observed to cause inhibition in the 50 mM KCl solution. In 150 mM KCl, some inhibition occurred at 400 µg/mL, however growth was still evident. ANOVA determined significant differences in the peptide's ability to cause yeast inhibition in the different salt concentrations (at the various peptide concentrations tested) (Figure 17).

Applying thermal treatment at 100 °C for 15 min did not change the activity of the peptide. Full inhibition was observed on the growth of *Z. bailii* at concentrations up to the MIC (100 µg/mL).

Modifying the media to the different pH of 3, 5 and 7 resulted in no change in the peptide's inhibitory activity. Full inhibition of the yeast was detected at the concentrations tested (200 and 100 µg/mL) due to the retention of its antiyeast activity. At pH 9 and 11, no yeast growth was seen in the controls.

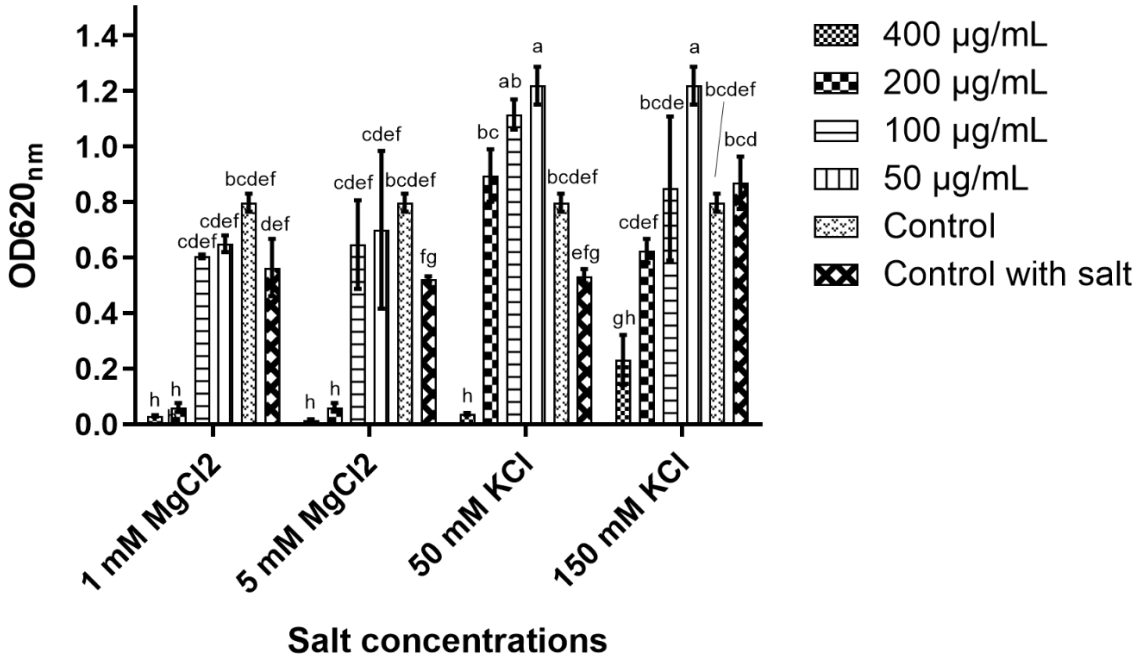

Figure 17. The effect of different salt concentrations ($MgCl_2$ and KCl) on the antiyeast activity of the peptide. ANOVA analysis was performed to determine the significant differences between the peptide's inhibitory activity in these salt concentrations (at various concentrations of the peptide). The bars containing the same letters are **not** significantly different and those allocated different letters were determined significantly different from one another. For example, the bars allocated "f" are all significantly similar to one another but are different to the bars allocated "g" or another letter. Bars allocated more than one letter indicates their similarity to more than one mean value.

5.4.5 Mechanism of Action

The peptide's mechanism of action was studied using laser scanning microscopy. The peptide was observed to cause both membrane permeabilisation and overproduction of reactive oxygen species (ROS) in a dose dependent manner. At the highest concentration of D-lp1 (400 μg/mL), complete permeabilization of the yeast cells was observed, with the level of permeabilisation decreasing as the peptide concentration was reduced (200 and 100 μg/mL) (Figure 18A, 18B and 18C). A similar effect was observed for the overproduction of ROS, with greater ROS detected at the higher concentration of 400 μg/mL (Figure 19A, 19B and 19C).

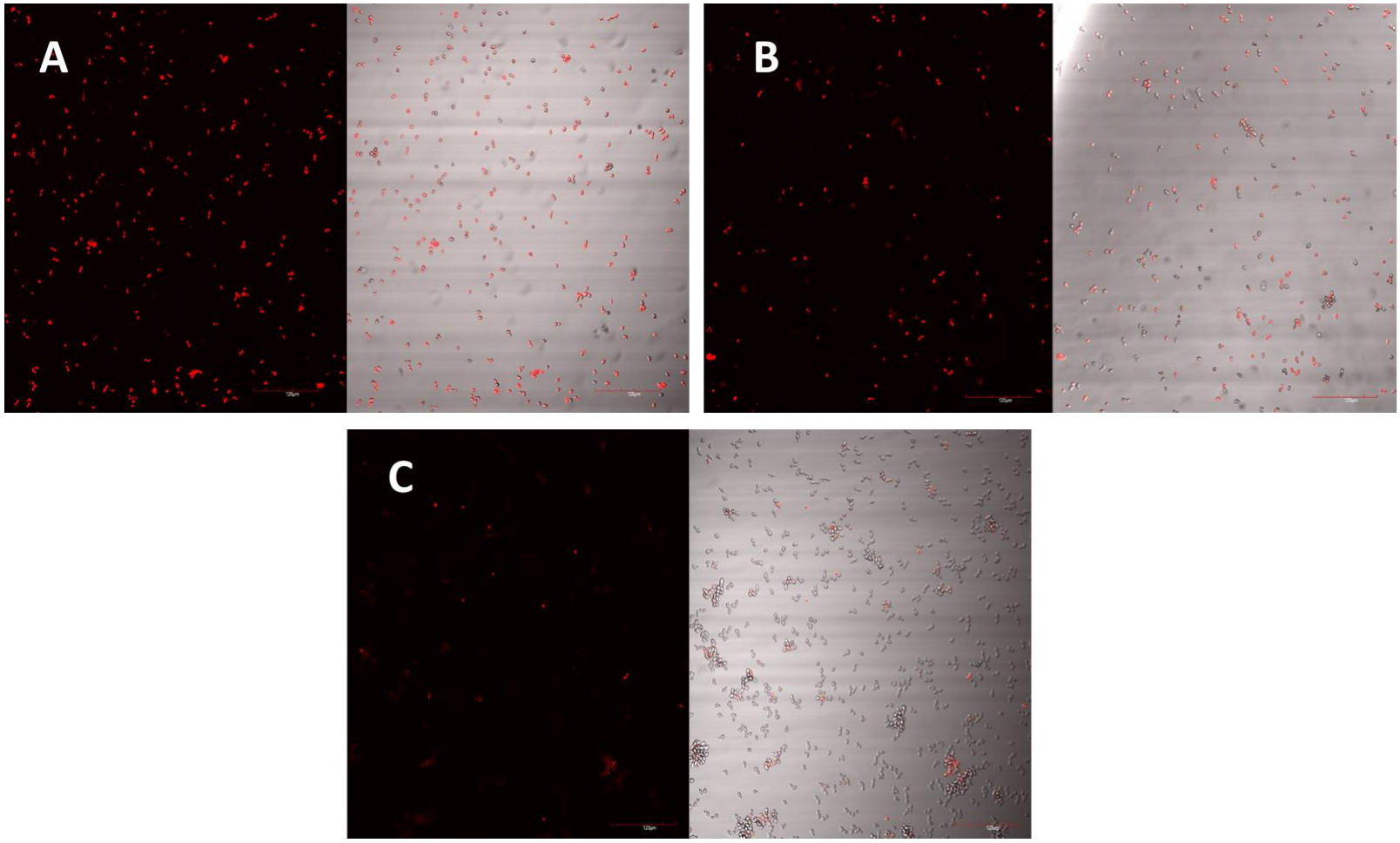

Figure 18: *Z. bailii m*embrane permeabilisation observed under the CLSM as a result of the peptide at concentrations of 400 µg/mL (A), 200 µg/mL (B) and 100 µg/mL (C).

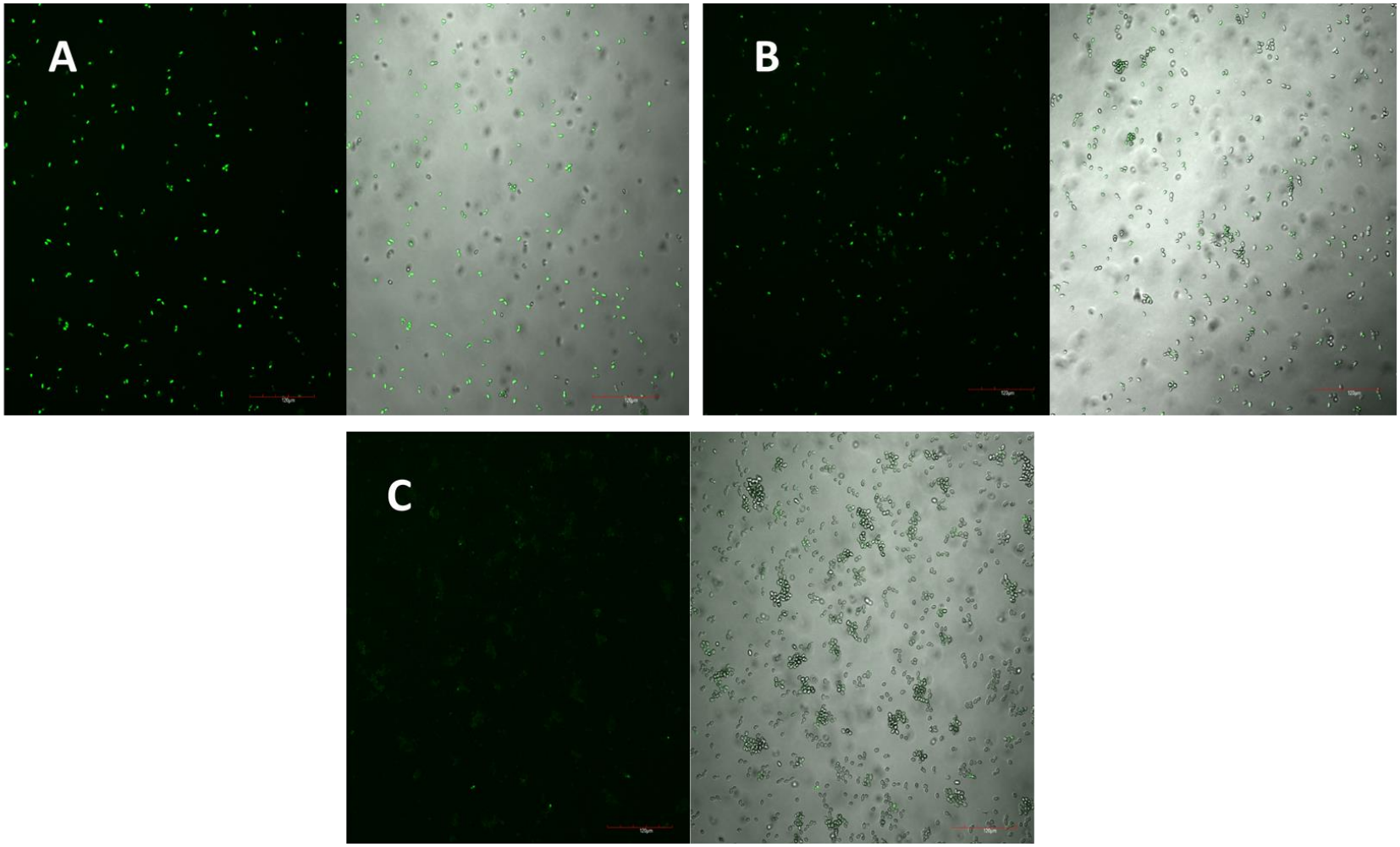

Figure 19: The overproduction of ROS generated by the presence of the peptide at the difference concentrations of 400 µg/mL (A), 200 µg/mL (B) and 100 µg/mL (C). A reduction in ROS overproduction is observed as the concentration of D-lp1 is reduced.

5.4.6 Total nucleotide leakage

The effect of D-lp1 on the cell membrane of *Z. bailli* was examined through a total nucleotide leakage assay (Figure 20). After a 4 h incubation period, an OD of 0.142 was measured at 400 µg/mL, a considerably higher OD compared to the negative control of *Z. bailii* with just distilled water (OD 0.003). The level of nucleotide leakage correlated with peptide concentration as 100 and 200 µg/mL resulted in ODs of 0.104 and 0.055, respectively.

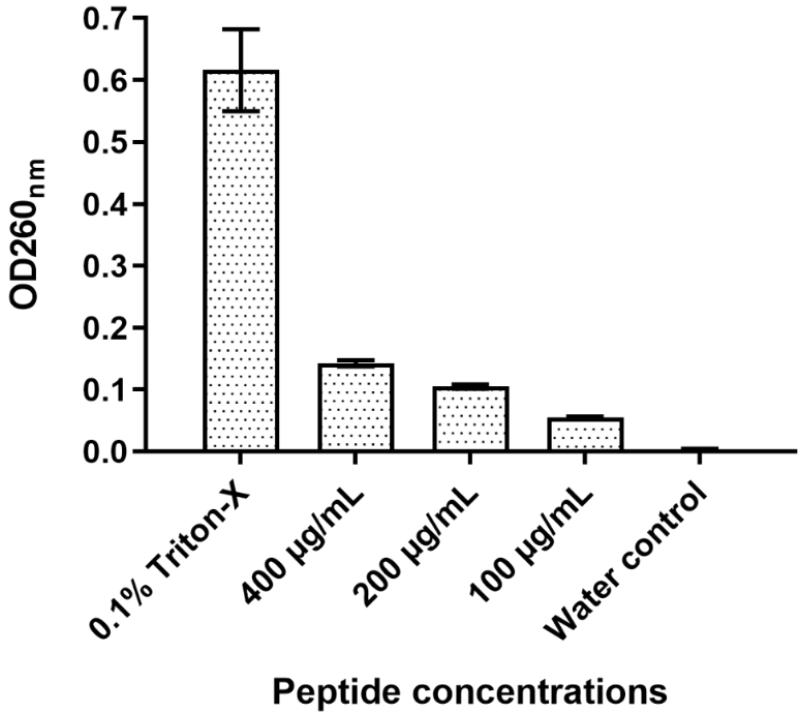

Figure 20. The total nucleotide leakage detected from *Z. bailii* 6 hr after inoculation and incubations with the peptide at different concentrations ranging from 100 to 400 µg/mL. Nucleotide leakage is observed to increase with peptide concentration.

5.4.7 Haemolytic assay

The haemolysis of red blood cells was used as an indicator for the safety of the peptide. D-lp1 was not found to cause haemolysis even at the highest concentration of 400 µg/mL, with less than 10% haemolysis observed.

5.4.8 Cytotoxicity assay

The viability of Caco-2 cells was measured to further determine the peptide's safety. At all the concentrations that were tested, D-lp1 caused a slight increase in the viability of the cells compared to the control cells containing only water (Figure 21).

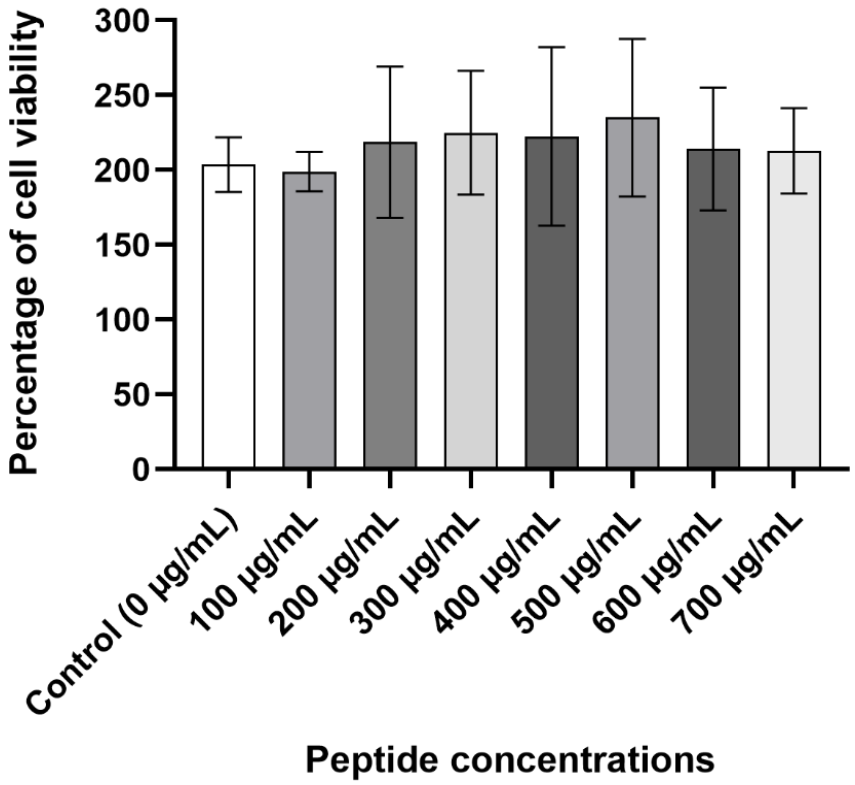

Figure 21. Percentage of cell viability caused by the different concentrations of peptide (from 100 to 700 µg/mL). A slight increase in cell viability is observed compared to the control.

5.4.9 Resistance to proteolytic digestion

D-lp1 was tested for its susceptibility to proteolytic digestion with the aim of investigating its safety as a preservative. Alpha-chymotrypsin was used as the proteolytic enzyme in order to mimic the environment commonly found in the gut. At all molar concentrations, the enzyme was capable of digesting the peptide. Yeast

growth was observed at all concentrations of peptide tested (400-100 µg/mL), indicating a reduction in its antiyeast activity.

5.4.10 Peptide activity in different beverages

D-lp1 was tested for its inhibitory effect against *Z. bailii* in apple juice, Fanta Orange and wine. In the apple juice, the peptide was fully effective at inhibiting *Z. bailli* at the known inhibitory concentrations (400, 200 and 100 µg/mL). In the Fanta Orange, only 200 and 400 µg/mL caused a reduction in yeast growth, while in the wine, only partial inhibition (at 400 µg/mL) was observed.

5.5 Discussion

The growing demand for natural preservatives has made plant-derived additives a popular alternative to the current chemical preservatives that consumers may find unappetising (Gould, 1995). In recent years, plant AMPs have risen in popularity for their application to counteract microbial pathogens. Due to the laborious processes involved in the purification of these peptides from plants, the chemical synthesis of AMPs can be a direct alternative. The current study examined the effect of a synthetic peptide derived from the barley endosperm, referred to here as D-lp1, on the growth of common food spoilage yeast. The peptide's amino acid sequence was previously discovered by Mendez *et al.*, 1990. Their work on its structure and characterisation led them to categorize the peptide into the thionin family, a family of potent AMPs whose structures are not dissimilar to plant defensins. Previously known as gamma-purothionin, D-lp1's 47 amino acid distribution within its sequence renders the peptide a structure homologous to plant defensins. The structure obtained from the RasMol programme illustrated the triple-stranded antiparallel beta-sheets and alpha helix present there. A cysteine stabilised alpha-helical motif is formed by these beta and alpha helix structures which are being held together by three disulfide bridges in the hydrophobic core (Bruix *et al.*, 1993). These characteristics are common amongst peptides in the family of plant defensins.

The peptide's high net charge (+9), the presence of 8 cysteine residues and its basic nature can be used to explain its strong antiyeast activity (Jindal *et al.*, 2014). Four of the five yeast tested were sensitive to the peptide, with MIC ranges as low as 50-100 µg/mL observed for *Z. bailii* and *D. hansenii.* The high net charge and rich presence of cysteine residues are common characteristics found in AMPs with potent antimicrobial activity (Haag *et al.*, 2012; SD, 2014; Chen *et al.*, 2018). The eight

arginine residues present in D-lp1's amino acid sequence contributes to the cationicity of the peptide, permitting easier interaction with the negatively charged yeast membrane, resulting in its disruption and cell death (Haag *et al.*, 2012).

The peptide's stability in the $MgCl_2$ concentrations revealed minimal changes to its antiyeast activity, while in 150 mM KCl, a reduction in its inhibitory activity was apparent. This effect could be hypothesised to have occurred due to the presence of K+ cations causing a reduction in the net charge of the yeast cell wall. This reduction in net charge could have caused the peptide to be repulsed from the yeast cell (Herbel and Wink, 2016). The shift to a more negatively charged yeast cell could have also caused the overall charge of the peptide to be modified. As a consequence, this could have triggered structural changes to the peptide that may explain the reduced antiyeast activity detected in the presence of KCl (Susa, Mortensen and Williams, 2014). Testing the stability of D-lp1 in the salts gives an insight on how the peptide might react in high salt environments frequently found to harbour spoilage yeast such as *Z. bailli*. Modifying the media to pH 3, 5 and 7 did not alter the peptide's antiyeast activity, while in the higher pH (9 and 11) no yeast growth was observed in the controls. This was likely due to the unsuitable growth conditions for the yeast, rather than the effect of the peptide (Peña *et al.*, 2015). It has been stated that the biological function of a peptide can be altered in a solution that holds a pH equal to a peptide's isoelectric point (pI) (Osorio, Rondón-Villarreal and Torres, 2015). The high pI of D-lp1 (9.9) may have resulted in the retention of its antiyeast activity this range of pH (pH 3, 5 and 7). Table 14 shows the effect that different pH have on the overall net charge of the peptide, with an increase observed in acidic conditions (pH 3 and 5), compared to the basic pH (9 and 11), which could further explain its ability to retain its antiyeast activity at pH 3, 5 and 7.

Table 14: The net charge of D-lp1 at the different ranges of pH. A reduction in the peptide's net charge is detected above neutral pH and in more basic conditions. (Data obtained using an online peptide net charge calculator).

	pH 3	**pH 5**	**pH 7**	**pH 9**	**pH 11**
Dlp-1 net charge	13.4	11.3	9.1	1.4	-5.3

Thermal treatment also resulted in its normal antiyeast activity, further supporting its potential for application as a preservative. The peptide's robust tertiary structure resulting from the various disulphide bonds formed by the 8 cysteine residues present in its sequence could explain this stability (Betz, 1993).

Both mechanisms of actions examined were found to be dose dependant, with the highest concentration of 400 µg/mL causing the most permeabilisation and overproduction of ROS. The cationic nature of the peptide causing an interaction with the negatively charged yeast membrane could explain the high levels of permeabilisation that was detected (Li *et al.*, 2017; Kumar, Kizhakkedathu and Straus, 2018). This mechanism of action has been widely studied in various plant AMPs (Thevissen, Terras and Broekaert, 1999; Abulimiti Yili *et al.*, 2014; Kraszewska *et al.*, 2016; Lei *et al.*, 2019). The total nucleotide leakage assay presents further evidence of the rate of membrane permeabilisation instigated by the peptide. The damage caused to the yeast membrane could have resulted in the nucleotide leakage from the cells (Hou *et al.*, 2007; Li *et al.*, 2016). The peptide's impact on the yeast membrane can also lead to a cascade of reactions, including the overproduction of ROS (Struyfs *et al.*, 2020). The generation of these species by yeast cells plays a role in normal cell function; however, the introduction of a stress factor like D-lp1 can ultimately lead to the overproduction of ROS, and subsequently to cell death (Wang *et al.*, 2015).

The peptide's safety was evaluated for its intended use as a potential preservative. Its haemolytic and cytotoxicity activity were studied, giving an indication of its safety if it were to be consumed. The haemolytic activity of a peptide can generally be linked to its cytotoxicity and to its ability to cause harmful effects on eukaryotic cells (Ruiz *et al.*, 2014). In the presence of D-lp1, the percentage of ruptured red blood cells measured was minimal, with less than 10% haemolysis observed at the highest concentration of 400 µg/mL. This low percentage of haemolysis signifies the peptide's safety and its suitability as a potential preservative. The cytotoxicity assays revealed a lack of toxicity towards the Caco-2 cells (a slight increase in cell viability was observed), further supporting the peptide's safety. This attribute is common amongst cationic AMPs whose affinity for negatively charged membranes makes them more attracted to the membranes of microbial cells than those of eukaryotic cells (that comprise of predominantly neutral and/or slightly positively charged membranes) (Chen *et al.*, 2016). In addition, the presence of cholesterol in eukaryotic cell membranes can act as a rigid barrier to the lipid bilayer structure of the cell that can impede the destruction of the membrane via the action of cationic AMPs not unlike D-lp1 (Brender, McHenry and Ramamoorthy, 2012).

D-lp1's sensitivity to α-chymotrypsin, a proteolytic digestive enzyme, was also examined to better characterise its safety if consumed. In the gastrointestinal tract, the presence of various digestive enzymes would make it an ideal environment for the breakdown of the peptide. D-lp1 was found to be sensitive to the α-chymotrypsin at all molar concentrations tested, providing additional evidence for its safety.

As a proof of concept, D-lp1 was applied into the different beverages. The peptide was found to retain its full antiyeast activity in the apple juice, while in the Fanta Orange and wine the higher concentrations still caused inhibition. The consistency of

beverages such as these makes it easier for the peptide to perform its inhibitory effects on the yeast. This matrix creates a suitable environment for the peptide to impart its effect on the cell membrane of the yeast, made possible by the consistency of the beverages. Nisin, an AMP that is known for its potent antimicrobial activity and by its successful incorporation into various food products has been applied in beverages with similar consistencies. Its application in alcoholic beverages has been found to inhibit the growth of common beer and wine microbial food spoilers (Rojo-Bezares *et al.*, 2007). The successful incorporation of the peptide into the beverages tested in this study helps to demonstrate its potential to perform as a novel preservative agent.

5.6 Conclusion

This study illustrates the potential of a synthetic peptide derived from barley endosperm to act as a novel food preservative. Consumers' opinions of the current forms of preservatives stems from the perception that foods should be more natural and unaffected by the addition of chemical preservatives. Taking advantage of plants and the AMPs that they produce may be an effective solution to this. Naturally extracting AMPs straight from the plant can be time consuming and costly. The current cost of synthesis for the production of peptides such as the one presented in this study can limit their application in food. The production of a peptide with similar length and amino acid composition as D-lp1 can amount to on average 10 US dollars per amino acid for a peptide of up to 4mg/mL and with a purity of >85% (price is based on general rates within the market of peptide synthesis). Although this cost of synthesis may be a disadvantage now, with time the cost of synthesis will reduce, making it more feasible to synthesise peptides. In addition to this, the time-effective production

process required to generate synthetic AMPs and the potential to develop a peptide of high purity, could make this the preferred method of production. The approach presented in this study, although not currently feasible for wide-scale production, demonstrates a proof of principle for the application of synthetic AMPs in food preservation.

5.7 Acknowledgments:

This work was supported by the Department of Agriculture, Food and the Marine (F.I.R.M.), Project Reference 15/F/731. A special thank you to Dr Yvonne O'Callaghan for all her help with the cell cultures.

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Chapter 6

Study on the characterisation and application of synthetic peptide Snakin-1 derived from potato tubers – action against food spoilage yeast

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Published in Food Control as:

Shwaiki, L. N., Arendt, E. K. and Lynch, K. M. (2020) 'Study on the characterisation and application of synthetic peptide Snakin-1 derived from potato tubers – Action against food spoilage yeast', *Food Control*. Elsevier Ltd, 118. doi: 10.1016/j.foodcont.2020.107362.

6.1 Abstract

The demand for more natural forms of food preservation by consumers has risen in recent years. Food spoilage by microorganisms, in particular food spoilage yeast, has been reported in different food matrices. *Zygosaccharomyces* species, for example, are well known for their spoilage in high sugar content foods. In the battle against food spoilage, natural antimicrobial peptides derived from plants could be a potential new source of food preservatives. The antimicrobial peptide investigated in this study was Snakin-1, derived from potato tubers. A synthetic form of Snakin-1 was synthesised based on the amino acid sequence of its natural peptide. The peptide was characterised for its inhibitory effect against a number of food spoilage yeast. The peptide's minimum inhibitory concentration was generated and its mechanism of action against *Zygosaccharomyces bailii* was examined. Membrane permeabilisation caused by the presence of the peptide was observed at the highest concentrations applied, and its ability to withstand high salt concentrations, thermal treatment and a range of pH was also investigated. The peptide's safety for human consumption was studied and no adverse effects were found. Finally, the peptide was applied into different beverages commonly spoiled by *Zygosaccharomyces bailii* in order to demonstrate the potential application of this synthetic plant antimicrobial peptide.

6.2 Introduction

Plant host defence proteins known as antimicrobial peptides (AMPs) constitute a large part of a plant's defence system, originating from the seeds, leaves, flowers, stems or roots (Nawrot *et al.*, 2014). Plant AMPs are becoming more widespread in research as potential sources of antimicrobial agents for the protection of foods against spoilage microorganisms (Tiwari *et al.*, 2009; Hintz, Matthews and Di, 2015; Shwaiki *et al.*, 2019; Thery *et al.*, 2019). The preservation of food has evolved from the incorporation of food additives to a demand for more biological-based, natural methods (Brul and Coote, 1999; Sharma, 2014). The demand from consumers for products without food additives, in particular chemical preservatives, comes from the negative perception towards chemicals and a general lack of consumer understanding as to the purpose and origin of such additives in foods. Therefore, there is a growing requirement for natural compounds that can be exploited for preventing the initial spoilage of food systems, and furthermore ensuring the continuous safety of the product over its shelf-life. Essential oils and organic acids are examples of natural preservative agents already established and being used in the food industry (Theron and Lues, 2011; Das, 2016).

The spoilage of food is caused by a wide range of microorganisms, one of which is yeast (Wang, Zhang and Fu, 2017). These microorganisms are able to contaminate food systems during the production process but also during storage, leading to spoilage. Such spoilage can lead to major economic losses in the food industry (Fleet, 2006). The major yeast spoilers in the food industry are *Zygosaccharomyces*, *Debaryomyces*, *Kluyveromyces*, and *Saccharomyces* species, affecting soft drinks, high salt content foods, dairy products and alcoholic beverages, respectively (Thomas and Davenport, 1985; Fleet and Mian, 1987; Blackburn, 2006; Breuer and Harms, 2006). Plant AMPs can be purified from any part of the plant through different

extraction processes; however, this can be time consuming and potentially costly. A possible solution could be the chemical synthesis of the target peptide, reducing the time and money spent on the resources required to perform the extraction process.

This paper aims to investigate the anti-yeast potential of the chemically synthesised peptide, Snakin-1 (SN-1). This peptide is responsible for the defence of the potato plant against external environmental pathogens and has been previously extracted and revealed to be a 63-amino acid residue peptide (Almasia *et al.*, 2008; Harris *et al.*, 2014). A previous study by Segura *et al.*, 1999 successfully isolated and characterised this peptide from potato tubers and demonstrated its antimicrobial activity against bacterial and fungal plant pathogens. Expression of the SN-1 gene was established and detected not only in the tubers, but in the stems, axillary buds and in young floral buds. SN-1 was found to contain a molecular weight of approximately 6.9 kDa and consists of two long α-helices with six disulphide bonds. It has structural similarity to thionins, another group of potent plant AMPs (Bohlmann and Apel, 1991; Kuddus *et al.*, 2016). This synthetic peptide was tested for its ability to inhibit a number of common food spoilage yeast, namely, *Zygosaccharomyces bailii, Zygosaccharmocyes rouxii, Debaryomyces hansenii, Kluyveromyces lactis* and *Saccharomyces cerevisiae.* This was accomplished through the investigation of its minimum inhibitory concentration (MIC). The potential application of the peptide in different beverages (Fanta Orange, cranberry and apple juice), its stability in different environments, as well as its mechanism of action against the yeast were investigated. Furthermore, the safety aspects of the peptide were also examined.

6.3 Material and Methods

6.3.1 Snakin synthesis

The 63 amino acid sequence of Snakin-1, hereafter termed SN-1 (Table 15), derived from *Solanum tuberosum* cv. *Desireé* (potato tubers), was chemically synthesised by GL Biochem (Shangai) Ldt to a purity of 80% as indicated by the supplier. The peptide was resuspended in water at a concentration of 2 mg/mL.

Table 15: Snakin peptide (SN-1) amino acid sequence derived from potato tubers (*Solanum tuberosum* cv. *Desireé*).

	Amino acid sequence
Snakin peptide SN-1	GSSFCDSKCKLRCSKAGLADRCLKYCGICCEECKCVPSGT YGNKHECPCYRDKKNSKGKSKCP

6.3.2 Peptide structure analysis

The programme RasMol (Valadon, 2009) was used to develop the secondary structure of the peptide. The amino acid sequence was obtained from the Protein Data Bank (Berman *et al.*, 2000) and used to build the model. The secondary structure was also analysed using circular dichroism (CD) spectroscopy as described by Schmidt, Arendt and Thery, 2019. Briefly, the peptide was diluted to a final concentration of 1 mg/mL in purified water or 20 mM sodium dodecyl sulfate (SDS). The measurements were performed on a Chirascan CD Spectrometer (Applied Photophysics), at 20°C, within wavelength ranges of 180 - 260 nm. This analysis was performed in triplicate, subtracting the measurements of the solvent CD from the sample CD.

6.3.3 Yeast strains

The five yeast strains used during the study were *Zygosaccharomyces bailli* Sa 1403, *Zygosaccharomyces rouxii* ATCC 14679, *Kluyveromyces lactis* ATCC 56498, *Debaryomyces hansenii* CBS 2334 (DMSZ, Germany), and *Saccharomyces cerevisiae* Baker's yeast (Puratos, Belgium). Each yeast was grown aerobically on Sabouraud dextrose (SD; Sigma-Aldrich) agar at 25°C and overnight cultures were performed in SD broth at the same temperature under gentle agitation. All media and reagents used were obtained from Sigma-Aldrich (MO, USA), unless otherwise stated.

6.3.4 Determination of minimum inhibitory concentration via anti-yeast assay

A microbroth dilution assay was performed to examine the anti-yeast activity of the peptide and determine its MIC against the different yeast. This was performed according to the National Committee for Clinical Laboratory Standards (NCCLS M-27A, NCCLS 2002). A yeast suspension of 10^4 cfu/mL was prepared from an overnight yeast culture in SD broth. Two hundred and ninety microlitres was transferred into the first well of a flat-bottom 96-well microtitre plate (Sarsdedt, Nümbrecht, Germany), after which 10 µL of the peptide at concentrations of 12.5 to 400 µg/mL was added. A serial dilution was performed by transferring 100 µL from the first well into subsequent wells containing 200 µL of yeast. The positive control consisted of yeast suspension in the absence of peptide. The microtitre plate was incubated for 48 h, at a constant temperature of 25 °C and under gentle agitation. Optical density (OD) was measured in 2 h intervals at a wavelength of 620 nm. This procedure was repeated in triplicate on three different plates.

The peptide's fungicidal and fungistatic activity against the yeast was examined by spotting 100 µL of the yeast suspension from an anti-yeast assay onto SD agar plates. The plates were incubated for 48-72 h, depending on the optimal incubation time for the yeast. *D. hansanii, S. cerevisiae* and *Z. bailii* were incubated for 48 h while *Z. rouxii* and *K. lactis* were incubated for 72 h.

6.3.5 Colony Count Assay

The peptide's ability to inhibit the growth of *Z. bailii* at the different concentrations observed in the anti-yeast assay was examined by performing a colony count assay. This was done to determine the time necessary for the peptide to cause inhibition of *Z. bailii.* A yeast suspension of 10^4 cfu/mL was prepared in conjunction with the peptide at concentrations of 50, 100, 200 and 400 µg/mL. One hundred microlitre of this suspension was spread onto SD agar plates at 1 h intervals for a period of 6 h. The control consisted of yeast with water alone. The plates were incubated for 48 h at 25 °C.

6.3.6 Peptide stability

The stability of the peptide was studied by changing the pH, salt and thermal conditions and testing for its anti-yeast activity. The salt stability assay was performed on using *Z. bailii* as the target yeast, with concentrations of peptide tested of 100, 200 and 400 µg/mL. The pH and thermal stability assays also used *Z. bailii* and was performed using concentration of 50, 100, 200 and 400 µg/mL.

The pH of the SD media was altered using 1 M sodium hydroxide and 0.1 M hydrochloric acid in order to increase or reduce the pH of the SD broth to obtain media with the following pH: pH 3, 5, 7, 9 and 11. An anti-yeast assay was performed as described above. Controls consisted of regular SD both pH-adjusted broth without added peptide.

The stability of the peptide in salt concentrations of 1 and 5 mM $MgCl_2$ and 50 and 150 mM KCl was examined through the addition of the respective salt into the medium, prior to performing an anti-yeast assay. A control of the yeast suspension in SD broth containing the salts, but without peptide, was used.

A study the peptide's thermal tolerance was performed by subjecting the peptide to 100 °C for 15 min, allowing it to cool for 30 minutes, followed by testing for its anti-yeast activity.

6.3.7 Total Nucleotide Leakage

Analysis of the total nucleotide leakage of *Z. bailii* resulting from the peptides' activity was performed according to Li *et al.*, 2016 with some modifications. A 10^6 cfu/mL suspension of *Z. bailii* was prepared from an overnight culture and washed twice using phosphate saline buffer (PBS). Peptide concentrations of 100, 200 and 400 µg/mL were added to the yeast suspension and incubated at 25 °C for 4 h, after which yeast cells were removed via filtration through a 0.22 µm filter. The OD^{260nm} of the filtrate was recorded. A 0.1% Triton X-100 and water were used as positive and negative controls, respectively.

6.3.8 Membrane Permeabilisation Assay

The peptide's ability to permeabilise the yeast membrane was studied as a potential mechanism of action. *Z. bailii* was used as the target yeast for this assay. The propidium iodide dye used in this assay is a binding agent that can stain the nucleic acid of yeast cells if membrane permeabilisation has occurred. This dye binding can only occur if the peptide has permeabilised the yeast membrane. The protocol of Shwaiki *et al.*, 2019 was followed, with some modifications. Briefly, a cell suspension of 10^6 cfu/mL was prepared from an overnight culture in SD broth and inoculated with peptide at concentrations of 100, 200 and 400 µg/mL. After a 2 h incubation period, 5 µM propidium iodide (SIGMA) was added and incubated for another 1 h before visualisation of the cells under a Confocal Laser Scanning Microsocope (CLSM) (Olympus FV1000, incorporating an IX81 inverted microscope, Germany), with maximal excitation (λEx) and maximum emission (λEm) wavelengths of 535nm and 617nm, respectively. Negative and positive controls consisting of water and Triton X-100, respectively, were used.

6.3.9 Haemolysis Assay

Haemolysis of red blood cells, that is, the release of haemoglobin from defibrinated sheep erythrocytes due to the presence of the peptide, was examined to determine its safety for human consumption. The assay was performed as outlined in Thery and Arendt, 2018. Briefly, a 4% red blood cell solution was incubated with peptide concentrations ranging from 12.5 to 400 µg/mL for 1 h at 37 °C. This suspension was then centrifuged at 1,000 *g* for 10 min and the OD^{405nm} of the supernatant was measured. A positive control of 0.1% Triton X-100 and negative control of PBS were

used. The percentage of haemolysis was calculated using the measured absorbance and the below formula.

$$\% \text{ Haemolysis} = \frac{(A405\ peptide\ treatment) - (A405\ PBS)}{(A405\ 0.1\%\ Triton\ X{-}100) - (A405\ PBS)}$$

6.3.10 Resistance to proteolytic digestion

To test the peptide's ability to resist proteolytic digestion different molar ratios of α-chymotrypsin (Sigma, St Louis, MO, USA) were incubated with the peptide. Alpha-chymotrypsin is a common digestive enzyme found in the human gut, and for the purpose of this assay, peptide to enzyme molar ratios of 60:1, 250:1, 2500:1 was tested. In an attempt to mimic the environment the peptide may encounter after its ingestion, the protocol by Thery *et al.*, 2019 was followed, with some modifications. The enzyme was incubated with the peptide for 4 hr at 37 °C, followed by inactivation of the enzyme at 80 °C for 10 min. An anti-yeast assay against *Z. bailii* was performed using the treated peptide, at concentrations of 100, 200 and 400 μg/mL. The α-chymotrypsin was prepared in a digestion buffer containing 50 mM Tris–HCl (pH 7.4) and 5 mM $CaCl_2$.

6.3.11 Peptide application in beverages

The peptide's application in different beverages was assessed by spiking them with *Z. bailii*, a yeast commonly found spoiling beverages such as soft drinks and fruit juices (Kuanyshev *et al.*, 2017). The beverages tested for the application of the peptide were Fanta Orange (Coca-Cola, Ireland) (pH 3.1), cranberry juice (*SuperValu*, Chilled Cranberry Juice) (pH 2.7) and apple juice (*CYPRINA*, Apple Juice) (pH 3.5). For each

beverage, a 10^4 cfu/mL yeast suspension was prepared from overnight cultures in SD broth and inoculated at this level. An anti-yeast assay was carried out on the inoculated beverages with peptide at concentrations of 50, 100, 200 and 400 µg/mL. A control consisting of the beverage inoculated with the yeast alone (no peptide) was also included for each assay. Yeast cell growth was monitored spectrophotometrically over 48 h.

6.4 Results

6.4.1 Peptide structure analysis

A proposed 3D structure of the peptide was modelled (Figure 22A) and compared to the results of the CD spectroscopy. The 3D model shows that the structure contains 3 alpha helices and 2 β-strands. In the CD spectra of the peptide (Figure 22B), the highest positive peak was observed at ~ 190 nm followed by a crossover at ~ 200 nm and a minimum of ~ 210 nm, in both water and SDS sample. However, as apparent in figure 1B, there are differences between the peptide in each solvent, as greater positive and negative peaks are observed in 20 mM SDS.

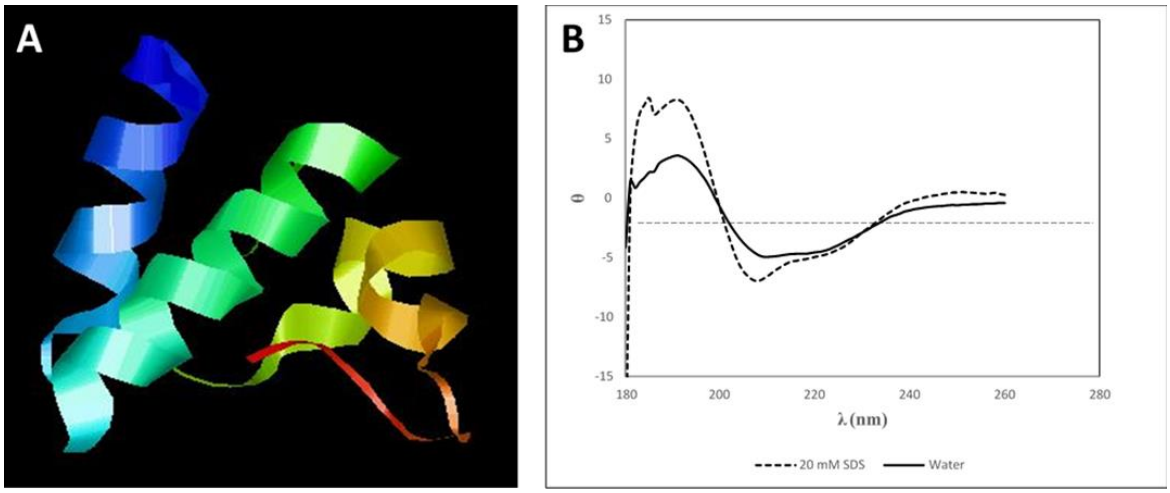

Figure 22: (A) RasMol 3D model of SN-1's secondary structure demonstrating the α-helical and β-sheet structural domains (B) CD spectroscopy graph illustrating the secondary structure profiles of the peptide in water (smooth line) and 20 mM SDS (dotted line). The highest positive peak obtained in the SDS solution of the peptide at ~190nm and two negative bands at ~200 and ~220nm are representative of its α-helical content, while the positive peak at ~190nm and negative peak at ~210 represent its β-sheet structural conformation.

6.4.2 Anti-yeast assay

The minimum inhibitory concentration of SN-1 was determined for all 5 yeast. Inhibition was observed against *Z. bailii* and *D. hansenii*, with MIC ranges of 50 to

100 µg/mL and 200 to 400 µg/mL, respectively. The peptide was found to be fungicidal against *Z. bailii*, at the highest concentration, while only fungistatic against *D. hansenii*, even at the highest concentration (Table 16).

Table 16: Ranges of minimum inhibitory concentrations of SN-1 against *Zygosaccharomyces bailli* Sa1403, *Zygosaccharomyces rouxii* ATCC14679, *Saccharomyces cerevisiae* Baker's yeast, *Kluyveromyces lactis* ATCC56498 and *Debaryomyces hansenii* CBS2334.

Yeast strain	Minimum inhibitory concentration (MIC)
Zygosaccharomyces bailli Sa1403	MIC range of 100 to 200 µg/mL – Fungicidal
Zygosaccharomyces rouxii ATCC14679	No inhibition
Saccharomyces cerevisiae Baker's yeast	No inhibition
Kluyveromyces lactis ATCC56498	No inhibition
Debaryomyces hansenii CBS2334	MIC range of 200 to 400 µg/mL – Fungistatic

6.4.3 Colony Count Assay

Observing the time course of inhibition in the presence of the peptide, at higher peptide concentrations (200 and 400 µg/mL) a rapid decrease in cell numbers was observed, after only 2 h incubation. This decrease was significantly delayed (between 4 and 5 h) at the lower peptide concentration of 100 µg/mL. In the presence of 50 µg/mL, the yeast is somewhat effected, but growth was observed to increase over time. The control with no peptide showed a steady increase in yeast growth, reaching $3.75x10^5$ cfu/mL after 6 h of incubation (Figure 23).

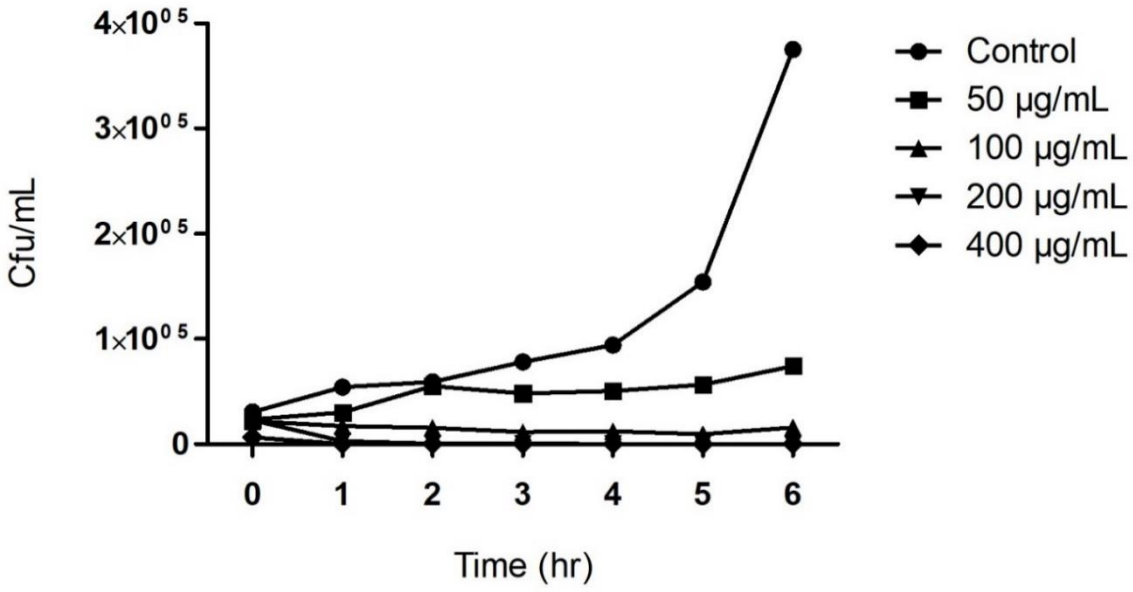

Figure 23: Colony count assay demonstrating the rate of *Z. bailii* inhibition by SN-1 at different concentrations ranging from 50 to 400 µg/mL

6.4.4 Peptide Stability

In the presence of different salts, the peptide was found to be sensitive to the higher concentrations of both $MgCl_2$ (5 mM) and KCl (150 mM), as no inhibition was observed, even at 400 µg/mL. In 1 mM $MgCl_2$ and 50 mM KCl, the peptide was resistant to these salt concentrations at its highest peptide concentration tested (400 µg/mL) with complete inhibition of *Z. bailii* being observed (Figure 24).

The pH change of the media caused a reduction in the peptide's anti-yeast activity at pH 7 as no inhibition was observed at any concentration of the peptide tested. pH 3 and 5 resulted in no change in the peptide's activity, as full inhibition was detected at 400, 200 and 100 µg/mL. The control, consisting of yeast alone in the absence of peptide showed that growth was completely inhibited at pH 9 and 11 (Table 17).

Heat treatment of SN-1 at 100 °C for 15 min resulted in no change in its anti-yeast activity, as all peptide concentrations tested fully inhibited *Z. bailii* growth.

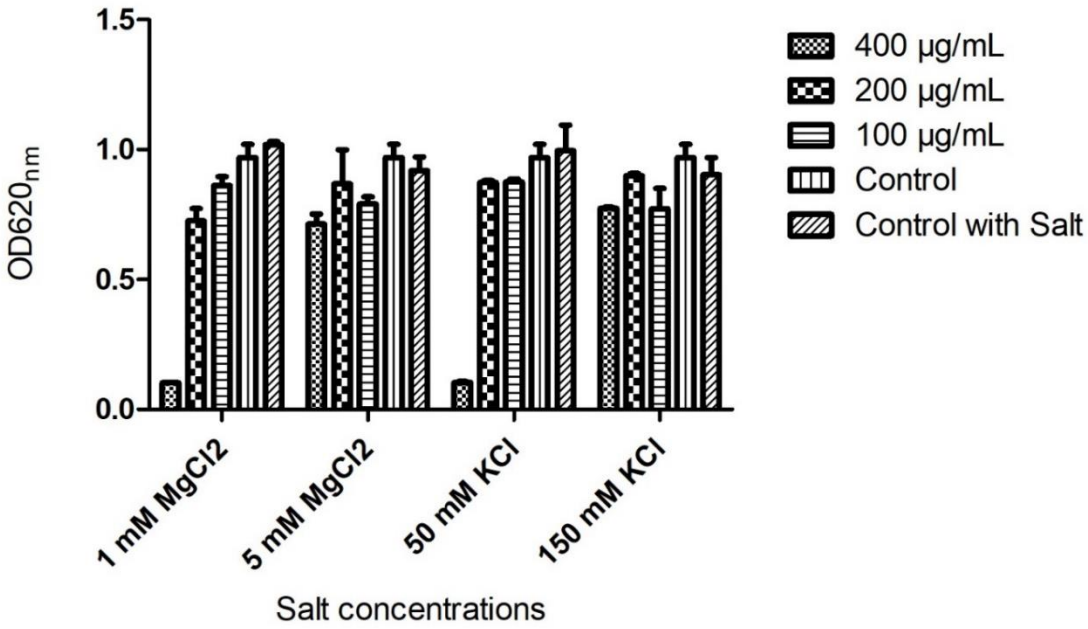

Figure 24: Stability of SN-1 in $MgCl_2$ and KCl at different salt concentrations.

Table 17: The effect of pH 3, 5, 7, 9 and 11 on SN-1's anti-yeast activity.

	SN-1 activity	**Control yeast**
pH 3	Full Inhibition	Full yeast growth
pH 5	Full Inhibition	Full yeast growth
pH 7	No inhibition	Full yeast growth
ph 9	Full Inhibition	No yeast growth
pH 11	Full Inhibition	No yeast growth

6.4.5 Total Nucleotide Leakage

This assay was carried out in order to observe the effect that the peptide has on the integrity of the yeast membrane by analysing the total amount of nucleotide displaced from the yeast cells. The total nucleotide leakage was measured after incubating *Z. bailii* with the peptide for 4 h. The measured OD at 260 nm showed a positive

correlation with peptide concentration (Figure 25). The highest concentration of peptide tested (400µg/mL) produced a considerably higher OD of 0.135 compared to the negative control of yeast alone (0.002). The OD measured with 200 and 100 µg/mL peptide was 0.064 and 0.027, respectively.

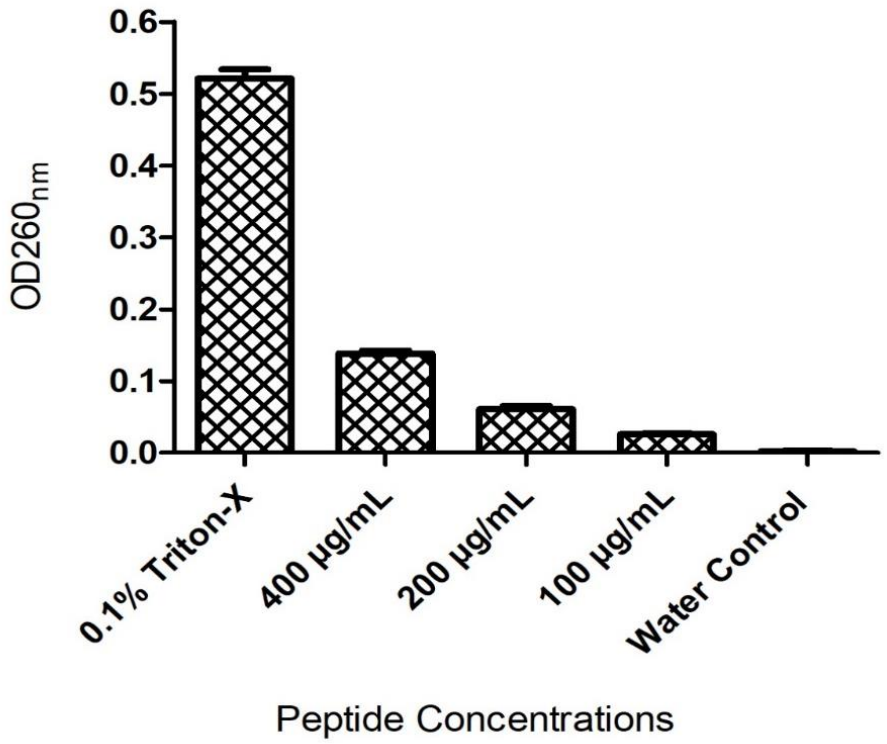

Figure 25: Measure of the total nucleotide leakage caused by the presence of SN-1 at different concentrations ranging from 100 to 400 µg/mL. Nucleotide leakage is observed to increase with increasing peptide concentration.

6.4.6 Membrane Permeabilisation

SN-1's mechanism of action was studied by observing its ability to cause yeast membrane permabilisation. The peptide's potential to cause permeabilisation can be detected by the ability of the PI dye to enter the cells through the damaged membrane. The peptide displayed high permabilisation activity against the membrane of *Z. bailii* at 400 µg/mL (Figure 26A). The level of permeabilisation was lower at reduced concentrations of peptide (Figure 26B and 26C).

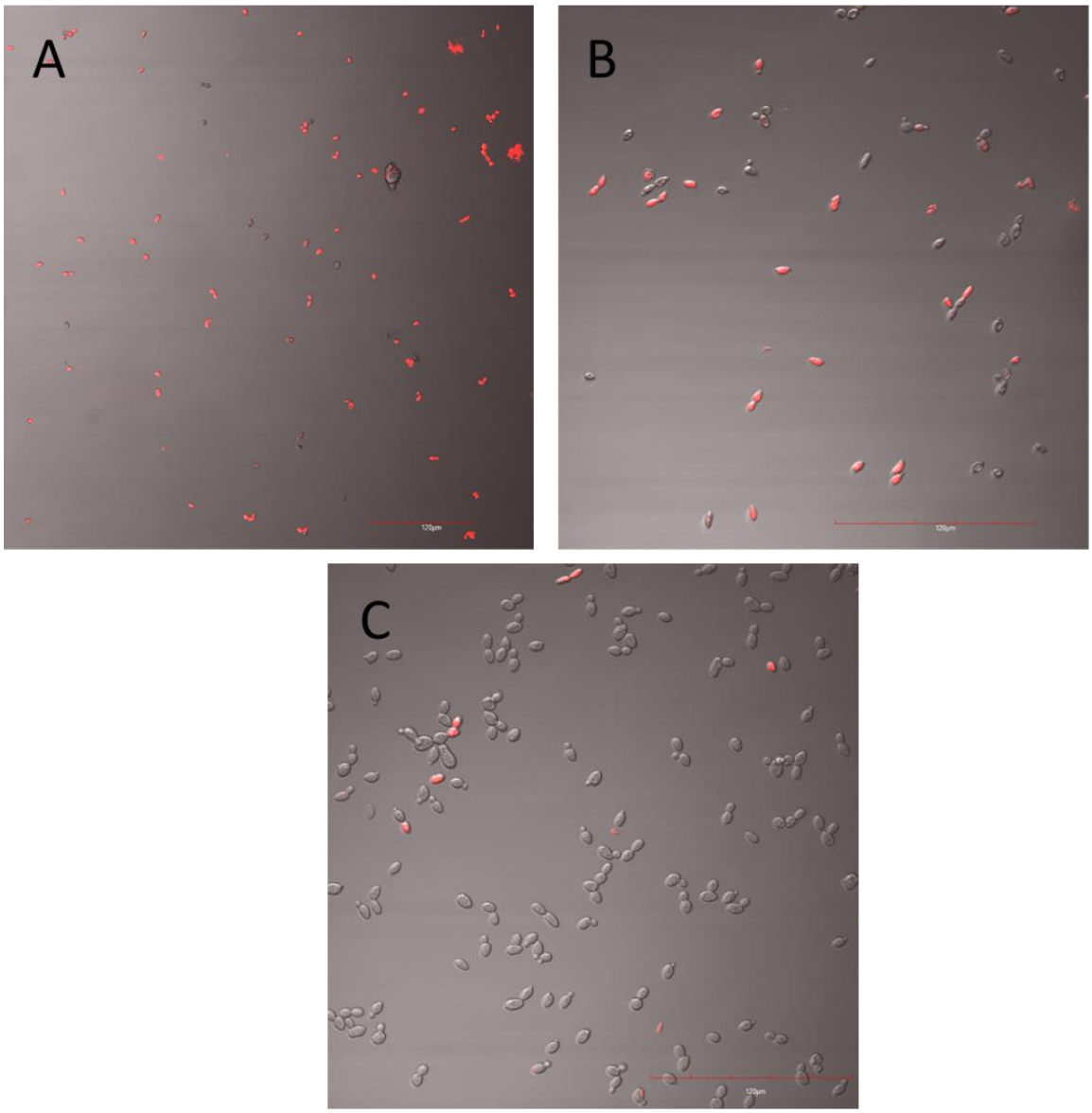

Figure 26: CLSM images showing the impact of SN-1 on *Z. bailii* cell membrane at 400 µg/mL (A), 200 µg/mL (B) and 100 µg/mL (C). Increasing rate of permeabilisation is observed with increasing peptide concentration.

6.4.7 Peptide's haemolytic activity against erythrocytes

This assay was carried out in order to observe the peptide's potential to rupture red blood cells and therefore characterise its safety in terms of human consumption. The haemolytic activity of SN-1 against erythrocytes was determined at different concentrations of the peptide (Figure 27). At the highest concentration of 400 µg/mL, the percentage of haemolysis was observed to be approximately 80%, while at 200 and 100 µg/mL (double the MIC and the MIC, respectively), haemolysis was significantly reduced, at less than 10%.

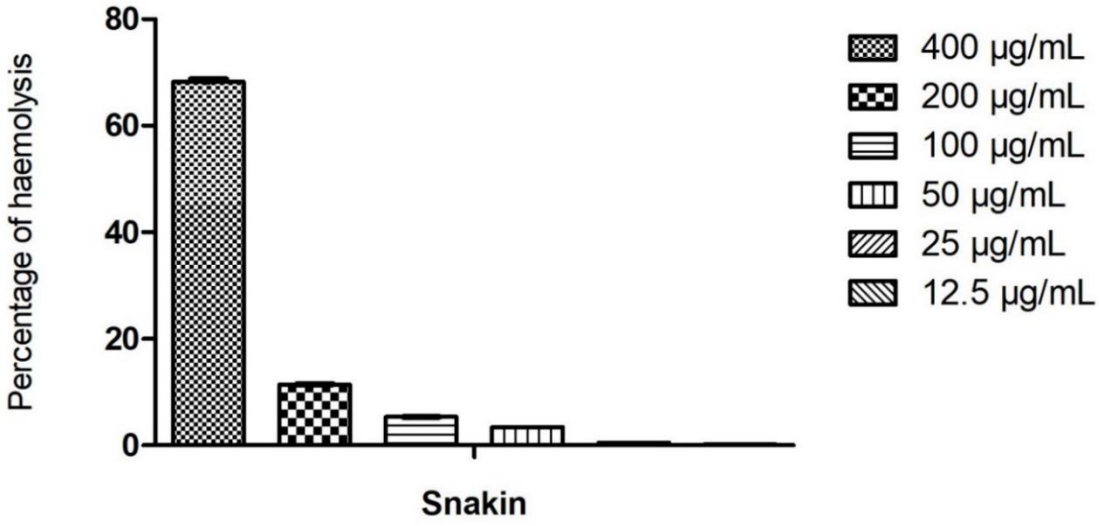

Figure 27: Percentage of haemolysis on sheep erythrocytes caused by SN-1 at concentrations from 12.5 to 400µg/mL.

6.4.8 Peptide's resistance to proteolytic digestion by α-chymotrypsin

The peptide's potential use as a preservative agent was tested by looking at its ability to be digested by a common proteolytic enzyme found in the gut. This trait allows it

to be considered a safe agent in terms of human consumption. The peptide was found to resist proteolysis by α-chymotrypsin at the enzyme's lowest concentration (peptide: enzyme molar ratio of 2500: 1). At this low concentration of α-chymotrypsin, SN-1 was able to cause inhibition against *Z. bailii* at 400 µg/mL but not at its MIC (100 µg/mL) and double its MIC (200 µg/mL). At the higher concentrations of the enzyme, the peptide was fully degraded as, even at the highest concentration of 400 µg/mL, no inhibition of the yeast was observed.

6.4.9 Application of SN-1 in beverages

The peptide's inhibitory activity against *Z. bailii* in different beverages was examined, with a view to potential applicability for the preservation of such products. Fanta Orange, cranberry juice and apple juice were tested. In both the Fanta Orange and cranberry juice, a complete inhibition of *Z. bailii* was observed at 100, 200 and 400 µg/mL, while in the apple juice, inhibition was only detected at 200 and 400 µg/mL.

6.5 Discussion

The interest for antimicrobial peptides as potential novel food preservatives has risen in recent years (Tiwari *et al.*, 2009; da Silva Malheiros, Daroit and Brandelli, 2010; Rai *et al.*, 2016; Shwaiki *et al.*, 2019; Thery *et al.*, 2019). According to the Food and Agriculture Organization of the United Nations (FAO), nearly 1.3 billion tonnes of food is wasted or thrown out each year because of microbial spoilage, which leads to major economic losses (Saucier, 2016). Chemical synthesis of peptides based on their natural amino acid sequence, offers potential for the development of a novel preservative agent to fight against food spoilage. This research examined the application of the antimicrobial peptide, Snakin-1 (SN-1), originating from potato tubers, for its inhibitory effect against some common food spoilage yeasts, and its potential safety for human consumption. SN-1's origin makes it an interesting candidate as it is naturally produced in one of the most widely grown crops in the world (Horton and Anderson, 1992) and potatoes are a staple food crop in many countries (Camire, Kubow and Donnelly, 2009). Thus, this peptide is both highly available and acceptable to consumers. A previous study by Segura *et al.*, 1999 successfully characterised the peptide as both antibacterial and antifungal against known potato plant pathogens.

In this study the secondary structure of the peptide was analysed using CD spectroscopy (Figure 22A). The peptide's structure was investigated in both purified water and 20 mM SDS – as a membrane mimicking model. The spectra obtained in water versus SDS shows differences in the size and profile of the peaks. The difference in the SDS and the water appears to be representative of a transition from β–sheet to a greater proportion of α-helical structure. The profile of both spectras indicate good, structured conformers which may be due to the presence of 6 disulphide bonds that

stabilise the peptide (Schmidt, Arendt and Thery, 2019). In addition, the low peaks of the graphs can be deciphered as helical structures, which is mirrored by the predicted RasMol 3D model of Snakin-1 (Figure 21B). The presence of the various disulphide bonds ensures a structured, stable conformation, meaning that the peptide conformation in both water and SDS are similar.

In this study, SN-1 inhibited two of the yeast tested, namely *Z. bailii* and *D. hansenii*, with a MIC ranging between 50 to 100 µg/mL and 200 to 400 µg/mL, respectively. The peptide's highly conserved cysteine residues, high net charge of + 9, and its basic nature could explain the observed anti-yeast activity. The antimicrobial characteristic of plant peptides has been linked to the presence of cysteine residues and their overall high net charge (Hong, Park and Lee, 2001; SD, 2014; Chen *et al.*, 2018). The SN-1 sequence contains a high number of lysine (Lys) residues (11), a positively charged amino acid, which increases the peptides' antimicrobial activity. The increase in cationicity allows for a better interaction with the negatively charged yeast cell, permitting the insertion of the peptide into, and disruption of, the yeast membrane leading to the eventual death of the cell (Haag *et al.*, 2012; Herbel and Wink, 2016).

A peptide's intended use in food products as a preservative requires that it has be stable under different environmental conditions, such as temperature, salt concentrations and pH. Heat exposure of the peptide (100 °C for 15 min) resulted in no loss of activity, as total inhibitory activity against *Z. bailii* was maintained. Previous studies on the thermostability of AMPs have shown similar findings (Hajji *et al.*, 2010; Ebbensgaard *et al.*, 2015; Dong *et al.*, 2018; Thery *et al.*, 2019). SN-1's stability in the presence of salts was performed in accordance with different studies that have looked at this characterisation in other AMPs (Kandasamy and Larson, 2006; Wu *et al.*, 2008; Thery *et al.*, 2019). The higher salt concentrations of $MgCl_2$ and KCl (5 and 50 mM,

respectively) caused a reduction in the peptide's anti-yeast activity, while lower concentrations had no effect. This reduced anti-yeast activity could be due to the presence of the cations causing a reduction in the net charge of the negatively charged yeast cells, thus leading to the repulsion of the peptide from the cell wall (Herbel and Wink, 2016). Another hypothesis could be the presence of the cations on the yeast membrane potentially modifying the peptide's overall charge and, as a consequence, its structure. This structural change could have caused observed reduced anti-yeast activity in the presence of salts (Baldauf *et al.*, 2013). The peptide's sensitivity to a neutral pH (pH 7), as observed from the peptide's reduced anti-yeast activity, could be explained by a modification of its net charge that can occur at neutral or basic pH, thus leading to a reduced interaction with the yeast membrane. Cationic AMPs similar to SN-1 have also been shown to be more positively charged below pH 7, possibly explaining this change in the peptide's activity (Walkenhorst *et al.*, 2013). Table 18 illustrates the effect that different pH have on the peptide's net charge, with an increase observed in more acidic conditions (pH 3 and 5), compared to the basic pH (9 and 11).

Table 18: The net charge of SN-1 at the different ranges of pH that were tested during the stability assay. A reduction in the peptides' net charge is observed above neutral pH and in more basic conditions. (Data obtained using an online peptide net charge calculator).

	pH 3	**pH 5**	**pH 7**	**pH 9**	**pH 11**
SN-1 net charge	15.3	10.18	9.0	-3.8	-18.3

The peptide's mechanism of action was explored through an examination of its ability to cause membrane permeabilisation of *Z. bailii* as one such mechanism proposed for many AMPs has been via such permeabilisation capacity (Shai, 1999; Pieters, Arnusch and Breukink, 2009; Pérez-Peinado *et al.*, 2018; Yang *et al.*, 2018; Yasir, Dutta and Willcox, 2019). SN-1's ability to permeabilise *Z. bailii*'s cell membrane was detected

at the highest concentration (400 µg/mL) with a reduced level of permeabilisation observed with decreasing peptide concentrations. Again, it is postulated that the peptide's high cationic nature allowed it to interact with the negatively charged yeast membrane and cause permeabilisation (Kumar, Kizhakkedathu and Straus, 2018). Measurement of the total nucleotide leakage was performed to further reinforce that membrane permeabilisation and damage was caused by the peptide. The assay showed an increase in the OD measured, proportional to the peptide concentration, indicating nucleotide leakage due to disruption of the yeast membrane. Previous studies that have looked at the release of bacterial cytoplasmic components by AMP showed similar findings (Hou *et al.*, 2007; Li *et al.*, 2016). For its application as a preservative in food, the safety of the peptide must be analysed to determine its suitability for human consumption. The haemolytic assay to determine the peptide's ability to rupture red blood cells, representative of the peptides effect on human cells, revealed less than 10% haemolysis at concentrations of 200 µg/mL and below; this is a positive attribute, suggesting that the peptide would be safe if consumed. Another aspect of safety is the ability of the peptide to be degraded in the human gastrointestinal tract. In this study, SN-1's sensitivity to the proteolytic digestive enzyme α-chymotrypsin was examined; the peptide was sensitive to degradation at all but the highest applied concentration (400 ug/mL) again indicating its safety for human consumption.

As a proof of concept, the application of the peptide in different beverages revealed that all beverage matrices were suitable for such an application of SN-1.

In conclusion, the synthetic peptide SN-1, based on the natural plant antimicrobial peptide, Snakin-1, represents a potential novel food preservative. The approach that taken in this study illustrates a possible solution for the development for more natural, consumer-acceptable preservatives. The chemical synthesis of peptides, as

demonstrated here, while currently being potentially economically prohibitive for translation to an industrial scale, must be considered against the time, cost, materials and processes for extraction and purification of such AMPs from natural plant material on such a scale. Although chemical synthesis may not be economically feasible currently, this study provides a proof of concept for the application of synthetic AMPs as novel food preservatives.

6.6 Acknowledgements

This work was supported by the Department of Agriculture, Food and the Marine (F.I.R.M.), Project Reference 15/F/731. A special thank you to Mr. Michael Cronin for his support with the CD spectroscopy, Dr. Thibaut Thery for his ideas on the topic and Ms. Lilit Ispiryan for her help with the confocal laser scanning microscope.

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Chapter 7

Inhibitory effect of four novel synthetic peptides on food spoilage yeasts

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Published in International Journal of Food Microbiology as:

Shwaiki, L. N. *et al.* (2019) 'Inhibitory effect of four novel synthetic peptides on food spoilage yeasts', *International Journal of Food Microbiology*. Elsevier, 300, pp. 43–52. doi: 10.1016/j.ijfoodmicro.2019.04.005.

7.1 Abstract

The spoilage of foods caused by the growth of undesirable yeast species is a problem in the food industry. Yeast species such as *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*, *Kluyveromyces lactis* and *Saccharomyces cerevisiae* have been encountered in foods such as high sugar products, fruit juices, wine, mayonnaise, chocolate and soft drinks. The demand for new methods of preservations have increased because of the negative association attached to chemical preservatives. The sequence of a novel short peptide (KKFFRAWWAPRFLK-NH2) was modified to generate three versions of this original peptide. These peptides were tested for the inhibition of the yeasts mentioned above, allowing for the better understanding of their residue modifications. The range of the minimum inhibitory concentration was between 25 and 200 µg/mL. *Zygosaccharomyces bailii* was the most sensitive strain to the peptides, while *Zygosaccharomyces rouxii* was the most resistant. Membrane permeabilisation was found to be responsible for yeast inhibition at a level which was a two-fold increase of the MIC (400 µg/mL). The possibility of the production of reactive oxygen species was also assessed but was not recognised as a factor involved for the peptides' mode of action. Their stability in different environments were also tested, focusing on high salt, pH and thermal stability. The newly designed peptides showed good antifungal activity against some common food spoilage yeasts and has been proven effective in the application in Fanta Orange. These efficient novel peptides represent a new source of food preservation that can be used as an alternative for current controversial preservatives used in the food industry.

7.2 Introduction

Yeasts are a diverse group of microorganisms that have been exploited for the production of foods and beverages for centuries. In the food industry, yeasts are an essential tool for the manufacture of bread, beer and wine, amongst other products (Gil-Rodríguez, Carrascosa and Requena, 2015; Hittinger, Steele and Ryder, 2018). Despite their positive impact in the food and beverage sector, the negative impact of undesirable spoilage yeasts is a significant problem in food production and continue to cause unnecessary waste leading to major economic losses (Fleet, 2006). A target of spoilage yeasts includes dairy products (Ledenbach and Marshall, 2009), mayonnaises, salad dressings (Kurtzman, Rogers and Hesseltine, 1971), fruit juices (Tournas, Heeres and Burgess, 2006), soft drinks (Wareing and Davenport, 2005), alcoholic beverages (Jespersen and Jakobsen, 1996), and even chocolate (Ho, Zhao and Fleet, 2014). The most common spoilage yeasts found in food belong to the genera *Zygosaccharomyces*, *Pichia*, *Debaryomyces*, *Kluyveromyces*, *Candida* and *Saccharomyces* (Loureiro and Malfeito-Ferreira, 2003). *Zygosaccharomyces* species can be categorised based on their high salt and sugar tolerance and their ability to cause spoilage in high sugar and salted foods. They tend to grow slowly and produce off-flavours and carbon dioxide (Sharma and Sharma, 2017). *Debaryomyces* species are the most common yeasts found in salad dressings and can grow in foods with salt concentrations as high as 20% (Breuer and Harms, 2006). *Kluyveromyces* spp., such as *Kluyveromyces lactis* and *Kluyveromyces marxianus* can hydrolyse fats and proteins and ferment lactose present in milk. This characteristic enables them to survive and grow in dairy products, in which they are common spoilage organisms (Valderrama *et al.*, 1999). Although *Saccharomyces* species represent one of the most important and applied microorganisms in the production of fermented foods, such as

bread and wine, some species can cause food spoilage. The spoilage of wine by S*accharomyces cerevisiae* is a common occurrence which is caused by the yeasts' tolerance to the high ethanol concentrations present in wine (Martorell, Querol and Fernández-Espinar, 2005; Rawat, 2015). In order to prevent the growth of such spoilage yeast, several methods of preservation have been adopted by food industries, ranging from chemical to physical preservation techniques (Gould, 1996). However, spoilage by yeast still occurs.

The popularity of chemical preservatives has been decreasing due to consumers' demand for more natural foods which contain less chemicals. The exploitation and application of antimicrobial peptides (AMP) derived from animal, plant or microbial sources has great potential for addressing this desire for more natural methods of food preservation (Rai *et al.*, 2016). AMPs are small proteins composed of 12 to 100 amino acid residues and are amphiphilic and generally positively charged; they are ubiquitous in animals, plants, insects and microorganisms (Jenssen, Hamill and Hancock, 2006; Mahlapuu *et al.*, 2016). Exploiting natural AMPs to prevent the growth of spoilage microorganisms can be expensive and also difficult to apply in foods due to the fact that any type of peptide used for food application must be safe for ingestion. Designing novel, synthetic peptides, based on natural, native peptides, can be an alternative option for the application of peptides in foods (Mohamed, Abdelkhalek and Seleem, 2016). The aim of this study was to design a number of novel synthetic peptides and investigate their inhibitory activity against yeast commonly found to cause food spoilage, namely, *Zygosaccharomyces* spp., (*Z. bailii* Sa 1403 and *Z. rouxii* ATCC 14679), *K. lactis* ATCC 56498, *D. hansenii* CBS 2334 and *S. cerevisiae* Baker's yeast. The synthetic peptide derivatives were designed through the modification of one original peptide sequence (KK-14). This peptide

displayed a broad spectrum of activity against fungi, consisting of a short sequence, which can be advantageous for future reproduction at a low cost of synthesis. The design of KK-14 and its derivatives were based on sequence frameworks of peptides with previously demonstrated activity against fungi. They were based on the designs of potent antifungal peptides **Indolicidin** (ILPWKWPWWPWRR) (a 13-mer Trp-rich peptide with a hydrophobic core), **LTX-315** (KKWWKKDipK) (a 9-mer cationic peptide with an unnatural β diphenylalanine residue), and **V13KL** (Ac-KWKSFLKTFKSAVKTVLHTALKAISS-amide) (a 26-residue amphipathic α-helical peptide) (Thery *et al.*, 2019). Thus, the attributes primarily hypothesised to be responsible for their antifungal activity were translated to, and exploited in the creation of this novel, original synthetic peptide and its derivatives. A hydrophobic centre, containing the hydrophobic residues Alanine (Ala), Tryptophan (Trp) and Proline (Pro) was chosen for the basis of this original peptide. In order to avoid a high cost of synthesis, the size of the peptide was limited to a short sequence of 14 amino acid residues. C-terminal amidation was incorporated in order to increase the peptides overall positive charge. Construction of a peptide with high haemolytic activity was avoided through the Lysine (Lys)/Arginine (Arg) and Trp/Phenylalanine (Phe) amino acids substitutions. The haemolytic activity is based on the measure of the peptides' ability to lyse erythrocytes (Ebbensgaard *et al.*, 2018). Previous studies have shown Arg/Phe rich peptides to contain low haemolytic activity against red blood cells (Strøm, Rekdal and Svendsen, 2002; Wessolowski, Bienert and Dathe, 2004; Ebbensgaard *et al.*, 2018).

These substitutions also enabled the generation of a peptide with antimicrobial activity. To be able to predict the antimicrobial potential of the peptide, an online calculator in conjunction with Support Vector Machine (SVM) methods were used.

The length of the peptide was also taken into consideration while constructing it; previous study has showed the potent properties of antifungal peptides to be linked to the length of the peptide, with shorter sequences resulting in better antifungal activity This original design was then used to generate 3 derivative peptides by modifying its sequence or structure; namely, substituting the Pro residue with an Arg, producing a D-enantiomer of the original peptide, and, through the insertion of an unnatural residue. In addition to investigating the inhibitory activity of the 4 peptides against the chosen spoilage yeasts, through the generation of minimum inhibitory concentration (MIC) values, the mode or mechanism of action and stability of the peptides was also examined. Finally, their application in two different food matrices was assessed.

7.3 Material and Methods

7.3.1 Synthetic peptides used in this study

The starting peptide (KK-14) was assembled in conjunction with sequences of numerous other antifungal peptides shown to be effective. This peptide was used to generate 3 others by modifying its structure and were synthetized by GL Biochem (Shangai) Ldt. The first modified peptide, termed Dip KK-14, was constructed by the addition of an unnatural ß-diphenylalanine residue as a substitution for Trp at position 8 (Trp8). The peptide termed, dKK-14, was generated by constructing a D-enantiomer of the KK-14 peptide, while the peptide, KK-14 (R10), was constructed via the replacement of the arginine residue with that of a proline. The sequences of the peptides are presented in Table 19. The purity of these peptides was >90% as indicated by the supplier. Each peptide was resuspended in sterile distilled water and at a concentration of 2 mg/mL.

Table 19: Amino acid sequences of the four synthetic peptides applied in this study. The residues in bold and underlined represent the modifications to the original peptide.

Peptide	**Amino Acid Sequence**	
Original Synthetic Peptide (KK14)	KKFFRAWWAPRFLK-NH2	Lys-Lys-Phe-Phe-Arg-Ala-Trp-Trp-Ala-Pro-Arg-Phe-Leu-Lys-NH_2
Dip KK-14 Peptide	KKFFRAW**<u>Dip</u>**APRFLK-NH2	Lys-Lys-Phe-Phe-Arg-Ala-Trp-**<u>Dip</u>**-Ala-Pro-Arg-Phe-Leu-Lys-NH_2
dKK-14 Peptide	***(D-enantiomer)*** KKFFRAWWAPRFLK-NH2	Lys-Lys-Phe-Phe-Arg-Ala-Trp-Trp-Ala-Pro-Arg-Phe-Leu-Lys-NH_2

KK-14 (R10) Peptide	KKFFRAWWA**R**RFLK-NH2	Lys-Lys-Phe-Phe-Arg-Ala-Trp-Trp-Ala-**Arg**-Arg-Phe-Leu-Lys-NH_2

7.3.2 Yeast strains

Five different strains of yeast were used in this study. *Kluyveromyces lactis* ATCC 56498, *Zygosaccharomyces bailli* Sa 1403, *Zygosaccharomyces rouxii* ATCC 14679, *Debaryomyces hansenii* CBS 2334 were obtained from DMSZ (Germany). *Saccharomyces cerevisiae* Baker's yeast was obtained from Puratos (Belgium). Each yeast was grown aerobically in Yeast Extract-Peptone-Dextrose (YPD; Sigma-Aldrich) agar at 25°C. Stock cultures were maintained at -80°C. Overnight incubation was performed in YPD broth at 25°C under gentle agitation. All media and reagents used in this study were obtained from Sigma-Aldrich (MO, USA), unless otherwise stated.

7.3.3 Antifungal activity by disc diffusion assay

The susceptibility of the yeast strains to each peptide was demonstrated using the disc diffusion assay as outlined by Cai *et al.,* 2016. In addition to the broth microdilution method, the disc diffusion assay can also be used to determine the yeast susceptibility to antimicrobial agents; however, some variability in the results is expected. Overnight cultures of each yeast were used to prepare a cell suspension of $5x10^6$ CFU/mL. Cells were centrifuged at 1000 *g* for 5 min and washed 3 times in sterile distilled water. A $1x10^5$ cell suspension (1 mL) was added to 5 mL of 1% molten agarose (Serva,

Heidelberg, Germany). This molten mixture was applied to YPD agar plates and allowed to set. Sterile paper discs, 6 mm in diameter were placed on the solidified agar and 50µl of peptide was applied to the disc at a concentration of 1) the MIC (as determined in the broth microdilution assay) and 2) two-fold the MIC, for each peptide. The plates were then incubated at 25°C for 3 days. Following incubation, the halo around the discs, representing the zone of inhibition, was measured in cm.

7.3.4 Antifungal assays and determination of Minimum Inhibitory Concentration

For each yeast strain, the MIC for each peptide was determined using a broth microdilution method outlined by the National Committee for Clinical Laboratory Standards (NCCLS M-27A, NCCLS 2002). Suspensions of the yeast strains were prepared from overnight cultures using yeast minimal media (YMM) [per 1 L; 0.8 g Complete Supplement Mixture (Formedium, Norfolk, United Kingdom) 6.5 g Yeast Nitrogen Base without amino acids (FORMEDIUM) and 10 g glucose] and adjusted to $1x10^5$ CFU/mL using a Marienfeld-Superior Haemocytometer. Two hundred and ninety microliters of this suspension was added to the first well of a flat-bottomed 96-well microtitre plate (Sarstedt, Nümbrecht, Germany). Peptide (10 µl) was added into the first well of the plate at a concentration of 400 µg/mL and serially diluted to the next wells (containing 200 µL of yeast) to yield five different concentrations of each peptide (12.5 to 200 µg/mL). Water, instead of peptide, was used as a control. The individual challenge experiment (yeast versus peptide) was performed on 2 different plates and in duplicate on each plate. Plates were incubated at 25 °C for 48 h with measurement of the optical density (620nm) at 2 h intervals under gentle agitation (Multiskan FC Microplate Photometer, Thermo Scientific, MA, USA). The MIC was

determined as the minimum concentration of peptide needed to inhibit the growth of the yeast. The peptides' ability to cause complete inhibition on the yeast was determined by subsequent spotting of 100 µl of yeast/peptide suspension from the microtitre plate onto YPD agar. This enabled the determination of whether a peptide was fungistatic or fungicidal. The minimum fungicidal concentration (MFC) was also determined as the lowest peptide concentration that showed either no growth or less than three colonies (per 100 µL) on the YPD agar plates. Yeast colonies were counted after 48- or 72-hours incubation, depending on the optimal incubation time of the yeast being tested; *S. cerevisiae, Z. bailii and D. hansanii* were incubated for 48 hours while *K. lactis* and *Z. rouxii* were incubated for 72 hours.

7.3.5 pH, salt and thermal stability of peptides

The stability of each peptide under varying pH and salt conditions was determined by examining the effect on its inhibitory activity against *Z. bailii*. *Z. bailii* was chosen for these stability assays because it was found to be the most sensitive to the peptides in the broth microdilution assay. Experiments were performed as described above using YMM adjusted to different pHs, i.e., 3, 5, 7, 9 and 11. The media was adjusted using 1M sodium hydroxide and 0.1 M hydrochloric acid to increase and decrease the pH accordingly. Media of different pHs without any added peptide was used as a control. The peptides were tested at their MIC, double and half the MIC.

The effect of two salts, $MgCl_2$ and KCl at two different concentrations in YMM (1mM and 5mM, and 50mM and 150mM, respectively) were examined. The peptides were assessed at the concentrations up the MIC (0 to 25µg/mL).

The resistance of the peptides to heat treatment, at 100°C for 15 min, was also evaluated, at the concentration up the MIC (0 to 25µg/mL).

7.3.6 Examination of membrane permeabilisation

Study of the peptides' mode of inhibitory action against the yeast, was examined by determining their potential to cause yeast cell membrane permeabilisation. Membrane permeabilisation is becoming increasingly recognised as one mechanism by which antimicrobial peptides target both yeast and both Gram positive and Gram negative bacterial cells (Lyu *et al.*, 2016; Dias *et al.*, 2017; Pérez-Peinado *et al.*, 2018). This was tested against *S. cerevisiae*, a yeast frequently used in membrane permeabilisation studies of anti-yeast agents (Diz *et al.*, 2006; Mayan, 2010; Cools *et al.*, 2017). A yeast cell suspension of 10^6 CFU/mL was prepared from overnight cultures. Peptide (10 µl) was added into 90 µl of yeast cell suspension and incubated for 2 h at 25°C, after which 5µM of propidium iodide (PI) (SIGMA) was added. This solution was incubated at room temperature under dark conditions for 20 min, before being washed with YMM to remove unbound dye, centrifuged at 3,000 *g* for 5 min and applied to glass slides. Triton X-100 (0.1%) was used as the positive control as it is a known to cause membrane permeabilisation. PI was used for this assay since it can only penetrate cells with compromised plasma membranes and subsequently binds to nucleic acids. It will only fluoresce when bound, therefore enabling detection of whether membrane permeabilisation has occurred. The extent of permeabilisation was measured using a confocal laser scanning microscope (CLSM) (OLYMPUS FV3-259) at the maximal excitation (λ_{Ex}) and maximum emission (λ_{Em}) wavelengths of 535nm and 617nm, respectively.

The kinetics of permeabilisation was performed as described above with the exception of adding the dye and peptide simultaneously. The fluorescence was measured, as above, every 10 min for up to 6 h. Fluorescence was corrected by subtracting the optical density of the YMM.

7.3.7 Examination of Reactive Oxygen Species generation

Overproduction of Reactive Oxygen Species (ROS) by yeast can be attributed, amongst other reasons, to the presence of an antimicrobial agent. To investigate whether the peptides act by this mechanism, an indicator for the generation of ROS was applied in an antifungal assay. This indicator was dihydrorhodamine 123 (Sigma-Aldrich), an uncharged ROS indicator that can pass across cell membranes and oxidize to the cationic rhodamine 123 (a green fluorescent compound) after cellular uptake (Djiadeu *et al.*, 2017). *S. cerevisiae* was used as the indicator yeast and the procedure was performed in accordance with the method established by (Hayes *et al.*, 2013). Dihydrorhodamine 123 (5 µg/mL) was added to a 10^6 cfu/mL yeast suspension for 2 h at 28°C. The cells were washed with YMM followed by centrifugation at 3000 *g* for 5 min and 10 µl of peptide (50 to 400 µg/mL) was subsequently added, maintaining the same temperature. The cells were then washed with 0.6 M potassium chloride and collected by centrifugation. A positive control of hydrogen peroxide (H_2O_2) was used at a concentration of 2 mM. Yeast cells were observed by CLSM. Fluorescence was measured at the maximal excitation (λ_{Ex}) and maximum emission (λ_{Em}) wavelengths of 488nmand 538nm, respectively.

7.3.8 Application of peptides in food matrices

The antifungal effect of the peptides was investigated in two food matrices (i.e., a soft drink and mayonnaise) in which fungal spoilage can occur, in particular with *Z. bailii*, which was therefore used for this assay. For the soft drink (Fanta Orange, Coca-Cola, Ireland) a microtitre plate method, as applied for the antifungal assay was used. The yeast was inoculated in YPD for overnight culture and a 10^2 cfu/mL solution was prepared in filter sterilised Fanta Orange from this overnight culture. This low concentration of yeast was used to represent the number of cells commonly found to spoil products such as soda drinks. All four peptides were tested using the same conditions and concentrations as performed for the microdilution plate assay (minimum of 12.5 µg/mL and maximum 200 µg/mL). The optical density (620 nm) was monitored over 48 h at 25°C. Controls consisted of Fanta Orange with 10^2 cfu/mL yeast in the absence of peptide, and Fanta Orange with no yeast. The ingredients of the Fanta Orange used for this application, as listed on the label, were as follows: carbonated water, sugar, orange fruit from concentrate (5%), citric acid, vegetable concentrates (carrot and pumpkin), natural orange flavouring with other natural flavourings, preservative (potassium sorbate), malic acid, acidity regulator (sodium citrate), sweeteners (acesulfame-K, aspartame), antioxidant (ascorbic acid), and stabilizer (guar gum). It also contained a source of phenylalanine. The pH of the Fanta Orange was recorded as 3.1; a pH lower than what was found for the YMM (pH 5.33).

The application of the peptides in mayonnaise (Hellman's, Unilever, United States) was also tested. The ingredients of the mayonnaise, as listed on the label, was as follows: Canola Oil, Water, Liquid Whole Egg, Vinegar, Liquid Yolk, Salt, Sugar, Spices, Concentrated Lemon Juice and Calcium Disodium EDTA. Overnight cultures of *Z. bailii* were added to mayonnaise at 10^2 cfu/mL in addition to peptide (12.5 to 200

µg/mL). This yeast, peptide and mayonnaise solution (100 µl) was spread onto YPD agar and incubated at 25°C for 48 h.

7.3.9 Statistics

Values are reported as mean ± standard deviation. To determine the significant difference between the results obtained for the salt stability assay, analysis of variance with ANOVA (SigmaStat, SPSS Inc., Chicago, USA), was performed. In all cases, a probability of $p < 0.05$ was considered statistically significant.

7.4 Results

7.4.1 Antifungal microtiter plate assay

The antifungal activity of the peptides was tested through incubation with the yeast in a microtitre plate. The growth of the yeast was observed over time to detect their antifungal properties. Each of the four peptides showed variation in the degree of their anti-yeast activity. Apart from *Z. rouxii,* each yeast strain was found to be sensitive to each of the peptides at one or more of the tested concentrations (Table 20). The most sensitive yeast was *Z. bailii*, displaying the lowest MIC, at 50 µg/mL, while the most active peptide was Dip KK-14 against all 5 yeast strains. The fungistatic or fungicidal ability of the peptides on the yeast varied and was dependant on the strain and peptide. The peptide with the highest fungicidal activity and completely inhibiting most yeast at its MIC was Dip KK-14. While KK-14 (R10) also displayed broad fungicidal activity at its MIC, this MIC was higher than that of Dip KK-14.

The Minimal Fungicidal Concentration (MFC) of the peptides which displayed only fungistatic activity at their MIC was found to be as high as 800 µg/mL. *Z. rouxii* and *S. cerevisiae* were the least susceptible to the fungistatic properties of Dip KK-14 and dKK-14, respectively. Table 21 shows a summary of the MFC of the peptides tested.

Table 20: MIC values of the peptides against the yeast strains. The nature of the inhibitory activity – fungistatic or fungicidal – is also shown.

	Kluyveromyces lactis ATCC 56498	*Zygosaccharomyces bailli* Sa 1403	*Zygosaccharomyces rouxii* ATCC 14679	*Debaryomyces hansanii* CBS 2334	*Saccharomyces cerevisiae Baker's yeast*
KK-14	100 µg/mL – **Fungicidal**	50 µg/mL – **Fungistatic**	No Inhibition	50 µg/mL - **Fungicidal**	200 µg/mL - **Fungicidal**
Dip KK-14	25 µg/mL – **Fungicidal**	25 µg/mL – **Fungicidal**	200 µg/mL - **Fungistatic**	50 µg/mL - **Fungicidal**	50 µg/mL - **Fungicidal**
dKK-14	100 µg/mL – **Fungistatic**	50 µg/mL – **Fungicidal**	No Inhibition	25 µg/mL - **Fungicidal**	200 µg/mL - **Fungistatic**
KK-14 (R10)	200 µg/mL – **Fungicidal**	50 µg/mL – **Fungicidal**	No Inhibition	100 µg/mL - **Fungicidal**	200 µg/mL - **Fungicidal**

Table 21: MFC values of the peptides shown to have fungistatic properties against the different yeast strains. Maximum concentration tested was 800 µg/mL.

Yeast strain	**Peptide**	**MFC (µg/mL)**
Z. bailii Sa 1403	KK-14	400
K. lactis ATCC 56498	dKK-14	400
S. cerevisiae - Baker's yeast	dKK-14	800
Z. rouxii ATCC 14679	Dip KK-14	800

7.4.2 Antifungal disc diffusion assay

The susceptibility of *Z. bailii*, a yeast already found be sensitive towards the peptides, was tested using a disc diffusion assay. Round sterile discs impregnated with each peptide at different concentrations was placed onto YPD plates inoculated with *Z. bailii*. The zones of inhibition (Table 22) against the respective peptide were evaluated. KK-14, Dip KK-14 and dKK-14 all showed zones of inhibition at

concentrations of 50 and 25 µg/mL. KK-14 (R10) had a halo around the disc containing 50 µg/mL peptide but not 25 µg/mL. As expected, all four peptides did not inhibit the yeasts at 12.5 µg/mL. 50 µg/mL of Dip KK-14 was not as prominent on the plate, even though this concentration of peptide was two-fold the MIC determined in the microdilution broth assay (Figure 28).

Table 22: *Z. bailii* Sa 1403 susceptibility in the disc diffusion assay. The table represents the degree of inhibition based on the measurement of the diameter of the zone of inhibition surrounding the discs. +++ = susceptible (16mm to 14mm); ++ and + = intermediate (14mm to 10mm); - = resistant (no zone of inhibition).

Concentration of peptide impregnated into disc	**KK-14**	**Dip KK-14**	**dKK-14**	**KK-14 (R10)**
12.5 µg/mL	-	-	-	-
25 µg/mL	+	+	+	-
50 µg/mL	+++	++	+++	+++

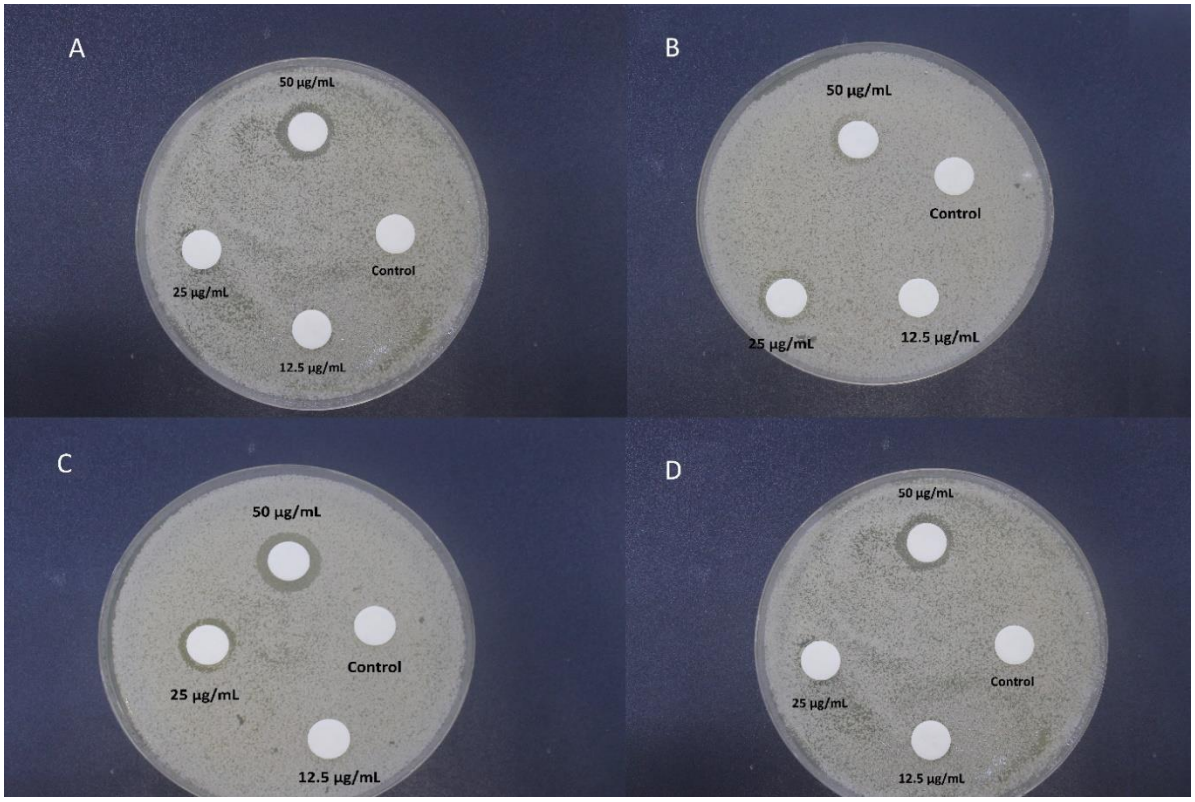

Figure 28: Results of the disc diffusion assay against *Z. bailii* Sa1403. Each peptide was tested at 50, 25 and 12.5 µg/mL. KK-14 (A), Dip KK-14 (B), dKK-14 (C) and KK-14 (R10) (D) can be seen to show varying degrees of inhibition.

7.4.3 Salt stability of the peptides

The antifungal assay was repeated with the addition of different salt concentrations ($MgCl_2$ and KCl) to test the stability of the each of the peptides (Figure 29). The presence of $MgCl_2$ at both concentrations tested (1 mM and 5mM) decreased the antifungal activity of KK-14 enabling *Z. bailii* to grow at the MIC (50 µg/mL). The higher concentration of 5 mM was more effective at disrupting the activity of the peptide, allowing growth to occur at double the MIC (i.e., 100 µg/mL). dKK-14 was more effected at the higher concentration of $MgCl_2$, with *Z. bailii* growing even at the MIC (50 µg/mL). KK-14 (R10) was least effected by $MgCl_2$, as observed by the inhibition of growth at its MIC, 50 µg/mL. Dip KK-14 were not affected by 1 mM

$MgCl_2$, as it still displayed inhibitory activity at its MIC (50ug/mL); however, 5 mM $MgCl_2$ affected the peptide to some extent, as growth was observed at the MIC.

The presence of KK-14 in 50 and 150 mM KCl resulted in its antifungal activity being lessened, both at its MIC and double that MIC (Figure 30). dKK-14 was more affected by the higher concentration of KCl in comparison to the lower concentration, with *Z. bailii* growing at both its MIC and double the MIC. The higher KCl concentration decreased the antifungal activity of KK-14 (R10), enabled the growth of *Z. bailii* at 50 and 100 µg/mL. The lower concentration of KCl (50 mM) was less effective at disrupting the antifungal activity. The MIC of Dip KK-14 was affected at both KCl concentrations, with 150 mM causing more disruption to the peptide's ability to inhibit *Z. bailii*.

After performing ANOVA, significant differences were observed between the peptide concentrations that were found to inhibit *Z. bailii* in the salt solutions, as can be seen from figures 29, 30 and 31.

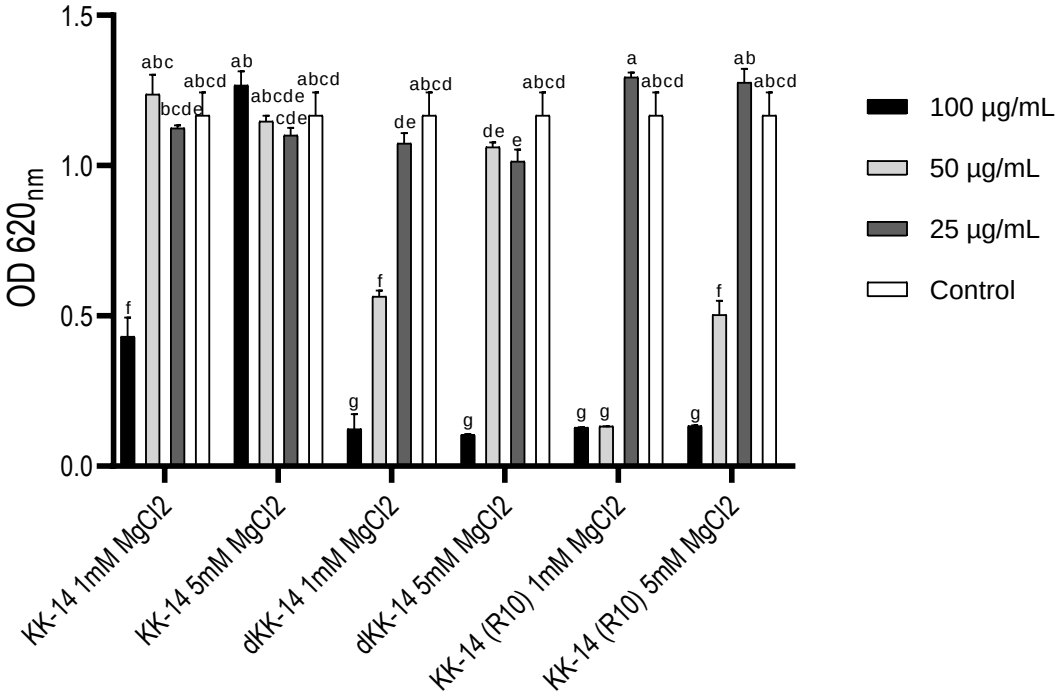

Figure 29: Effect of MgCl2 on the inhibitory activity of the peptides KK-14, dKK-14 and KK-14 (R10) against *Z. bailii* Sa1403, at concentrations of 1 and 5 mM. Means sharing the same letter do not differ significantly at the 95% confidence level based on the Tukey mean comparison method; $p < 0.05$. The bars containing the same letters are **not** significantly different while those allocated different letters were determined significantly different from one another. For example, the bars allocated "f" are all significantly similar to one another but are different to the bars allocated "g" or another letter. Bars allocated more than one letter indicates their similarity to more than one mean value.

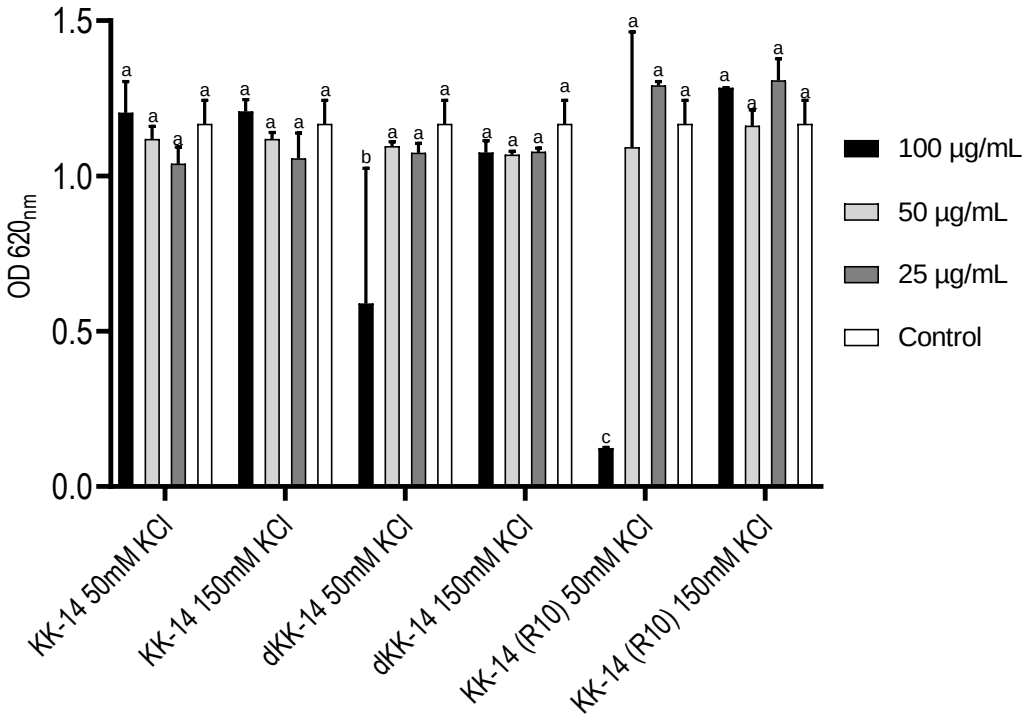

Figure 30: Effect of KCl on the inhibitory activity of the peptides KK-14, dKK-14 and KK-14 (R10) against *Z. bailii* Sa1403, at concentrations of 50 and 150 mM. Values with different letters vary statistically. Tukey Test; $p < 0.05$.

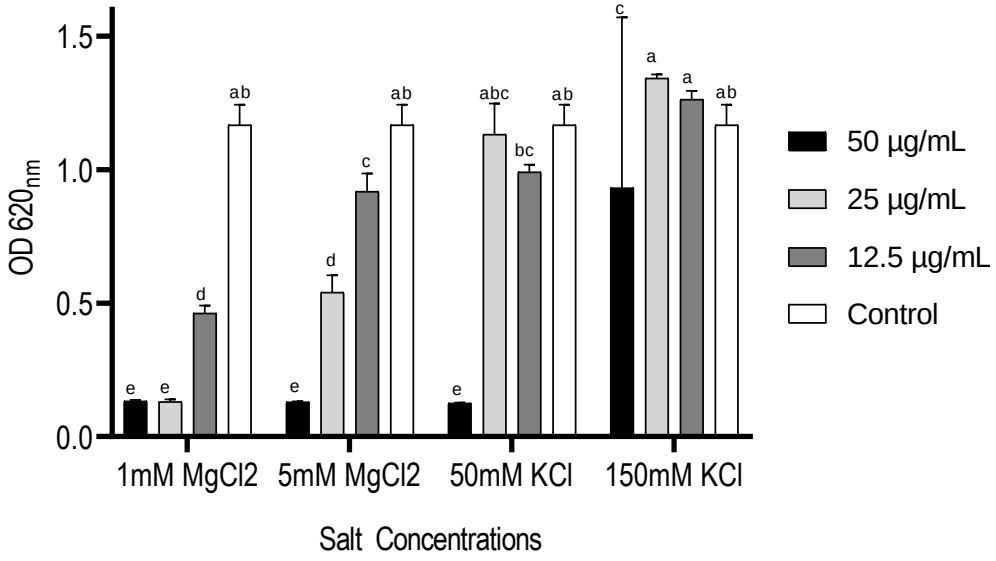

Figure 31: Effect of KCl and MgCl2 at concentrations of 50 and 150 mM, and 1 and 5 mM, respectively, on the inhibitory activity of Dip KK-14 against *Z. bailii* Sa1403. Values with different letters vary statistically. Tukey Test; $p < 0.05$.

7.4.4 Temperature stability of the peptides

High temperatures of 100°C were subjected onto the peptides and tested in an antifungal assay for their inhibitory effect against the yeast. Heating of KK-14 at 100°C for 15 min caused a reduction in the antifungal properties of the peptide, resulting in the increased growth of *Z. bailii* in the presence of this peptide. At both the MIC (50 µg/mL) and double the MIC (100 µg/mL), the peptide caused no inhibition of *Z. bailii* as was originally observed in the antifungal assay. dKK-14, KK-14 (R10) and Dip KK-14 were not affected by this increase in temperature, evident by the inhibition caused at the MIC. The inhibition of the yeast at all 3 concentrations (double the MIC, its MIC and half the MIC) was observed (Table 23).

Table 23: Effect of heat treatment (100°C for 15 min) on each peptide and the resulting antifungal activity against *Z. bailii* Sa 1403 post-heat treatment.

Concentration	**KK-14**	**dKK-14**	**Dip KK-14**	**KK-14 (R10)**
100 µg/mL	No inhibition	Inhibition	n.d.	Inhibition
50 µg/mL	No inhibition *[MIC]*	Inhibition *[MIC]*	Inhibition	Inhibition *[MIC]*
25 µg/mL	No inhibition	No inhibition	Inhibition *[MIC]*	No inhibition
12.5 µg/mL	n.d.	n.d.	No Inhibition	n.d.

7.4.5 Effect of pH on antifungal activity of the peptides

The antifungal assay was repeated using YMM changed to different pH to observe the inhibitory effect of each peptide under such pH stress (Table 24). Changing the pH of the media to pH 3 (control pH 5.33) resulted in a decrease in *Z. bailii* inhibition caused

by the four peptides. Increasing the pH to 9 and 11 resulted in complete inhibition of the yeast. The greatest effect caused by change in pH was seen in pH 5 and 7. KK-14 was affected at pH 5 with a decrease in its antifungal activity, thus enabling growth of *Z. bailii* at the MIC and even at double that MIC. At pH 7, inhibition was only observed at the higher peptide concentration tested (100 µg/mL, double the MIC), while growth was observed at its MIC. For the dKK-14 peptide, at pH 5, inhibition was only observed in double its MIC and not at its MIC, indicating a decrease in its antifungal activity. pH 7 did not affect the antifungal activity of dKK-14. An increase in antifungal activity was observed for Dip KK-14 at pH 5 and 7. KK-14 (R10) was more effective at pH 7, compared to the control (unadjusted) medium, where complete inhibition was observed at half the MIC. At pH 5, use of the MIC and double that resulted in a similar level of inhibition to that observed in the microtitre plate antifungal assay.

Table 24. The effect of different pH on the stability and antifungal activity of the four peptides against *Z. bailii* Sa 1403. Inhibition = No growth in the MIC and double the MIC; Complete Inhibition = No growth in all three concentrations tested; No inhibition = Growth in all three concentrations.

	pH 3	**pH 5**	**pH 7**	**pH 9**	**pH 11**
KK-14	No Inhibition	No Inhibition	Inhibition only at 100 µg/mL	Complete Inhibition	Complete Inhibition
dKK-14	No Inhibition	Inhibition only at 100 µg/mL	Inhibition	Complete Inhibition	Complete Inhibition
Dip KK-14	No Inhibition	Complete Inhibition	Complete Inhibition	Complete Inhibition	Complete Inhibition
KK-14 (R10)	No Inhibition	Inhibition	Complete Inhibition	Complete Inhibition	Complete Inhibition

7.4.6 Membrane permeabilisation activity

S. cerevisiae was incubated with the different concentrations of peptide found to be inhibitory and viewed under the CLSM to observe the mode of action of the peptides. Membrane permeabilisation of *S. cerevisiae* was assessed in the presence of the peptides. The yeast showed strong fluorescence when treated with 400 µg/mL of the peptides, Dip KK-14, dKK-14 and KK-14 (R10). KK-14, however did not affect the cell membrane at 400 µg/mL (Figure 32). The degree of permeabilisation decreased as the concentration of peptides decreased, in a dose-dependent manner. The lowest concentration for all four peptides (25 µg/mL) showed almost no permeability. The original synthetic peptide, KK-14, in general, caused less permeabilisation of *S. cerevisiae* (Figures for KK-14, Dip KK-14 and dKK-14 not shown).

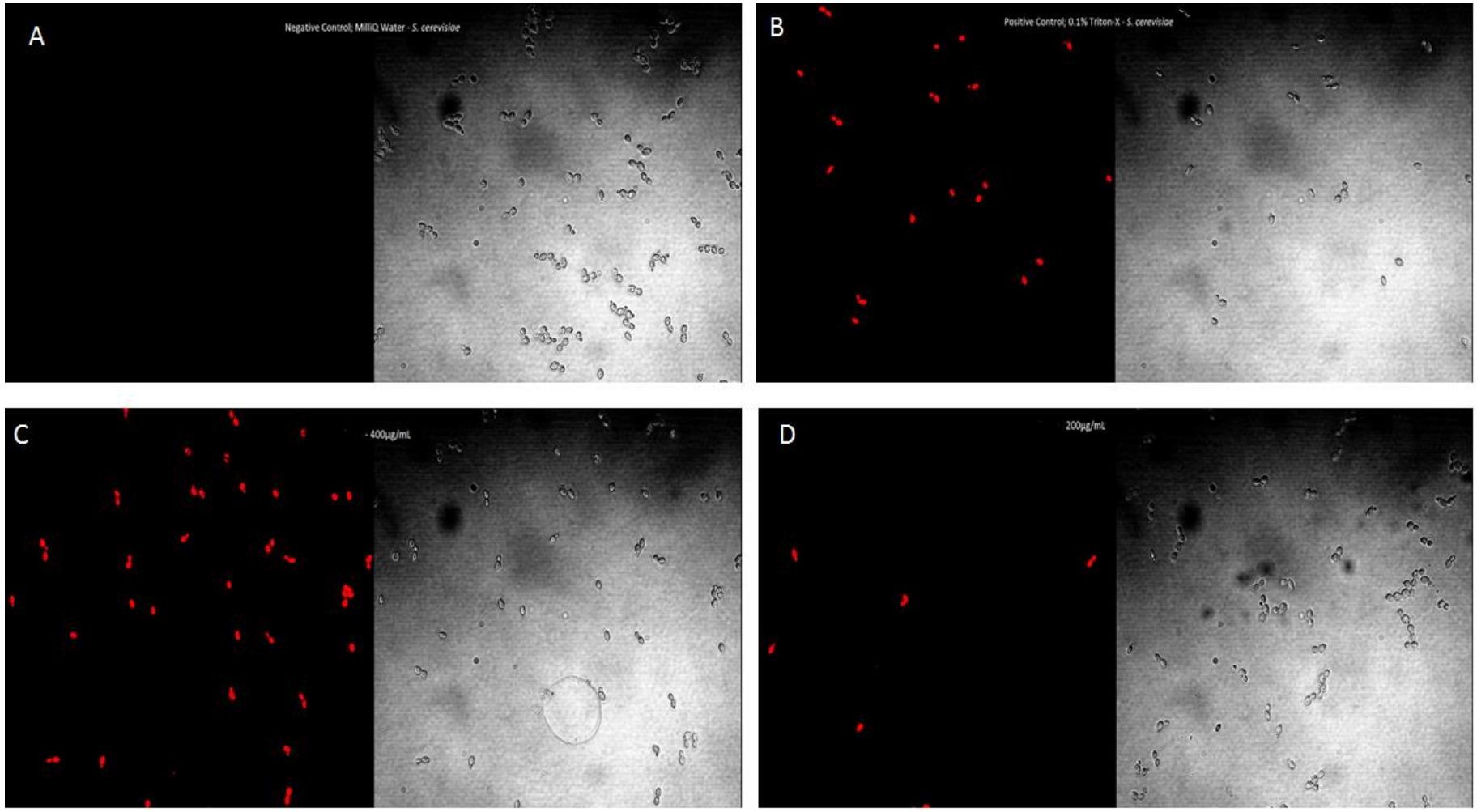

Figure 32: Results of the permabilisation effects of KK-14 (R10) on *S. cerevisiae* – Baker's yeast. A, B, C and D show the negative control, positive control, 400 µg/mL and 200 µg/mL peptide, respectively.

7.4.7 Kinetics of membrane permeabilisation

Each peptide was incubated with *S. cerevisiae* and propidium iodide in a microtitre to follow the progress of permeabilisation of the yeast in the presence of the peptides. Analysis of the kinetics of the membrane permeabilisation of the four peptides was performed with *S. cerevisiae.* For KK-14 (R10) and dKK-14, the kinetics revealed faster permeabilisation over 6 hrs. As predicted, both peptides resulted in a steady increase in the measured fluorescence over the period of 6 hrs, indicating the occurrence of membrane permabilisation (Figure 33). KK-14 (R10) at the concentration of 400, 200 and 100 µg/mL all showed permeabilisation while only 400 and 200 µg/mL of dKK-14 caused observable permeabilisation (Figure 34). Significantly lower permeability was observed after 6 hours with KK-14 and Dip KK-14 (data not shown).

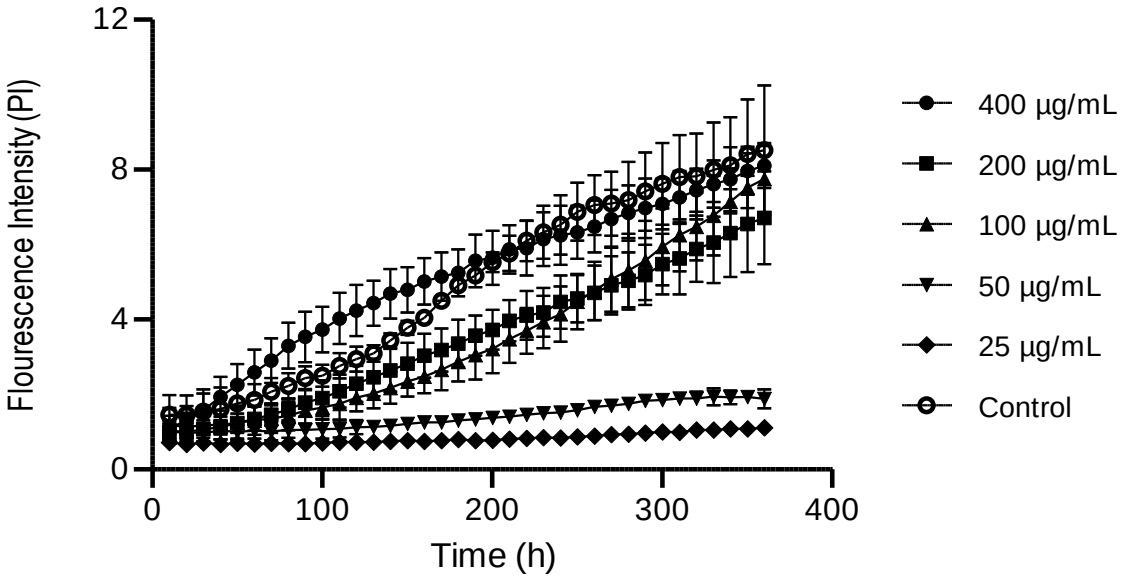

Figure 33: Kinetics indicating the rate of membrane permeabilisation of *S. cerevisiae* – Baker's yeast in the presence of KK-14 (R10) at different concentrations (25 – 400 µg/mL). A positive control consisting of *S. cerevisiae* in the presence of 0.1% Triton-X was also included to demonstrate a positive rate of membrane permeabilisation.

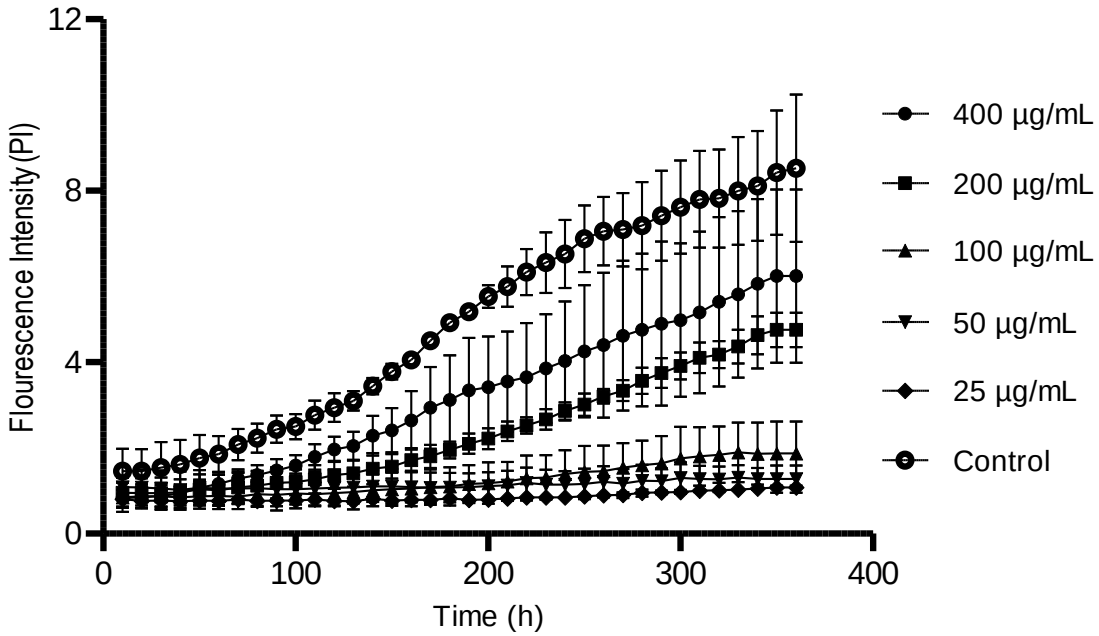

Figure 34: Kinetics indicating the rate of membrane permeabilisation of *S. cerevisiae* – Baker's yeast in the presence of dKK-14 at different concentrations (25 – 400 µg/mL). A positive control consisting of *S. cerevisiae* in the presence of 0.1% Triton-X was also included to demonstrate a positive rate of membrane permeabilisation.

7.4.8 Stimulation of reactive oxygen species production

The yeast was incubated with the peptides and dihydrorhodamine 123, a dye capable of detecting ROS, and viewed under the CLSM. Although *S. cerevisiae* treated with the peptides showed a degree of inhibition in the antifungal assays, no ROS production was observed in this assay; even at the highest concentration tested (400 µg/mL) for each of the four peptides. The positive control, consisting of H_2O_2, showed the production of ROS, in comparison to the negative control consisting of yeast alone in YMM (data not shown).

7.4.9 Application of peptides in a food matrix

The antifungal assay was repeated using Fanta Orange as the main medium for the yeast to grow. Investigation of the application and effect of the peptides in a soft drink (Fanta Orange) found that they caused complete inhibition of *Z. bailii*, inoculated at 10^2 cfu/mL, at all concentrations tested. Figure 35 shows the extent of inhibition that each peptide caused at 12.5 µg/mL.

None of the peptides caused inhibition of *Z. bailii* in mayonnaise. Growth was observed at each of the concentrations tested (12.5 to 200 µg/mL). The negative control consisting of mayonnaise alone without peptides showed growth.

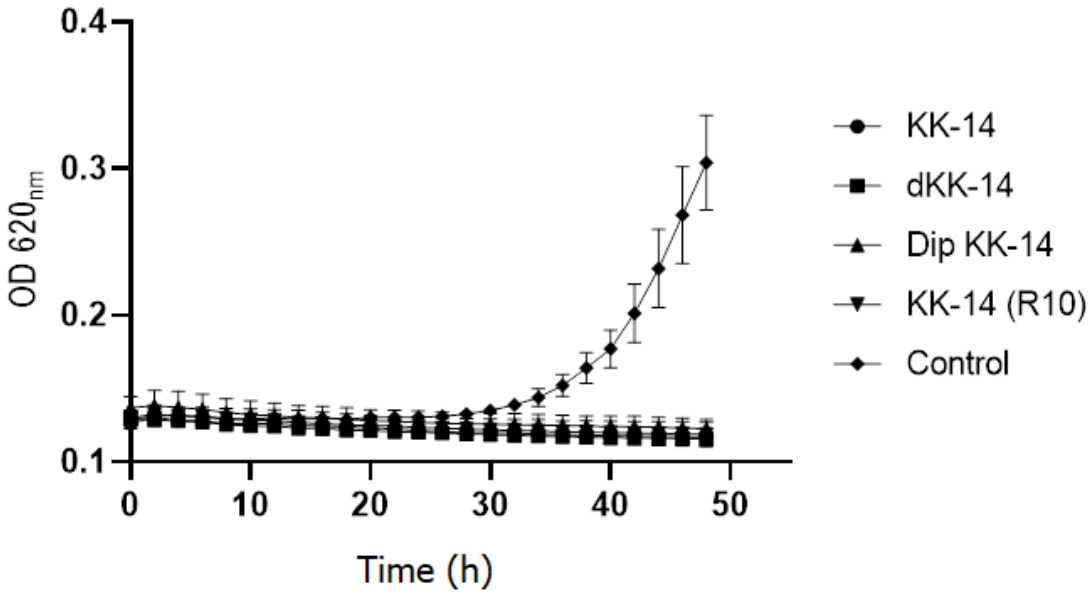

Figure 35: Antifungal assay of peptide KK-14, Dip KK-14, dKK-14 and KK-14 (R10) against *Z. bailii* Sa1403 in Fanta Orange at concentration of 12.5 µg/mL.

7.5 Discussion

The introduction of synthetic peptides in the food industry to combat food spoilage caused by yeasts represents a novel alternative for the replacement of chemical preservatives. This study deals with the production and modification of several peptides proven to be effective against yeast species commonly encountered in food spoilage.

The generation of a novel peptide sequence based on numerous antifungal peptides was modified to generate three derivative peptides. This was accomplished by altering specific residues in the original sequence. Their ability to cause inhibition of the yeast species tested was found to vary, being strain- and peptide-dependant. The original peptide, KK-14, inhibited 4 of the 5 yeasts, with *Z. rouxii* showing complete resistance even at 200 μg/mL. *K. lactis*, *Z. bailii*, *D. hansenii* and S. *cerevisiae* showed inhibition at concentrations of 100, 50, 50 and 200 μg/ mL, respectively. The incorporation of Ala, Trp, Pro, Phe and Leucine (Leu) in the primary sequence served to impart hydrophobicity on the peptide. In general, an increase in hydrophobicity promotes the spontaneous insertion of such peptides into cell membranes (Engelman and Steitz, 1981). In addition, the presence of two Lys residues on the N- and C-terminal of the sequence helped to provide the peptide a position on the yeast cell membrane into which it could better infiltrate (Stark *et al.*, 2002). It has previously been shown that peptides which displayed antimicrobial activity against Gram positive and Gram negative bacteria contained these residues which imparted a hydrophobic nature on these peptides, and likely contributed to their antimicrobial activity. Thus, the incorporation of such residues in novel peptides should promote antimicrobial activity,

making them suitable for the generation of novel AMPs. Dip KK-14 consisted of the same amino acid sequence as the original peptide with the exception of a β-di-phenylalanine (Dip) residue substituted for Trp10. β-di-phenylalanine is an amino acid with a similar structure to alanine and phenylalanine and has its amino group bonded to the β carbon (Cheng *et al.,* 1992). The antifungal activity of this peptide was found to be higher than KK-14 against all yeast may be attributed to the insertion of the Dip residue. A previous study showed that the presence of two Phe residues in a modified peptide sequence significantly improved the antimicrobial activity of the peptide by causing a greater disruption to bacterial cell membranes (Shahmiri *et al.,* 2015). In the present study, β-di-phenylalanine replaced a non-hydrophobic Trp, thus increasing the hydrophobicity of the peptide, which could explain the observed increase in antifungal activity. The addition of the β diphenylalanine as a modification of the original KK-14 peptide produced peptide with longer side chains, which could have promoted cell membrane penetration in comparison to a peptide with the Trp residue in that position. The use of a β- instead of an α-diphenylalanine was applied to increase the proteolytic resistance of the peptide, as it is less likely to be degraded in this form (Cabrele *et al.,* 2014).

The antimicrobial activity of the D-enantiomeric peptide showed similar inhibitory capacity to that of KK-14 against all yeasts, except *D. hansenii*. This decrease in yeast resistance of *D. hansenii* could be attributed to an increased ability of the D-enantiomer to cause membrane permeabilisation or it could potentially be due to this peptide's increased resistance to proteases secreted by this yeast (de la Fuente-Núñez *et al.,* 2015). It has been shown that the D-enantiomeric form of studied peptides not

only display an increase in antifungal activity compared to their original counterpart, but also demonstrate proteolytic resistance, including to human serum proteases (Jung *et al.,* 2007; Tugyi *et al.,* 2005). Another reason for the increase in activity against *D. hansenii* could be due to the backbone of the D-enantiomer; having different rotations in three-dimensional space may lead to its side chains having different topologies, therefore effecting its antimicrobial activity (Mohamed *et al.,* 2017).

The peptide with the substitution of its Pro amino acid for an Arginine (Arg), KK-14 (R10), was expected to display higher antimicrobial activity. While the same antimicrobial activity as the KK-14 peptide was attained against three of the yeasts, for *K. lactis* and *D. hansenii* a higher MIC was obtained than with the original prototype. This was unexpected since a substitution of Pro to Arg increases the net positive charge, which would be expected to increase the antifungal activity of the peptide. Previous studies on cationic peptides demonstrated improved antimicrobial activity through the production of arginine-substituted analogues (Muñoz *et al.,* 2007; Taniguchi *et al.,* 2016). This insertion of an extra Arg, in combination with successive Trp residues should have produced a peptide with a better interfacial affinity for lipid membranes, leading to increased inhibition (Fjell *et al.,* 2012). Cationic peptides consisting of Arg residues followed by Trp generated an antifungal peptide capable of inhibiting the growth of *Candida albicans* (Jin *et al.,* 2016). For those peptides that only showed fungistatic activity at their MIC, the concentration of peptide required to have a fungicidal effect was investigated further. At the concentration of 800 μg/mL, *S. cerevisiae* and *Z. rouxii* were completely inhibited by dKK-14 and Dip KK-14, respectively, in both the disc diffusion assay and microdilution broth assay. This

indicates that the minimum amount of these peptides needed to cause complete killing of the yeast was as high as 800 μg/mL. For KK-14 and dKK-14 against *Z. bailii* and *K. lactis*, respectively, 400 μg/mL was the minimum fungicidal concentration.

The peptides' mode of action towards *S. cerevisiae* was verified using propidium iodide (PI) as the indicator for the detection of compromised plasma membranes. PI is a fluorescent molecule that can bind to DNA intracellularly (Crowley *et al.*, 2016). Membrane permeabilisation towards *S. cerevisiae* was difficult to detect even at the highest concentration of peptide tested in the antifungal assays (200 μg/mL). A two-fold higher concentration of each peptide was therefore tested (400 μg/mL). At this dose, strong permeabilisation was detected for peptides Dip KK-14, dKK-14 and KK-14 (R10). Peptide KK-14, however, was found to be the least effective at causing permeabilisation. This assay confirmed that one possible mode of action of the peptides is via the permeabilisation of yeast membranes. The application of these peptides for use in human consumption can be assumed to be safe, with respect to the peptides' ability permeabilise mammalian membranes. The cationicity and amphipathicity of the peptides enable interaction with the yeast membrane, a negatively charged structure. The neutral nature of mammalian membranes renders them unaffected by these peptides (Bechinger and Gorr, 2017; Kumar *et al.*, 2018; Wimley, 2010). The kinetics of the membrane permeabilisation demonstrated that KK-14 (R10) caused permeabilisation at a faster rate than KK-14 and Dip KK-14. The higher helicity caused by the Arg residue, as well as the Trp/Arg succession in this peptide sequence could have resulted in the increased permeabilisation of the yeast (Deslouches *et al.*, 2016). Many well studied AMPs are recognised for their ability to

induce cell death via a number of different mechanisms. One of which is through the stimulation of ROS overproduction (Bondaryk *et al.*, 2017). This was not detected, even at the highest concentration of 400 μg/mL. This suggests that ROS production was not a factor in the antifungal activity of the peptides. Thus, membrane permeabilisation was the more likely mode of action of the peptides. Interactions between the peptides and the binding sites on the yeast's membrane known as sphingolipids, could have facilitated such membrane permeabilisation (Bondaryk *et al.*, 2017). The hydrophobicity of the peptides also contributed to this mode of action. It has been shown that the amino acid residues Phe, Trp, Leu and Ala, present in each the peptides, contain hydrophobicities above a threshold value (a value obtained from the calculated indices of a peptide's amino acid residues and which determines the peptide's hydrophobicity and thus its ability to integrate into cell membranes) enabling them to spontaneously insert into membranes (Liu *et al.*, 1996; Stark *et al.*, 2002). Thevissen *et al.*, 1999 demonstrated that the primary cause of growth inhibition by plant defensins is through binding-site mediated insertion.

The stability of the peptides was tested under high salt concentrations, the concentrations of which were tested being based on previous research (Betts *et al.*, 1999; Kandasamy and Larson, 2006; Wu *et al.*, 2008). Both salts showed a similar negative effect on the antifungal activity, particularly at the higher concentration; however, the modified peptides were affected to a lesser extent than the original KK-14 peptide. There was a statistical difference in the peptides' inability to inhibit *Z. bailii* under the influence of the different salt concentrations, in particular for $MgCl_2$. The presence of cations in the medium may be a factor that could have prevented the

peptides from interacting with the cell membrane of the yeast, a significant step required for optimal effect. It has been shown that at a higher salt concentration, peptides are less likely to interact with the cell membrane. In addition, the presence of salts may have altered the peptides' overall charge causing a modification to their structure (Baldauf *et al.,* 2013). This interference could be due to a reduction of the available head group area for the lipids, which leads to their tighter packing, making it more difficult for the peptides to penetrate and cause effect (Wu *et al.,* 2008). The sensitivity of these peptides to the monovalent cation K+, in the form of KCl, reduced their antifungal activity at high salt concentration. Testing the peptides against the salts enabled for the better understanding of the peptides' response to a high salt matrix, an environment commonly found in food spoiled by such yeast (Rawat, 2015).

Subjecting KK-14 to a heat treatment caused a decrease in peptide activity against *Z. bailii*. Heating could have caused irreversible changes to its secondary or tertiary structure. This loss of structure may have consequently caused a reduction in its antifungal activity (Ozkan *et al.,* 2016). The stability of dKK-14, KK-14 (R10) and Dip KK-14 was unaffected, indicating their stability in high temperature. One potential reason for this could be the lack of tertiary structure and the flexibility of the peptide. It has been shown that the greater the flexibility of some cationic peptides, the more enhanced the antimicrobial effects (Liu *et al.,* 2013). Adjusting the medium to pH 3 resulted in no inhibition of *Z. bailii* growth in the presence of any peptide. This could be due to the ability of yeast to thrive in acidic environments. *Z. bailii* is a yeast notorious for its resistance to acidic, low pH, environmental stresses (Palma *et al.,* 2017). In addition, significantly changing the pH of the medium could have

modified the charge on the peptides, leading to the alteration in peptide folding. This could have then resulted in a decrease in their activity (Di Russo *et al.*, 2012). This change in peptide structure coupled with *Z. bailii*'s resistance to the low pH media may explain this complete lack of inhibition observed (Yang and Honig, 1993). At high pH, there was complete inhibition of growth; however, this was likely due to the unfavourably high pH being unsuitable for growth, rather than peptide activity (Dang *et al.,* 2010). Nonetheless, the peptides' net charge would have been altered at such basic pH conditions, undoubtedly resulting in the decrease of their antiyeast activity. Table 25 illustrates the effect that different pH have on the overall net charge of the peptides, with an increase observed in acidic conditions (pH 3 and 5), compared to the basic pH (9 and 11).

Table 25: The net charge of KK-14 and its derivatives at the different ranges of pH. A reduction in the peptides' net charge is detected above neutral pH and in more basic conditions. (Data obtained using an online peptide net charge calculator).

	pH 3	**pH 5**	**pH 7**	**pH 9**	**pH 11**
KK-14, Dip KK-14, dKK-14 net charge	5.5	5.01	4.9	3.6	1.0
KK-14 (R10)	6.5	6.0	5.9	4.6	1.9

Overall, compared to its derivatives, KK-14 was observed to be most affected by the addition of the salts, as well as to the changes of media pH to basic and acidic conditions. Additionally, the KK-14 peptide was not thermostable after subjecting it to 100°C for 15 minutes, resulting in the reduction of its antiyeast activity. Contrarily, the derivative peptides (Dip KK-14, dKK-14 &KK-14 (R10)) displayed good thermostability, as well as good stability in the media containing the $MgCl_2$. Adjusting

the media to the different ranges of pH also allowed the peptides to predominantly retain their inhibitory activity. These assays provide good indication of their stability in different conditions, as opposed to the original KK-14 peptide, making them more suited for the proposed application in food as potential preservatives.

In a separate study by Thery et al., 2019, KK-14 and its derivatives were found non-haemolytic at the concentrations relevant for their antiyeast activity. However, the authors also tested the peptides' cytotoxicity against Caco-2 cells, a human epithelial cell line, and observed a decrease in cell viability by the peptides KK-14, KK-14 (R10) and Dip KK-14 at the highest concentrations (100 and 200 µg/mL), and a consistent survival rate of the cells in the presence of dkk-14. Therefore, this cytotoxic activity can be seen as a limitation of use of these peptides. However, their ability to withstand the action of the digestive enzyme trypsin was also demonstrated in this study via a proteolytic digestion assay, revealing dKK-14 to be the only peptide that was not degraded by the enzyme. This attribute, although encouraged in the field of therapeutics, is a drawback for its application in food products. However, KK-14, Dip KK-14 and KK-14 (R10) were successfully degraded by the trypsin, revealing their safety in terms of their digestibility as potential food preservatives

Dip KK-14 was the most favourable peptide that could be considered in the application as an antifungal agent. The combination of its low MIC, good membrane permeability, and stability against most of the salt concentrations, make for a promising antifungal agent. Application of the peptides to two food group with which *Z. bailii* is commonly associated, enabled investigation of the peptides for real world applications. In the soft drink (Fanta Orange) the growth of *Z. bailii* was completely inhibited by all peptides

at all concentrations tested, even at the lowest concentration (12.5 μg/mL). This could indicate a potential synergistic effect between the peptides and a component of the soft drink, which may have enhanced their inhibitory effect. Indeed, growth was observed for the control, consisting of *Z. bailii* alone inoculated in the Fanta Orange, indicating that the presence of preservatives in the soft drink had no effect. Testing of the peptides against *Z. bailii* in mayonnaise resulted in no inhibition. This could be attributed to the viscosity of the mayonnaise, physically inhibiting the interaction between peptide and yeast. This highlights a limitation of use of these peptides when applied to such a viscous matrix, making them unsuitable for use in certain types of foods.

Although limitations may arise, the overall ability of each peptide to cause inhibition of yeast makes them an appealing substitute for chemical preservation of certain food types. This study highlights the potential for using peptide modification as a means of creating synthetic peptides suitable for application as anti-yeast agents in food. The cost of synthesis of such synthetic peptides may, however, currently be a limitation to their incorporation as food preservatives. An alternative to this challenge associated with synthetic peptides can be the incorporation of natural peptides extracted from plants.

7.6 Acknowledgements

This work was supported by the Department of Agriculture, Food and the Marine, Food Institutional Research Measure (F.I.R.M.), Project Reference 15/F/731

7.7 References

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Chapter 8

General Discussion

8.1 General Discussion

The rise in the global population over the years has forced an increase in food production worldwide. In order to continue to feed the expected growing population, a requirement to reduce the amount of food waste and food loss must be met. The waste and loss of food can be attributed in large part to the undesirable growth of spoilage microorganisms (Rawat, 2015). The occurrence of these microbial spoilers in food and agriculturally significant crops can have significant economic consequences to food industries worldwide (Bautista, 2014). The existing forms of food preservatives that are employed have been used to reduce the growth of these microorganisms (ranging from bacterial to fungal (mould and yeast) species) (Wedzicha, 2003). Despite effective preservation methods and agents developed within the last century, food industries are constantly aiming to lengthen the shelf life of products so that they last longer either on the shop shelve or the consumer's home. For this reason, there is a necessity to develop preservative agents with increased antimicrobial activity that can be applied to food products with the purpose of reducing and slowing the rate of food spoilage, subsequently with the aim to reduce food waste. In addition to this, consumer demand for less processed and more natural occurring food additives is driving the demand for alternative forms of food preservatives. In recent decades, there has been an increasing interest on the exploitation of plant compounds for their application to reduce food spoilage and increase the shelf life of food products due to their natural origin. Plant antimicrobial peptides (AMPs) are compounds produced by the innate immune system of plants that serve to protect against pathogenic microorganisms. These peptides have shown potential for their

application in causing inhibition and reducing the growth of numerous plant and food spoilage microorganisms. The overall aim of this thesis was to study the inhibitory potential of various plant AMPs and their ability to reduce the growth of food spoilage yeast. Therefore, two literature reviews have been compiled as part of this thesis to outline the potential application of plant AMPs in the aim of reducing spoilage in food and agriculturally important crops. Both natural **(chapter 2)** and synthetic **(chapter 3)** approaches of plant AMP production, for their application in reducing the growth of spoilage microorganisms and food spoilage are discussed.

The approach discussed in **chapter 2** highlights the potential exploitation of plant AMPs for their ability to reduce the growth and presence of spoilage microorganisms. A typical method of peptide purification is also reviewed here, together with the different steps of peptide extraction, isolation and purification being described. In most extraction processes, the plant of interest is extracted using a buffer of specific pH, and put through various purification processes, such as ion exchange chromatography and high-performance liquid chromatography (HPLC). These purification steps allow for a pure peptide solution to be attained and further tested for antimicrobial activity. There are many studies that emphasize the antimicrobial activity of AMPs purified from plant sources to combat pathogens associated with plant disease. The study by Cammue *et al.*, 1992 is a prime example of this strong activity against various fungal species. Two potent AMPs were purified from the *Mirabilis jalapa* (marvel of Peru) plant, both of which demonstrated inhibitory activity against 13 different fungal plant pathogens, including the common fungal pathogens *Fusarium culmorum* and *Alternaria brassicicola,* both known to cause serious disease

to cereal crops and various vegetables in the brassica family, respectively. This strong inhibitory activity has been observed from a number of AMPs purified from plants (Fujimura *et al.*, 2003, 2004; Diz *et al.*, 2006; Mandal *et al.*, 2009). Most studies that focus on purified plant AMPs focus on reducing the growth of bacterial and fungal pathogens typically associated with crop infections. Out of this research there is growing interest for the investigation of plant AMPs and their application in food products as novel preservatives to reduce spoilage, especially given the natural sources from which they are derived. This chapter also highlights the spectrum of antimicrobial activity of these purified plant AMPs, further emphasising the degree of potency that they possess. Throughout, the safety and the mechanism of action of AMPs are emphasised due to the central importance of these aspects when considering application of such novel compounds in food products. The safety of these peptides is commonly associated with their ability to target microbial cells instead of eukaryotic cells. The mechanism by which AMPs elicit their antimicrobial activity via the targeting cell membranes of microbial cells is well understood. Due to their cationic nature, these plant peptides target the negatively charged membranes of microbial cells, instead of the neutral/slightly positively charged mammalian cells (Bacalum and Radu, 2015). This mechanism, known as membrane permeabilisation, can take place via several different approaches (Figure 4). Due to this attribute, the mode of action of AMPs is typically associated with the selective inhibition of microbial cells. A different approach for the production of AMPs from the purification method described above is also introduced here. The synthetic development of peptides based on known plant peptide amino acid sequences is presented as an alternative to production via extraction and purification methods discussed in this chapter. Both the natural

extraction and the chemical synthesis of AMPs result in the production a peptide of interest. However, the approach of natural extraction can prove to be time consuming and can result in a low concentration and yield of peptide. During peptide chemical synthesis, the end-product yields a peptide with higher purity and a more concentrated form than the naturally extracted ones. Moreover, the antimicrobial activity of naturally extracted and purified AMPs from plant hosts has been found to be less effective than its chemically synthesised counterpart (Thery and Arendt, 2018; Schmidt, Arendt and Thery, 2019).

Chapter 3 delves into this approach of the synthetic production of plant derived peptides based on the amino acid sequence of natural peptides. The time-consuming processes involved in the extraction and purification of plant AMPs from the original plant can be significantly reduced when synthetic means of peptide production are applied instead. In this chapter, the development of peptide sequences based on natural plant AMPs is discussed in detail as an alternative method for peptide production with the aim of utilising them as potential preservative agents in food. In particular, their modulation and modification for the development of a peptide with increased potency and stability while also reducing the potentiality of toxic characteristics are highlighted. It is important that a balance between the antimicrobial activity and the toxicity of a peptide is met when developing a peptide. The cationicity of a peptide and the distribution of amino acids in a sequence are factors that can determine the toxicity/haemolytic activity but also the antimicrobial activity, therefore a balance must be met. The addition of basic amino acids such as Arg or Lys can cause an increase in the antimicrobial activity of a peptide, and depending on the position of

such residues within the sequence, can cause a reduction in the haemolytic activity (Jin *et al.*, 2016). The hydrophobicity, amphipathicity and length are also significant contributors that define the antimicrobial activity of a peptide. Increasing both the hydrophobicity and amphipathicity can affect the overall toxicity (as well as the antimicrobial activity) (Thery, Lynch and Arendt, 2019). Shorter peptide sequences have also been found to display increased antimicrobial activity, stability, and safety characteristics (Lyu *et al.*, 2016; Mohanram and Bhattacharjya, 2016). Peptide synthesis can be performed through either chemical or biological methods. However, chemical synthesis is often the preferred approach due to production of high purity peptides derived from this.

The initial objective of the research performed in this thesis was to test various peptides whose sequences were derived from known plant AMPs for their inhibitory activity against the growth of common food spoilage yeast. The production of these peptides was through solid phase peptide synthesis (SPPS), which is the more conventional method of peptide chemical synthesis due it its simplicity, efficacy and its compatibility with automated synthesis (Goodwin, Simerska and Toth, 2012). **Chapter 4** explores the antimicrobial activity of two defensin peptides derived from the seeds of the radish plant (Terras *et al.*, 1992). Both peptides (Rs-AFP1 and Rs-AFP2) were chosen for this analysis as previous research showed a range of antimicrobial activity against fungal species. Terras *et al.*, 1992 observed antifungal activity against common fungal pathogens that can cause infections and disease to major crop plants including wheat, barley, potato, and beans. Evidence for their antimicrobial activity against yeast species is however limited (Vriens *et al.*, 2016),

particularly against yeast species associated with food spoilage. The research outlined in this chapter found that both peptides inhibited yeast growth, with Rs-AFP2 displaying better antiyeast activity compared to Rs-AFP1 (Figure 7). The higher net charge and more positively charged residues found within Rs-AFP2 compared to Rs-AFP1 may have resulted in this increased antiyeast activity. These characteristics have been identified as contributing factors to a peptide's antimicrobial activity (Jiang *et al.*, 2008). Their safety was determined via haemolytic assay which showed the peptides to only be haemolytic at concentrations 4-fold higher than the MICs determined for their yeast inhibitory activity (Figure 11). Both peptides were applied in various food matrices and successfully reduced the growth of *Z. bailii* in apple juice (at concentrations 3-fold higher than the MIC), Fanta Orange and cranberry juice (full inhibition at recorded MIC), all beverages that are susceptible to spoilage by this yeast (Fleet, 1992). Their application in mayonnaise was also demonstrated.

The characteristics that permitted Rs-AFP2's more potent antiyeast activity (compared to Rs-AFP1) were explored further and applied in the selection of the subsequent peptide, research on which is detailed in **chapter 5**. The characteristics that distinguished Rs-AFP2 as the more potent peptide were applied for the selection of this synthetic peptide. Thus, a peptide with greater number of positively charged residues and a higher net charge resulting in a more basic nature was chosen for analysis. The amino acid sequence of the barley peptide (D-lp1) examined in this chapter originated from barley endosperm (Molina *et al.*, 1993). This AMP is part of the family of plant defensins. However, the characteristics of this peptide was first thought to belong to the family of plant thionins. Further analysis proved that its

cationic and positively charged nature, 47 amino acid residue length and existence of 4 disulfide bridges formed by the 8 cysteine residues present, placed this peptide in the defensin family instead (Colilla, Rocher and Mendez, 1990). Plant defensins are characterised by their cationic, basic nature, forming cysteine stabilised alpha/beta motifs (CSαβ motifs). Gamma-thionins was the name previously designated to these peptides; however, the lack of evolutionary similarities to other thionins and their similarities to animal and insect defensins resulted in the name change to plant defensins (Broekaert *et al.*, 1995). The characteristics of plant defensins make them ideal candidates for the inhibition of microbial cells due to their affinity to the negatively charged membranes typically associated with bacterial/fungal cells (Abulimiti Yili *et al.*, 2014). Chapter 5 reveals the strong antiyeast activity that D-lp1 possesses, causing inhibition to four of the five yeast strains tested (Table 13). In addition, an increase of both safety and stability characteristics is observed by D-lp1 (Figures 21 and 17), in comparison to what was observed for Rs-AFP2 (Figures 11, 12 and 7). Although both are potent AMPs, the more basic nature of D-lp1 caused by the higher net charge (+9) (compared to Rs-AFP2 (+6)) may have resulted in the higher antimicrobial activity. In addition, the presence of positively charged cysteine (8), glycine (7) and arginine (8) residues in D-lp1's sequence could have produced the higher activity that was observed compared to Rs-AFP2 (8 cysteine, 4 glycine and 3 arginine residues). Studies have found that peptides containing a high net charge and a high number of cysteine, arginine and/or glycine residues have significant antimicrobial properties due to the increased interaction with the microbial cell membrane (Yeaman and Yount, 2003; Haag *et al.*, 2012; Jindal *et al.*, 2014; Maróti, Downie and Kondorosi, 2015; Goyal and Mattoo, 2016). The increased safety

characteristics observed in the barley peptide was hypothesised to be due to its lower hydrophobicity compared to Rs-AFP2. It has been previously reported that peptides with higher hydrophobicity can more readily enter neutral-charged membranes typically associated with eukaryotic cells, resulting in the haemolytic effect that was observed by Rs-AFP2 and less so by D-lp1 (Gopal *et al.*, 2009; Hollmann *et al.*, 2016). D-lp1 was observed to be more resistant to the salt concentrations (in particular to the $MgCl_2$ and lower concentration of KCl) compared to Rs-AFP2, better maintaining its antiyeast activity in the salts. This difference in the antiyeast activity detected for the two peptides could be explained by the higher net charge found on D-lp1. The disruption to the overall net charge of the peptides caused by the presence of the salts on the yeast cell membrane could have led to the reduction in activity. The higher net charge of D-lp1 could have resulted in it maintaining its overall charge and thus its antiyeast activity in the high salt concentrations to a greater extent in comparison to Rs-AFP2 (Terras *et al.*, 1992). This characteristic, together with the high structural stability provided by the intrachain disulfide bonds, could also explain D-lp1's stability in a greater range of pH (pH 3, 5 and 7) compared to Rs-AFP2 (pH 5). D-lp1 was shown to prevent the growth of *Z. bailii* in various beverages, supporting its application as a potential food preservative.

Chapters 4 and 5 demonstrated the efficacy of plant defensins in reducing the growth of food spoilage yeast in various environmental conditions and in different food products. The spectrum of activity and the variability that peptides in the same family of plant AMPs possess is also evident in these chapters. These observed differences in the properties of the peptides show the necessity for testing each peptide for their

application in various food matrices and environments, as certain peptides may be more suitable for application in various foods than others, depending on their characteristics and that the environment and of the food matrix. This information leads into **chapter 6**, which deals with the study of a peptide whose sequence was derived from a family of plant AMPs called snakins. AMPs from this family are characterised by the presence of a GASA domain in the C-terminal region, with twelve conserved cysteine residues that contribute to their overall stability and activity (Nahirñak *et al.*, 2012). A peptide derived from potato tubers (SN-1) was selected for analysis in this chapter. SN-1 was chosen on account of the bioavailability of potatoes worldwide, their particular importance as a staple food source in this country and their cheap production costs (Horton and Anderson, 1992). The literature and data already available on SN-1 provided good evidence for its antimicrobial activity, with strong inhibition observed against both bacterial and fungal plant pathogens (Segura *et al.*, 1999; Almasia *et al.*, 2008; Darqui *et al.*, 2018). Research on activity against yeast, in particular food spoilage yeast, was however limited, encouraging the research performed in this chapter. SN-1's antiyeast activity was detected against two of the five yeast investigated (*Z. bailii* and *D. hansenii*). This antiyeast activity is lower than what was observed with the defensin peptides Rs-AFP1, Rs-AFP2, and D-lp1. Comparison of the SN-1 (snakin) peptide with the defensin peptides reveals weaker antiyeast activity, and reduced stability (salt and pH) and safety characteristics. The CSαβ motif present in the defensin peptides could explain their increased stability in comparison to the snakin peptide that does not have this motif within its structure. The presence of this CSαβ motif in AMPs has been shown to be a factor in the development of stable and potent antimicrobial properties in plant defensins (Dias and Franco,

2015). However, the cysteine-rich amino acid sequence of SN-1 (12 cysteine residues) was sufficient to produce the inhibitory activity observed against the two yeast species. In plant AMPs, the presence of numerous cysteine residues is often a good indicator of antimicrobial activity (Srivastava *et al.*, 2021). Moreover, the formation of a stable peptide as a consequence of the multiple disulfide bonds present, resulted in SN-1's good stability in the different salt, pH and thermal conditions (Figure 24 and Table 17). However, its stability was not any better than that observed for the defensin peptides. Even though the properties of SN-1 differ from the defensin peptides, the data generated can nevertheless provide a proof of concept for its application as a food preservative. Its inhibitory activity against *Z. bailii* in the beverages tested (Fanta Orange, cranberry juice, and apple juice) providing further evidence for this proof of concept. However, SN-1's activity in the beverages presented less inhibitory power than the defensin peptides.

Throughout this thesis, each peptides' mechanism of action is explored. Chapter 2 and 3 illustrate the manner by which AMPs can impart their antimicrobial activity. Onemode of action described is by the permeabilisation of microbial cell membranes. This mechanism is instigated by the positive charges found on cationic AMPs such as the defensin and snakin peptides discussed in chapters 4, 5 and 6. The negatively charged membranes of the yeast cells result in their permeabilisation caused by the attraction of the cationic peptides. The accumulation of a peptide on the membrane can compromise the integrity of the cell, resulting in the leakage of cytoplasmic components and ultimately cell death (Nawrot *et al.*, 2014). All four peptides tested (Rs-AFP1, Rs-AFP2, D-lp1 and SN-1) were found to trigger this response (Figures 8,

18 and 26). This mechanism of action of AMPs has also been linked to stimulation of the excessive production of reactive oxygen species (ROS) through a cascade of reactions (initiated by the permeabilisation of the cell membrane) (Struyfs *et al.*, 2020). The defensin peptides (Rs-AFP1, Rs-AFP2 and D-lp1) were all found to cause this overproduction in ROS, while the snakin peptide (SN-1) did not. The reason for this is not yet clear. The mechanism of action of snakin peptides (such as SN-1) have not been well established and are not yet completely understood, therefore a reason for this lack of ROS overproduction cannot be hypothesised (Su *et al.*, 2020). Figure 10 reveals another effect caused by the radish defensins Rs-AFP1 and Rs-AFP2. The scanning electron microscopy figures (figure 10) illustrates shrunken yeast cells. This may have been caused by potassium efflux due to the cell permeablisiation caused by the peptide. As the safety of these peptide is paramount for their application in food products, understanding their mechanism of action and their selectivity towards microbial cells can help validate their use as food preservatives. As described earlier, the cationic nature of plant AMPs increases their attraction to negatively charged surfaces, in particular to microbial cell membranes. The neutral/slightly positive nature of eukaryotic cells makes them less effected by the activity of AMPs (Chen *et al.*, 2016). However, some weak hydrophobic interactions between an AMP and the mammalian cell membrane can still occur (Mahlapuu *et al.*, 2016), resulting in the haemolytic activity observed in chapters 4 and 6 (Figures 11 and 27). The effect of the defensin peptides (Rs-AFP1, Rs-AFP2 and D-lp1) on human cells was investigated. While D-lp1 did not cause any harmful effects on the human cells, both radish defensins were actually found to produce the opposite effect – an increase cell viability through cell proliferation was observed. This mechanism, although beneficial for cases

such as wound repair or chemotaxis of leukocyte cells, can be a marker for tumour development in mammalian cells (López-Sáez, 1998; Ackermann, 2016). This is a characteristic that would need to be avoided and further investigation would be necessary when considering their application in food preservation.

As reviewed in chapter 3, the development of synthetic peptides can also be achieved through the assembly and targeted modification of amino acid sequences derived from natural, 'wild-type' peptides. This method of peptide development can create a peptide with improved characteristics, such as enhanced antimocrobial activity and increased safety and stability. **Chapter 7** demonstrates this approach and illustrates how peptide modifications can result in modulation of properties and activity of the derived peptide. A novel peptide (KK-14) developed based on known AMPs was analysed for its antiyeast activity and further modified into 3 additional peptide derivatives (Dip KK-14, dKK-14 and KK-14 (R10)). This chapter deviates from the cysteine-rich peptides that chapters 4, 5 and 6 characterised in order to highlight the level of activity that peptides with similar properties (hydrophobicity, cationicity, amphipathicity) can have, even those that lack the cysteine residues that can be essential for activity in some plant peptides. The KK-14 peptide was developed to encompass a hydrophobic centre as a result of the cationic residues chosen and a medium size to prevent dimerization but to also provide the peptide with a defined structure. A C-terminus amidation was also applied to increase the peptide's net charge and subsequently boost its antimicrobial activity. One form of modification on this KK-14 peptide was performed through the substitution of specific residues. This was accomplished by substituting the eighth tryptophan residue for a β-di-phenylalanine residue (Dip KK-

14) and the tenth proline residue for an arginine residue (KK-14 (R10)). These substitutions were performed to improve their antimicrobial activity and their salt stability (Dip KK-14) and to increase the net charge of the peptide in order to increase its antimicrobial activity (KK-14-R(10)). The D-enantiomer form of KK-14 was also created (dKK-14). The results obtained from this analysis demonstrated that the modified peptides displayed greater antiyeast activity compared to the original peptide (KK-14), in addition to varying degrees of improved salt, pH and thermal stability (Figures 29-31 and Table 23 and 24). Similar to the natural, unmodified peptides described already, the mechanism of action of these derivative peptides was shown to be via membrane permeabilisation. In general, the Dip KK-14 peptide with the β-di-phenylalanine residue was found to display the most potent antiyeast activity, possibly due to the now longer side chain as a consequence of this substitution; the longer side chain has been shown previously to promote better cell membrane penetration (Thery *et al.*, 2019). In addition, the increase in its hydrophobicity caused by this substitution could have also improved its antiyeast activity. The hydrophobicity of a peptide has been found to be correlated to its antimicrobial activity and can result in better potency (but also increased cytotoxic effects) (Zhou *et al.*, 2019). Interactions with salt bridges and hydrogen bonds (known to confer better antimicrobial activity in peptides) would have been facilitated through the additional side chain presented by the β-di-phenylalanine residue (Olli and Kirti, 2006; Nguyen *et al.*, 2011). Both dKK-14 and KK-14 (R10)) also displayed good antiyeast activity, with dKK-14 (and even KK-14) demonstrating better activity than KK-14(R10). Although the difference in the antiyeast activity would not be considered significant, this reduced antimicrobial activity is unexpected as the addition of an Arg residue (in combination with

successive Trp residues) should have resulted in a peptide with increased affinity to microbial membrane and therefore improved antimicrobial activity. This method of peptide development has been used in numerous studies (Sotirova *et al.*, 2014; Jindal *et al.*, 2015; Lee *et al.*, 2019; Klubthawee *et al.*, 2020), where novel peptides are modulated for the development of potent antimicrobial agents against bacterial pathogens (e.g., *Pseudomonas aeruginosa* and *Bacillus subtilis*) for application in biomedicine for potential new drug therapy. Chapter 7 clearly illustrated the potential that novel peptides, whose sequences are designed based on native plant AMPs, have for the reduction of food spoilage yeast in a soft drink. Furthermore, the capacity of sequence modification for the development of peptides with increased antimicrobial activity compared to an original sequence, was also confirmed (as illustrated by the modification of KK-14 and the development of the three more potent peptide derivatives).

Explored in chapters 4 to 7 is the integration of each peptide into various foods to investigate their potential application as novel food preservatives. This was performed to observe the ability of each peptide to reduce the growth of common spoilage yeast in various food products. *Z. bailii* was used in every study of the food application trials because it is a very common food spoilage yeast. It can grow in and spoil foods containing high concentrations of fermentable sugars, such as carbonated drinks, syrups, fruit juices and wine. Other food products such as salad dressings, mayonnaise, vinegar and sweet pickle can also be susceptible to *Z. bailii* growth (Tudor and Board, 1993). It was for this reason that a carbonated drink, fruit juices, wine, salad dressing and mayonnaise were the foods chosen for the product application trials. Each peptide

revealed varying degrees of yeast inhibition in each beverage/food, however their ability to perform better in liquid matrices was clearly observed. Rs-AFP1 ad Rs-AFP2 were the only peptides that were successful in causing inhibition of *Z. bailii* in a viscous matrix (salad dressing). D-lp1, SN-1, the novel KK-14 peptide and its derivatives, were incapable of reducing yeast growth in either the mayonnaise or salad dressing. The peptides' increased effectiveness in beverages can be due to the favourable consistency of these liquids, in comparison to the salad dressing and mayonnaise. The latter high viscous matrices could have physically inhibited the interaction between peptide and yeast, resulting in the lack of inhibition observed. What this illustrates is that, although their inhibition can be hindered in foods with thicker consistencies, their application in beverages (such as the ones presented in this thesis) can be taken as a proof of concept for their further development as potential novel food preservatives. This also highlights that in such trials, the peptide and the food matrix must be tested and investigated to find the most optimal peptide for a particular food product, based on the properties of the peptide, but also on the intrinsic properties of the food product.

Each AMP that was tested in this thesis originated from plants, either through the selection of wild type natural amino acid sequences from known plant AMPs (chapters 4, 5 and 6), or through the development of a novel peptide (and its derivatives) whose sequence was established on the basis of plant AMP sequences with the integration of specific amino acids or motifs known to confer enhanced antimicrobial activity (chapter 7). The motive for selecting radish-, barley- and potato- derived peptides was because of their availability and sustainable production costs. Highlighting and

promoting this link of inexpensive production costs and plant-based sources with consumers, would aid the in promoting the acceptance of the concept of plant-derived synthetic peptides as food preservatives.

The general aim of this thesis was to study plant AMPs (that have been chemically synthesised) and to understand how their antimicrobial activity can be exploited for the development of potential new preservative agents. Chapter 4 to 6 explored this approach and established each peptides' antiyeast activity, revealing the barley defensin (D-lp1) as the most potent and suitable natural wild-type peptide for the potential development of a novel preservative agent. Chapter 7 deals with another side, that of the use of modified peptides. The modification of a peptide to generate derivates with improved antiyeast activity and stability was demonstrated, alongside their suitability as potential preservative agents. Using both the natural wild-type and peptide modification approach enabled a comparison of the various levels of antiyeast activity and properties of the various peptides obtained with each approach. Considering the antiyeast activity of all the peptides analysed throughout this thesis, the *de novo* KK-14 derivatives could be seen as the most potent agents for the purpose of inhibiting food spoilage yeast and further applying them as preservative agents. Lower minimum inhibitory concentrations were observed from these derivatives, in comparison to the peptides based on natural peptides, indicating that a lesser quantity would need to be used for the same preservative power. However, it can be argued that the degree of added benefit in terms of the increased potency might not be considered enough to select this approach of peptide modification, over the use of wild-type natural peptide sequences. One of the main motivations for evaluating

sequences derived from plants was to eliminate the negative perception that consumers have developed on preservatives (there is an increase desire for more natural forms of preservatives). Although the modification of the KK-14 peptide was able to generate derivatives with slightly better activity and stability, such peptides may therefore not be the most suitable preservative agents. This is because, given consumers perception of preservatives, their ideals may lead to the belief that such purposely modified derivatives are less natural, and may therefore oppose the idea of modified peptides in their food. In addition to this, the production processes, costs and the research and development required to investigate and develop peptides like these derivatives may be cost-ineffective and thus counterproductive for their application in food preservation when considering the economics of food production.

Overall, this thesis highlights the potential of plant derived synthetic peptides as novel and potent preservative agents that could, in the future be applied into food products to increase their shelf life. Increasing the shelf life of foods is intended to reduce the burden that food waste and food loss puts on both the global economy and the environment. The research presented here can be taken as a proof of concept for what these peptides can achieve in reducing the spoilage of food/beverages, and subsequently in minimising the occurrence of food waste/food loss. Nevertheless, it is also important to consider the expenses associated with the production of synthetic peptides. This high cost of synthesis currently makes these approaches less appealing for the purpose outlined in this thesis. However, with time and as the technology develops further, this cost of synthesis will drop, at which point synthetic peptides could become part of the much-needed solution for the control of food waste.

8.2 References

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Appendix

List of publications

First author publications in peer-reviewed Journals

Shwaiki, L. N. *et al.* (2019) 'Inhibitory effect of four novel synthetic peptides on food spoilage yeasts', *International Journal of Food Microbiology*. Elsevier, 300, pp. 43–52. doi: 10.1016/J.IJFOODMICRO.2019.04.005.

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Shwaiki, L. N., Lynch, K. M. and Arendt, E. K. Future of antimicrobial peptides derived from plants in food application – a focus on synthetic peptides. Trends in Food Science and Technology. (under review). Date of submission 19/11/2020

Other Publications

Thery, T. *et al.* (2019) 'Antifungal activity of a de novo synthetic peptide and derivatives against fungal food contaminants', *Journal of Peptide Science*. John Wiley and Sons Ltd, 25(1), p. e3137. doi: 10.1002/psc.3137.

List of poster presentations

Shwaiki LN, Arendt EK, Lynch KM, Thery TLC (2018). Inhibitory effect of four novel synthetic peptides on food spoilage yeasts. The Food Safety Authority of Ireland Science Conference, Dublin, Ireland, August 2019.

Shwaiki LN, Arendt EK, Lynch KM, Thery TLC (2018). Inhibitory effect of four novel synthetic peptides on food spoilage yeasts. 35th European Peptide Symposium, Dublin, Ireland, August 2018.

Shwaiki LN, Arendt EK, Lynch KM, Thery TLC (2018). Inhibitory effect of four novel synthetic peptides on food spoilage yeasts. 8th International Meeting on Antimicrobial Peptides, Edinburgh, UK September 2018.

List of oral presentations

Shwaiki LN, Arendt EK, Lynch KM, Thery TLC (2018). Inhibitory effect of four novel synthetic peptides on food spoilage yeasts. 47th Annual Food Research Conference, Cork, Ireland, December 2018.

Shwaiki LN, Arendt EK (2019). Characterisation and application of novel and plant derived antimicrobial peptides to combat food spoilage yeast. School of Food and Nutritional Sciences Internal Conference, Cork, Ireland, December 2019.