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UNIVERSITY COLLEGE CORK

School of Food and Nutritional Sciences



FUNDAMENTAL STUDY OF NATURAL AND SYNTHETIC ANTIMICROBIAL PEPTIDES AS NOVEL PRESERVATIVES AGAINST FUNGAL FOOD SPOILAGE

Thesis presented by

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To obtain the degree of

Doctor of Philosophy - PhD in Food Science and Technology

Head of School

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March 2019

A ma famille, mes amis, mes amours, mes emmerdes…

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Abstract

A large part of food loss is associated with the development of fungal contamination at early or late stages of production. This spoilage, via the production of mycotoxins, has potentially disastrous consequences on consumers' health. The overuse of chemical antimicrobials in foods (e.g., sorbates) and pesticides (e.g., azoles) in crops has also led to the development of resistant strains, including human pathogens. This thesis aims to consider the potential of antimicrobial peptides as novel preservatives against spoilage fungi of food products. Antimicrobial peptides are short (<100 amino acids) components of the innate immune system widely spread amongst eukaryotic and prokaryotic kingdoms. A literature review about the diversity of antimicrobial peptides and proteins (AMPs) and their efficacy against fungal food spoilage is presented in Chapter 2. The studies presented in Chapters 3 and 4 highlight the activity of synthetic analogues of a human defensin, HBD-3, and a cowpea defensin-like peptide, Cp-thionin II, against selected species representing food contaminants, *Fusarium culmorum*, *Penicillium expansum* and *Aspergillus niger*. The peptides inhibit conidial germination, potentially with morphogenic changes and killing activity against *F. culmorum* hyphae, but suffer salt sensitivity. Mechanistic studies reveal membrane permeabilization and overgeneration of reactive oxygen species. The potential of the peptides for food applications has been exploited either with a recombinant yeast, able to express a recombinant HBD-3, or with the addition of synthetic Cp-thionin II in bakery products. However, the cost of synthesis of natural-based peptides is a major drawback to economically viable applications. To overcome this obstacle, a 4-residue peptide has been successfully tested against mould and yeast species. After conjugation with a lauric acid, synergistic antifungal activity is observed with strong improvements of peptide resistance. However, the price to pay for such improvement is a strong increase of toxicity. Based on rational design with key determinants of antifungal activity, a novel 14-residue peptide, made of the sequence KKFFRAWWAPRFLK-NH_2, has been constructed in order to find a balance between activity and toxicity. Three analogues, created to improve the peptide's properties, consist in residue substitution or enantiomerization. The different modifications hardly alter the antifungal potency but significant improvements in resistance are obtained with the enantiomerization or a Pro to Arg substitution. However, those modifications are associated with an increase of toxicity. In spite of continuous improvements and cost reduction, the launch of new synthetic compounds faces regulations and consumers' demand for natural products. The natural approach consists in the isolation of an AMP from broccoli, a healthy raw material that belongs to the *Brassicacea* family, an AMP-rich plant family. Extracts of broccoli seeds show activity against the three fungal species studied. An antifungal napin-like protein has been isolated and purified via chromatographic methods. In addition, it shows no cytotoxicity and reduces fungal development on contaminated barley grains. Due its extraction and purification via "non-chemical", food-grade, processes, this antifungal compound could represent a fully natural novel preservative. The world is facing a major crisis, with a boom of population worldwide, associated with a rise of undernutrition while food production is sufficient. The results of this work offer novel perspectives in the research of large spectrum food preservatives in a clean-label approach.

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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.

_____

Signature

Date: 13/03/2019

Abbreviations

AFP	antifungal peptide/protein
AMP	antimicrobial peptide/protein
ANN	artificial neural network
B. olereacea	*Brassica oleracea*
BBI	Bowman-Birk inhibitors
BoNap	*Brassica oleracea* var. *italica* napin
Bzl	benzyl
CaM	calmodulin
cAMP	cationic antimicrobial peptide
CC_{50}	cytotoxic concentration 50%
CCK	cysteine-knot
CD	circular dichroism
cfu	colony forming unit
CLSM	confocal laser scanning microscopy
Cp-thionin II	cowpea thionin II
CSαβ	cysteine-stabilized α, β
CSM-URA	complete supplement mixture – URA
Dab	2,4-diaminobutanoic acid
DC	dendritic cell
Dip	β,β-diphenylalanine
DIW	deionized water
DMEM	Dulbecco's modified Eagle's medium
DT	decision tree
EDTA	ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization of the United Nations
FBS	foetal bovine serum
Fmoc	fluorenylmethyloxycarbonyl chloride
FST	fungal strain
GlCer	glucosylceramide
GMM	genetically modified microorganism
HB	hydrogen bonds

HBD-3	human β-defensin-3
HDP	host defence peptide
HIC	hydrophobic interaction chromatography
HIV	human immunodeficiency virus
HM	hydrophobic moment
HPLC	high performance liquid chromatography
IC_{50}	inhibitory concentration 50%
IL	interleukin
KK14	KKFFRAWWAPRFLK-NH_2
KT43C	synthetic Cp-thionin II
Lfcin	lactoferricin
LEAP	liver-expressed antimicrobial peptide
-mer	monomer
MAPK	mitogen-activated protein kinase
MALDI-TOF	matrix assisted laser desorption ionization-time of flight
MBHA	methylbenzhydrylamine
MRSA	ethicillin-resistant *Staphylococcus aureus*
MIC	minimum inhibitory concentration
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
Nal	β-naphthylalanine
NEAA	non-essential amino acids
NO	nitric oxide
O3TR	OOWW-NH_2
PAS	periodic acid Schiff
PDA	potato dextrose agar
PDB	potato dextrose broth
PBS	phosphate-buffered saline
pI	isoelectric point
PI	propidium iodide
PMSF	phenylmethylsulfonyl fluoride
PR	pathogenesis-related

PS-SCL	positional scanning synthetic combinatorial library
PTM	post-translational modification
PVDF	polyvinylidene difluoride
QSAR	quantitative structure-activity relationship
RBC	red blood cell
ROS	reactive oxygen species
RP- HPLC	reverse phase-HPLC
rpm	round per minute
SG	SYTOX Green
sHBD-3	synthetic HBD-3
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SFM	synthetic fungal media
TFA	trifluoroacetic acid
TFE	trifluoroethanol
TL	thaumatin-like
TMR	tetramethylrhodamine
TNF-α	tumor necrosis factor α
URA	uracil
USP	ultrashort peptide
WHO	World Health Organization
YMM	yeast minimal media
YNB	yeast nitrogen base
YPS	yeast peptone sucrose
YEPD	yeast Potato Dextrose Agar

Chapter 1
Introduction

In our galaxy and in a future not so far, far away…

Here we are, in 2050, over 9 billion people live on Earth, with about 70 percent of the world's population being urban. For the last 35 years, an increase of 2 billion people has occurred, mostly in developing countries. Despite the multiplication of income levels and a 70 percent increase in food production, sustainable food systems have failed to provide enough safe and nutritious food for all people (FAO [Food and Agriculture Organization of the United Nations] 2017, UNICEF / WHO / World Bank Group 2018). In the midst of adequate supplies, the insufficient raise of capital make hunger persist, with immediate and long-term physical, social and economic consequences on the poorest and most vulnerable people. The reallocation in developing country budgets, the increase of governmental investments in agricultural R&D in developing countries, or the policy changes to enhance access to new technologies to smallholder farmers, have all failed to fight poverty. Evidentially, within regions, the gap between potential crop yield and actual crop yield can be as large as 76 percent (FAO [Food and Agriculture Organization of the United Nations] 2017). In addition, many countries continue to depend on international trade to ensure their food security. The import of cereals into developing countries, for instance, doubled the last 40 years (FAO [Food and Agriculture Organization of the United Nations] 2017).

The principal cause of hunger is poverty, which includes the deficit of resources, unequal income distribution, war and, in a cyclical way, hunger itself. Lastly, the extreme weather events due to climate change and the increased biofuel production (due to the use of land and economic resources) have led to a higher number of undernourished children, especially in Africa and South Asia.

Food loss and waste, which represents 2.5 billion tonnes every year in 2050, is one major reason for the inability of people to have access to food. A beneficial and sufficient distribution could feed those nearly 25 percent of undernourished people in developing countries. Food waste has crucial economic consequences for those people whose livelihood depends on food value chains. Moreover, it threatens sustainable food production, with a negative impact on food security and nutrition for current and future generations. In developed countries, food spoilage is more associated with high consumer standards, reflected by the cheap cost of discarding products and, hereby, the fact that people can afford to waste food. In developing countries, food waste still occurs mostly during production processes, because of poor storage, transport and processing facilities, or inefficient harvesting practices. Industry has continuously overused chemical preservatives to fight this microbial spoilage, despite a limited spectrum of activity, in terms of targets and conditions, and consumers' awareness of health issues.

The overuse of chemicals has led to a tremendous increase of resistant food contaminants and the appearance of new species of superbugs. Globally, in 2050, superbugs, such as Methicillin-Resistant *Staphylococcus aureus* (MRSA), cause 10 million deaths per year, which is more than cancer, with an estimated cost of $100.2 trillion (de Kraker et al. 2016). Moreover, pesticides, such as azoles, spread massively to protect crops, had led to the development of fungal resistant strains. Resistance has been found in fungal human pathogenic strains, azoles being the first treatment to fungal infections. In 2050, the number of deaths due to fungal which leads to 1.6 million deaths per year, from several diseases such as chronic pulmonary or invasive aspergillosis, cryptococcal meningitis complicating HIV or invasive candidiasis (Rautenbach et al. 2016). Just like the mosquito is by far the fiercest animal, the real killers of fungal kingdom are not the death caps but species belonging to the genera *Cryptococcus*, *Aspergillus* or *Candida*, killing almost as much as tuberculosis, the most lethal infectious disease. Moreover, food disorders associated with fungal attacks are noteworthy.

However, in the years 2015-2018, scandals of super-villain superbugs and food contamination were already headlines of newspapers and intensive research was

being carried out to solve the different issues. What is a better way to fight super-villains than using superheroes? Thus, novel compounds, more efficient and with a broader spectrum of activity than traditional products, were investigated. In foods, natural antimicrobials appeared to be the leading compounds, both, to satisfy consumers' demand in developed countries and to limit spoilage-associated food waste in developing countries. At this time, a big hope was placed on compounds called antimicrobial peptides and proteins (AMPs) to fight multi-drug resistant bacteria.

Broadly, AMPs include three categories: gene encoded antimicrobial peptides, antimicrobial proteins and non-gene encoded peptide antibiotics. AMPs are among the first compounds to meet pathogens upon infection and are considered as a type of super hero of the innate immune system, acting as pre-barrier before the activation of the adaptive immunity. The beginning of the antimicrobial peptide/protein field is usually attributed to the discovery of lysozyme by Sir Alexander Fleming, in 1922 (Wang 2017). Ninety years before this discovery, Niels Ryberg Finsen (who was awarded the Nobel Prize in Medicine and Physiology) used light therapy against skin tuberculosis. The connection between light therapy, vitamin D and AMPs followed years later and since 1980s, the research on AMPs has continuously grown. A discovery timeline of important AMPs is accessible online (access address: http://aps.unmc.edu/AP/timeline.php, (Wang et al. 2016)). Beyond their antimicrobial activities, the term "host defence peptide" emphasizes the immunomodulatory functions of these peptides, such as chemotactic properties, wound healing and their role in immune disorders, such as Alzheimer's disease, multiple sclerosis, or diabetes mellitus type 1. As defensive tools, in mammals, AMPs have been found in higher concentrations in environments commonly exposed to pathogens such as skin, gastrointestinal tract, or lungs. Due to their diversity, the classification of AMPs is challenging, but several parameters are generally recognized of major importance. Although most AMPs have a positive net charge, neutral and anionic peptides exist. The positive charge of AMPs is thought to favour the electrostatic interaction between the peptide and the negatively charged membrane of microbes, especially bacteria. The hydrophobic content, which is the ratio between hydrophobic amino acids and the total number of amino acids, is an important feature of AMPs. Although, the hydrophobic content can vary from 0 to 100, a large

proportion (50-78%) of AMPs possesses a hydrophobic content of 30-60% (Wang et al. 2015). AMPs made only of hydrophobic residues are thought to have a long residence time in microbial membranes, whereas the absence of these residues would strongly reduce the binding to membranes. AMPs are found in the three domains of life and are generally organized into four structural classes: α-helices, β-sheets, mixed αβ structures and non-αβ structures. Like all super heroes, AMPs have a weakness, namely, sensitivity to high salt concentrations. Thus, at physiological salt concentrations, AMPs generally lose their antimicrobial properties despite the ability of the so-called HDPs to retain their immunomodulatory properties in such conditions (Hancock et al. 2016).

In mid 2010s, the antifungal properties of several antimicrobial peptides and proteins (so called antifungal peptides and antifungal proteins (abbreviated AFPs, indifferently)) were studied against fungal food spoilage. The aim of the research, presented in this thesis, was to offer novel potent alternatives to traditional chemical preservatives, to extend the shelf life of foods. The potential of natural AMPs or those derived from foods themselves had been considered as alternatives to traditional antimicrobial compounds in foods (Hintz et al. 2015, Rai et al. 2016, Rydlo et al. 2006). However, most of the research was focused on limiting food-borne pathogens. Thus, the first natural AMP considered for food application was the bacteriocin nisin A, a bacterial peptide active against Gram-positive bacteria (and several Gram-negative species when combined with other treatments), discovered in 1928 and approved by the FDA in 1988 (Gharsallaoui et al. 2016). Other AMPs have been approved as food preservative, such as the class IIa bactericon Pediocin PA-1, discovered in 1992 or the bovine AFP lactofferin (Henderson et al. 1992).

Chapter 2 presents an overview of natural AFPs, from a large range of organisms, which display activity against fungal (moulds and yeasts) food contaminants. Plants, for which fungi represent a bigger threat than bacteria, are one of the most potent sources of AFPs. Thus, plant AFPs are extensively described. The key parameters of the antifungal activity and an in-depth understanding of the multiple mechanisms of action are also presented. The study of residue substitutions enables the design of many efficient peptides with improved characteristics. In addition, the

potential application and effects of these peptides and peptide-based compounds in food matrices is discussed. Among the numerous potential AMPs, the initial interest focused on antifungal defensins, especially the human β-defensin 3 (HBD-3) and a defensin-like peptide isolated from cowpea (*Vigna unguiculata*), seeds, termed cowpea thionin II (Cp-thionin II). Due to its high positive net charge, responsible (at least partially) for its strong antimicrobial activity and resistance in salt solutions, HBD-3 has been broadly studied for therapeutic applications. This peptide has also been shown to be active against the fungi, *Candida albicans* and *Aspergillus niger*, while a recombinant and a synthetic version of HBD-3 has previously been shown to reduce the bacterial spoilage of wort and beer (James et al. 2014). The use of recombinant microorganisms allows a higher yield of peptide during production, at a lower cost. The studies, presented in **Chapters 3** and **4**, focus on the understanding of the antifungal activity of synthetic and recombinant versions of these AFPs against food contaminants and their potential role as novel preservative for baked goods. However, the high costs and a certain sensitivity (*e.g.*, salt sensitivity) in food matrices of synthetic AMPs represent limiting factors for their application in food and/or fungal spoilage. Based on the previous identification of the key determinants of the antimicrobial activity, researchers have endeavoured to design peptides with optimized properties. **Chapter 5** evaluates the potential of a previously described ultrashort peptide (USP) made of 2 Orn to impact the cationic nature and 2 Trp residues to impact the hydrophobic nature on the peptide. The antimicrobial activity of this 4-mer peptide has been shown to be improved by a superpower, consisting of lipidation at its N-terminus. In this chapter, the effect of lipidation of O3TR on the antifungal activity and stability of the peptide was described. Fatty acids are known to possess antifungal activity, but with a lower efficacy than AMPs. They have also been considered as potential natural food preservatives, but their application is limited by the negative impact on flavour properties (Hyldgaard et al. 2012). However, the beneficial effect of the lipopeptide on antimicrobial activity is balanced by a higher haemolytic and cytotoxic activity.

The easy access to peptide synthesis (and forecasting the price decrease in peptide synthesis in the future) and the identification of key determinants has opened up the scope of possibilities for the design of safe and potent peptides. Thus, the study presented in **Chapter 6** aims at showing the efficacy and safety of a novel peptide. To

our knowledge, the 14-mer peptide, termed KK14, consisting of the sequence KKFFRAWWAPRFLK, has not been found in nature or studied before. Three derivatives of KK14 were also designed, in order to highlight the role of specific residues on the activity, structural features and stability of the peptide.

Nevertheless, in addition to consumer's desire, cost and economic cost issues, the applications of synthetic peptides as novel food preservatives are limited by regulatory authorities. Thus, natural compounds are more likely to be accepted for food applications. Based on previous works on *Brassicacea* plants, the study presented in **Chapter 7** relates to the richness of antifungal peptides in broccoli seeds (*Brassica oleracea* var. *italica*). Antifungal compounds from broccoli seeds were isolated and characterized, in terms of spectrum, structure and mechanism of action. Broccoli is well perceived by consumers, which could facilitate its acceptance as a new preservative. The efficacy of these natural antifungal compounds and the facilitated methods of extraction offered promising perspectives.

The aim of this doctoral dissertation is to highlight the potential use of AMPs with antifungal activity as novel preservatives for food products. The knowledge gained regarding the modes of action and the role of residues could generate the design of efficient peptides, presenting optimized properties, such as non-specific mechanisms or low toxicity. The use of a synthetic, a genetic and a natural approach shine a light on the advantages and flaws of each approach.

Since the end of the 19th century, major progress has been achieved in the treatment of infections and in the protection of foods. These considerable achievements have drastically reduced mortality. However, facing the rising of new threats, this research aims at proposing new lines of research and at showing the efficacy of AFPs to reduce fungal contamination…

…And to avoid the human catastrophe, partly due to food waste crisis, which was looming on the horizon of 2050.

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Chapter 2

Natural Antifungal Peptides/Proteins as Model for Novel Food Preservatives

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Abstract

A large range of ingredients and food products are subject to fungal contamination, which is a major cause of destruction of crops, exposure to invasive mycotoxins, and food spoilage. The resistance of fungal species to common preservation methods highlights the necessity of new ways to increase the shelf life of raw material and food products. Antimicrobial peptides and proteins (AMPs) are essential members of the immune system of most living organisms. Due to their broad range of activity and their stability, they represent promising alternatives to traditional preservatives. However, despite the growing number of reports of potential food applications of these AMPs, the number of approved peptides is low. Poor solubility, toxicity, and a time-consuming extraction are hurdles that limit their application in food products. Thanks to a deep understanding of the key determinants of their activity, the development of optimized synthetic peptides has reduced these drawbacks. This review presents natural and synthetic antifungal peptides/proteins (AFPs), effective against food-related fungi, with particular emphasis on AFPs from plant sources. The design of novel antifungal peptides via key elements of antifungal activity is also reviewed. The potential applications of natural and synthetic AFPs as novel antifungal food preservatives are finally discussed.

2.1 Introduction

Fungi (moulds and yeasts) play a major role in the food industry. They are involved, for example, in the manufacture of fermented products and in the ripening of cheeses. However, they are also responsible for spoilage, which causes economic losses and may affect human health. Estimates are that food poisoning affects 1 in 10 people worldwide every year (WHO [World Health Organization] 2015). Besides bacteria, mycotoxin-producing fungi are also known to cause foodborne diseases and their growth must be limited to provide a constant supply of safe food. Mycotoxins are a cause of human liver cancer and provoke damage of the nervous system; they also affect the immune system and fertility. The poorest regions of the world, in particular sub-Saharan regions, are affected with approximately 500 million people exposed to the natural mycotoxins of cereals. Fungal contamination is also responsible for the destruction of a third of the crops worldwide. This loss must be added to another third of the food production wasted every year, which represents 1.3 billion tonnes of edible goods discarded, with an average cost of $750 billion (Stenmarck et al. 2016). A quarter of this total loss would be sufficient to feed the 900 million people currently suffering from hunger. Although the current food production is 1.5-2 times sufficient to feed everyone on the planet, the Food and Agriculture Organization (FAO) assumes a required increase in food production of 70% by 2050 to feed 9.1 billion people. The problem of food access cannot only be attributed to microbial spoilage (Holt-Giménez et al. 2012). Nevertheless, reduction of contamination in raw materials and food waste is urgently needed.

Fungal spoilage and limitations of preservation methods

A broad range of food products can be damaged and lost due to fungal spoilage, as foods provide an ideal substrate for the growth of fungi if left untreated (Stratford et al. 2013). In general, growth of important food-spoilage microorganisms occurs over the range of pH 4-8, whereas spoilage yeasts and moulds can survive and expand in the pH range of 2-5 (Booth and Stratford 2003). In addition to health issues, due to the presence of mycotoxins, fungi lead to deterioration of flavours and appearance

(Oliveira et al. 2012). Mould and yeast spoilage are a serious issue of baking and beverage industries.

Losses of bakery products due to mould spoilage vary between 1-5 per cent, for an estimated cost of 500 million euros per year (Hernández et al. 2018). Although bacterial species such as *Salmonella* spp., *Listeria monocytogenes*, *Bacillus cereus*, *Clostridium botulinum* have been implicated in baking products foodborne illnesses, mould and yeast growth is by far the major factor limiting shelf life of high and intermediate bakery products (Smith et al. 2004). Due to heat treatment, contamination occurs from environmental spores, contact with contaminated surfaces and potentially heat-resistant spores. Furthermore, fungal mycotoxins are able to withstand traditional treatments (Battey, Duffy, and Schaffner 2002). Amongst the 40 species of fungi, usually described as contaminant agent of baked foods, *Rhizopus nigricans*, *Penicillium expansum* or *P. stolonifer*, and *Aspergillus niger* are the most commonly moulds involved in bread spoilage (Table 2.1). Other common molds include *Rhizopus* sp., *Aspergillus* sp., *Penicillium* sp., *Monilia* sp., *Mucor* sp. and *Eurotium* sp. *Eurotium* species are usually the first fungi to colonize improperly dried, stored commodities and their presence on baking products results in an increase of water availability, leading to colonisation by other species. Wild yeast of the genera *Trichosporon*, *Saccharomyces*, *Candida*, *Pichia*, *Torulaspora* and *Zygosaccharomyces* could occur as spoilage, resulting generally from unclean utensils and equipment (Hernández et al. 2018). The growth of filamentous yeast can give a "chalky bread" occurs, mostly occurring due to *Saccharomyces* sp. or *Pichia burtonii*, a particularly resistant yeast to preservatives, low, white, spreading colonies. In addition to unpleasant aspect, mould and yeast spoilage cause undesirable odours (Hager et al. 2012).

In soft drinks, despite the presence of sugars, used as a source of energy by microbes, a low pH and a high volume of carbon dioxide represent major hurdles to microbial growth (Wareing and Davenport 2007). Yeasts are the main contaminants and spoilers of soft drinks because of their natural occurrence in ingredients used for soft drink production, such as sucrose and fruit juices, and their ability to grow in these selective conditions. Although yeasts do not represent a risk to consumer's health, they can lead to serious economic losses (Hernández et al. 2018). The spoilage of soft drinks by yeasts is usually characterized by abundant CO_2 production, turbidity and the

production of unpleasant flavours (Hernández et al. 2018). *Dekkera bruxellensis, Debaromyces hansenii, Issatchenkia orientalis, Kloeckera apiculata, Pichia membranifaciens, Zygosaccharomyces bisporus, Saccharomyces* spp., *Schizosaccharomyces pombe,* are commonly associated with soft drinks spoilage (Pitt and Hocking 2012), as well as preservative-resistant yeasts, such as *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* (Hernández et al. 2018). Conidia present in the environment can contaminate soft drink environment in response to hygienic failures or contaminated raw material/packaging contaminated packaging (Azeredo et al. 2016). However, most molds are aerobic and therefore inhibited in low oxygen tension environment. The presence of visible mycelium, discoloration, off-flavours are the main reasons of consumer rejection of spoiled soft drinks and some fungi strains are able to produce toxigenic compounds, such as aflatoxins or patulin (Kregiel 2015; Hernández et al. 2018; Pitt and Hocking 2012).

Chemical preservatives and limitations

In order to prevent fungal growth, heat treatment and acid-based preservatives are applied as the most common techniques. Traditional methods to inhibit mould growth on baking products also include physical treatments (e.g., UV light or IR radiation) (Melini and Melini 2018). Drinks containing fruit juices, teas, containing no chemical preservatives, are usually pasteurized at temperatures below 90∘C. The combination of low pH greatly and heat treatment emphasizes the antimicrobial barrier of each treatment (Kregiel 2015) .

Chemical preservatives are defined as “any chemical that, when added to food, tends to prevent or retard deterioration thereof, but does not include common salt, sugars, vinegars, spices, or oils extracted from spices, substances added to food by direct exposure thereof to wood smoke, or chemicals applied for their insecticidal or herbicidal properties”. Preservatives comprise antimicrobials, antioxidants, anti-browning agents and aimed at preventing or delaying chemical and biological food deterioration. The use of antimicrobials is clearly defined with the identification of the target microorganisms and demonstrated efficacy and safety in food systems via model studies, including toxicological studies. The parameter used to set the human safety standard of food additives, including antimicrobials, is the No Observed Adverse

Effect Level (NOAEL) in milligrams per kilogram of body weight per day. NOAEL is the highest dose that induce no adverse effect in the most susceptible animal species. In 1961, a Joint FAO/WHO Expert Committee on Food also introduced the acceptable daily intake (ADI, expressed as milligrams of additive per kilogram body weight), the amount of an additive in food that could be ingested orally on a daily basis over a lifetime without an appreciable health risk. Although widely used, certain problems and limitations of the NOAEL have been pointed out by several authors (Carocho et al. 2014).

The most common used preservatives of baking industry are propionic and sorbic acid (and respective salts). Their efficacy as mould inhibitor has been shown, especially against *Aspergillus niger* rand *Penicillium* species (Melini and Melini 2018; Saranraj and Geetha 2012) . The levels of sorbate used in bakery products ranges from 0.001-0.3 per cent have generally low impact on food quality and organoleptic properties. Sorbate acts synergistically with sodium chloride, calcium propionate, sodium propionate, citric acid and sucrose achieving a longer shelf life. Propionic acid is a naturally occurring carboxylic acid used especially bakery products but with an unpleasant rancid flavour. There are not many studies regarding the toxicity of propionic acid or its salts. However, some studies have showed some adverse effects, such as sleep disturbance or immune response suppression (Carocho et al. 2014).

Sorbates (E 200–203), benzoates (E 210–219), and dimethyl dicarbonate (DMDC) (E 242) are permitted in ready-to-drink beverages in Europe. Benzoic acid (E210) occurs naturally in certain fruits (e.g. cranberries) and has been used to inhibit microbial growth of foods. Due to higher water solubility and stability than benzoic acid, benzoate salts are particularly well suited for use in carbonated, non-alcoholic, and juice beverages. Although regarded as nontoxic, benzoates can react with ascorbic acid and form benzene, a known human carcinogen, and some authors identified adverse effects such as cytotoxicity to human blood lymphocytes, obesity prevalence or hyperactivity behaviour (Beezhold and others 2014). DMDC and sulphites are also commonly used as a preservative in cold-sterilized soft drinks. However, sulfites, sorbates and benzoates, have been associated with allergic reactions in some people, especially suffering of asthma (Kregiel 2015) . Chemical preservatives inhibit most yeast and mould species, but a few species are able to proliferate in preserved foods (Azeredo et al. 2016; Kregiel 2015).

Natural alternatives

Due to the misuse of various preservatives, an increase of food contaminants that are more tolerant to food processing and preservation methods has occurred. In addition, consumers are demanding healthier products, with high-quality properties and less preservative for an extended shelf life (Rydlo, Miltz, and Mor 2006). Plants, animals and microorganisms are potential sources of natural antimicrobials (Gyawali and Ibrahim 2014). Several natural antimicrobials are generally recognized as safe and could represent a novel alternative to traditional chemical preservatives. However, a limited spectrum and/or adverse changes in organoleptic characteristics have been noticed with these natural antimicrobials (Ahmed et al. 2013). The use of microorganisms and their metabolites has gained strong interest to prevent spoilage and to extend the shelf life of foods. In baking industry, lactic acid bacteria used for the preparation of sourdoughs affected the outgrowth of *Aspergillus niger* or *Penicillium expansum* spores in bread systems. Novel technologies include active packaging comprising oxygen absorbers have been recently reviewed. Active packaging can be defined as packaging intended to extend the shelf-life of packaged foods or to maintain and/or improve the condition thereof by releasing or absorbing substances into or from the food or its surroundings. So far, the application of nanotechnology to extend bread and bakery products shelf-life has been poorly exploited.

Table 2.1. Main fungal contaminants of bakery products and product defects

Spoilage agents		Properties of colony and product defects
Mould	*Penicillium spp.*	Blue/green, flat, spread rather slowly
	Aspergillus niger	Black, fluffy, visible sporeheads
	Aspergillus flavus *Aspergillus candidus* *Aspergillus glaucus* *Cladosporium* spp. *Eurotium* spp. *Fusarium* spp.	Cream to green fluffy colonies
	Neurospora stophila	Pink, fluffy and spread fast
	Rhizopus nigricans	Grey/black, fluffy and spread fast
	Mucor spp.	Grey
Yeasts	*Hyphophichia burtonii* *Pichia anomala* *Scopsisfi buligera*	Slow growth on bread surface, low, white, spreading colonies
	Pichia burtonii	Very fast growth on bread Chalk mould white spots at surface of bread
	Zygosaccharomyces bailii *Torulaspora delbrueckii* *Pichia membranifaciens* *Candida* spp.	Smooth, round, convex and white to cream patches Alcoholic essence smells
	Trichosporon variable	White or pink colonies

Table 2.2. Main fungal contaminant of beverages and product defects

Spoilage agents		Properties of colony and product defects
Moulds	*Aspergillus* spp. *Cladosporum sphaerospermum* *Fusarium oxysporum* *Eupenicillium brefeldianum* *Mucor* spp. *Neosartorya fischeri* *Penicillium glabrum* *Rhizopus stolonifer* *Talaromyces flavus* *Mucor* spp.	Mycelial white to grey mats, discoloration, swollen packages Musty, stale
Yeasts	*Aureobasidium pullulans* *Candida spp,* *Cryptococcus* spp. *Debaryomyces* spp. *Dekkera* spp. *Kluyveromyces* spp. *Pichia* spp. *Rhodotorula glutinis* *Saccharomyces* spp. *Schizosaccharomyces pombe* *Zygosaccharomyces* spp.	Haze, clouds, surface films, swollen packages Abundant CO_2 production Yeasty, aldehyde, vinegar, pineapple note flavours

Natural antifungal peptides and proteins

Natural antifungal agents are extensively present in the organisms of the different kingdoms, which over time have developed a complex immune system to face the attacks from environmental fungal pathogens (Ganz 2003; Cederlund, Gudmundsson, and Agerberth 2011). Amongst the defence system, antimicrobial peptides are small, mostly cationic, peptides widespread within the different prokaryotic and eukaryotic kingdoms (Hassan et al. 2012; Adem Bahar and Ren 2013; Beisswenger and Bals 2005; Wiesner and Vilcinskas 2010). These molecules have been the focus of recent reviews, especially their antimicrobial action (Håvard Jenssen, Hamill, and Hancock 2006; Wiesner and Vilcinskas 2010), their involvement in inflammatory diseases (Felício et al. 2017; Arias et al. 2017), and their immunomodulatory activity (Hancock, Haney, and Gill 2016). The term AMP is commonly used to design antimicrobial peptides, e.g. comprising less than 100 amino acids. In this review, the abbreviation AMPs refers to both antimicrobial peptides and proteins, regardless. While many AMPs inhibit the growth of bacteria and fungi, some peptides, including histatins or bacterial lipopeptides, specifically target fungi (Matejuk et al. 2011). Natural AMP research mainly focuses on novel specific antifungal and antibacterial drugs against emerging infections (Matejuk et al. 2010) and also on a biotechnological use of AMP genes to create genetically modified crops (Lacerda et al. 2014).

Some AMPs are particularly effective against fungi, so called antifungal peptides and proteins (AFPs). Despite structural and sequence diversity, antifungal peptides present similar features, including small size and presence of cationic and hydrophobic residues. A common way to group antifungal peptides is their specific structure: linear peptides forming α-helix in a hydrophobic environment; peptides appearing in β-sheet structures with several bonds; CSαβ peptides comprised of helix (α or 3_{10}) and β-sheets stabilized by disulfide bonds (SS bond); α-hairpin or SS bond-containing loop; linear peptides, comprising a high amount of specific residues; random structured small peptides. Other classifications based on covalent bondings, hydrophobicity, net charge, or targets have been proposed to improve peptide design (G. Wang 2015). The peptide net charge is controlled intrinsically by the presence of polar residues, positively (Arg, Lys, His) or negatively (Glu, Gln, Asp) charged. A positive net charge (cationicity) is generally associated with a strong interaction with the negatively charged membrane of microbes. Hydrophobicity, or hydrophobic content, is based on

the proportion of hydrophobic residues (Val, Ile, Trp, Phe, Leu, Cys, Met, and Ala) in the peptide. A minimal hydrophobicity is required for the penetration and binding of peptides into the bilayer.

In this review, AFPs are presented on a family basis, with a particular interest on AFPs from plants, whose extract is more likely to be accepted for food applications. In addition, most of AFPs isolated from plants have been shown to be non-cytotoxic, unlike peptides isolated from invertebrates.

The diversity of AFPs is also marked by different sensitivities to salt concentrations, pH variation, and heat treatment. The comparison of peptide sequences and structures led to a better understanding of requirements for antifungal activity. Thus, efficient peptides with improved characteristics, such as solubility, stability, or toxicity, have been developed in a time-effective manner. Currently, some 1200 antifungal peptides have been identified in different databases (antimicrobial peptide database apd3, collection of antimicrobial peptides camp, data repository of antimicrobial peptides dramp), and many others are under patent submission.

This review gives an overview of active AFPs against food-related fungi and discusses the effects of food system environments on AFPs and their promising use as novel food preservatives. Peptides and proteins which are active only against *C. albicans* were deliberately omitted and the reader is referred to complementary reviews about AFPs (De Lucca and Walsh 1999; Desbois, Tschorner, and Coote 2011; Matejuk et al. 2011; Håvard Jenssen, Hamill, and Hancock 2006). In addition, many AFPs, particularly those originating from insect venom, are not discussed due to a too high hemolytic activity/cytotoxicity.

2.2 Plant sources

Due to environmental stresses and constant threats of pathogen attacks, plants have adapted with a broad range of defence tools. Facing pathogen invasion, plants are able to generate pathogenesis-related (PR) proteins in the different plant organs. At least seventeen different families have been created to group PR proteins possessing a wide range of defence-related properties (Christensen et al. 2002; van Loon and van Strien 1999). Several of these families, such as plant defensins (PR-12), thionins (PR-13) or

lipid transfer proteins (PR-14), display broad antimicrobial activities (Selitrennikoof 2001) and belong to the class of AMP. The classification of plant AMP and their subsequent segregation into PR families is based on several characteristics, such as nature and distribution of amino acids, particularly cysteine residues (Lay and Anderson 2005). In agreement with most of the literature about plant AMPs, the PR classification will not be further discussed in this review. The diversity of plant AMPs is illustrated by the absence of conserved sequences or structural patterns. The sequences and structural motifs of the different plant AFP families are presented in Table 2.1 and Figure 2.1, respectively. Despite a great overall diversity, many AFPs share specific biochemical characteristics. Plant AFPs are generally rich in cysteine and associated SS bonds, which confers them a high stability to heat treatment or protease digestion. They are also characterized by a high proportion (around 17%) of charged, mainly positively, amino acids (Barbosa Pelegrini et al. 2011). They also share mechanisms of antifungal activity, such as binding abilities to specific domains on the fungal plasma membrane (K Thevissen et al. 2005; Karin Thevissen et al. 2003). The main mechanisms of plant AFPs are summarized in Figure 2.2. In addition to strong antifungal activities (presented in Table 2.2), the antibacterial activity of AMPs from plants (Nawrot et al. 2014; James P. Tam et al. 2015), and fruits (Meneguetti et al. 2017) has been reviewed.

Thaumatin-like proteins

Antifungal **thaumatin-like proteins** (TLPs) are produced by diverse plant species such as barley (Van Der Weerden, Bleackley, and Anderson 2013), or chestnut (Garcia-Casado et al. 2000). **Thaumatin** is a 22-kDa protein, stabilized by 8 disulfide bonds, isolated from *Thaumatococcus daniellii*, an African plant species. This extremely sweet protein (100,000 sweeter than sucrose) is allowed as a sweetener in several food products. In general, TLPs are 21-26 kDa proteins, with sixteen conserved cysteine residues forming eight disulfide linkages. Their structure is made of three folded domains, with an interesting basic cleft, probably involved in the antifungal action. TLPs range of activity includes yeasts and filamentous fungi, such as *Fusarium* spp. or *S. cerevisiae*. **Osmotin** and **zeamatin,** expressed in tobacco and maize, respectively, are among the most described TL proteins. They provide protection towards abiotic stresses and invading phytopathogens. Osmotin inhibits the growth of

S. cerevisiae (IC_{50} = 0.3 μM) (D. J. Yun et al. 1998), *Fusarium solani*, and *Fusarium oxysporum* (IC_{50} = 67 $\mu g.ml^{-1}$) (C. D. T. De Freitas et al. 2011) by using different mechanisms depending on the fungal targets. TL proteins' main mechanism is believed to be due to their ability to create transmembrane pores. Thus, osmotin permeabilizes the membranes of *F. oxysporum* (Abad et al. 1996), whereas it induces signaling cascades and activates pheromone-response MAPK and stress response pathways in *S. cerevisiae* (D. J. Yun et al. 1997). Unlike zeamatin, osmotin docks specifically on cell wall phosphomannans to facilitate diffusion across the cell wall and subsequent cell damage (Ibeas et al. 2001). TL proteins demonstrate variable activities against fungal species, which has been attributed to specific composition of fungal cell wall and membrane. In addition to their low concentration required for activity, the structure of TL proteins imparts a great stability to heat- and proteolytic treatment of under varied pH (Smole et al. 2008; Roberts and Selitrennikoff 1990).

Thionins

Thionins are isolated from various plant species including barley and cowpea. All thionins are present in almost every crucial plant tissue from endosperm to leaves. These small proteins of around 5 kDa contain a high proportion of Arg, Lys and Cys residues, positively charged at neutral pH, and divided into 2 distinct groups: α-/β-thionins and γ-thionins. The group α-/β-thionins is subdivided into five classes based on the overall net charge and number of disulfide bonds. They contain two antiparallel α-helices and an antiparallel double-stranded β-sheet with 3 or 4 disulfide bridges, forming a characteristic stable gamma fold. Thionins are hydrophobic and display their antifungal activity via interactions of the hydrophobic and/or polar residues with membrane, with a possible lysis of cell membranes (De Lucca et al., 2005). *Capsicum annum* thionin-like peptide (CaThi) is an inhibitor of several *Candida* species and *S. cerevisiae* (Taveira et al. 2014, 2016). Yeasts treated with CaThi show structural damage to their membrane and eventual permeabilization. In *S. cerevisiae*, CaThi interacts with components of the plasma membrane, which alters the proton gradient, inhibiting the acidification of the medium, and inducing an increase in reactive oxygen species (ROS) formation. CaThi has been suggested to have cytoplasmic targets in *C. tropicalis* cells (Taveira et al. 2016). Although most antifungal thionins, such as viscotoxins (Giudici et al. 2006), show toxicity to mammalian cells, some thionins and

pseudothionins are safe. The stability of thionins is ensured by the SS bonds and the residue arginine in position 10, as it is critically involved in hydrogen bonding (HB) with associated secondary structures (Rao, Hassan, and Hemple 1994). However, thionins' ability to permeabilize fungal membrane is inhibited by the presence of salts, a property that might limit their utility as food agents.

Plant defensins

Plant defensins belong to the largest group of plant AMPs. These Cys-rich peptides (45-54 amino acids), with a mass of ~5 kDa, possess a characterisitc CSαβ motif, which comprises a β-sheet made of 3 strands, an α-helix and connecting disulfide bonds. They were first identified as γ-thionins from wheat and barley grains, and later grouped as plant defensins based on similarities with defensins from insects and mammals (Franky R G Terras et al. 1992). However, unlike mammalian defensins, plant defensins possess a fourth disulfide bond joining the two termini, which makes these peptides extremely stable. Some plant defensins have been shown to possess a fifth disulfide bond. Plant defensins consist of over 100 members and are present in multiple tissues, especially seeds (Nawrot et al. 2014). However, sequence homology of plant defensins from different species is limited to a number of characteristically conserved residues: eight Cys, two Gly, one Glu and an aromatic residue, at specific positions in the amino acid chain (Kovaleva et al. 2009). This diversity emphasizes the diversity of targets and antifungal mechanisms (Valente, De Paula, and Almeida 2013). These defensins are considered as members of the plant immune system, since they are not only overexpressed during microbial invasion, but also in response to several attacks and during plant development (Stotz, Thomson, and Wang 2009). Although they are active mainly active against phytopathogens, there is an increasing interest to combat fungal infection and biofilms formation from *C. albicans* in humans (Cools et al. 2017).

The radish defensin Rs-AFP2 is one of the most characterized defensins. It is active against a broad range of filamentous fungi (F. R. G. Terras et al. 1992). Rs-AFP2 binds to the sphingolipid glucosylceramide (GlCer), permeabilizing the fungal membrane and causing a Ca^{2+} influx and K^{+} efflux which results in alkalinisation of growth media (Vriens, Cammue, and Thevissen 2014). The activity of Rs-AFP2 appears to be dose-dependent with stronger permeabilization and induction of ROS generation at high

concentrations (Aerts et al. 2007). Like Rs-AFP2, MsDef1, isolated from *Medicago sativa*, requires the presence of GlCer to perform antifungal activity against *Fusarium graminearum*. MsDef1 interacts with the fungal membrane, blocks L-type calcium channels, and activates MAPK signalling in *F. graminearum*. Another sphingolipid, MIP2C, is also the binding site of the plant defensin DmAMP1, isolated from *Dahlia merkii*. DmAMP1 causes a Ca^{2+} influx, a K^{+} efflux, and alkalinisation of the growth media. *Fusarium* species are particularly sensitive to the tomato defensin, TPP3, or the tobacco defensin, NaD1 (MIC = 1 µM) (Baxter et al. 2015; Van Der Weerden, Hancock, and Anderson 2010). TPP3 permeabilizes fungal membrane after specifically binding to phosphatidylinositol (4,5)-bisphosphate (PIP2) (Baxter et al., 2015). Following interaction with the cell wall, NaD1 enters the hyphae via membrane permeabilization and kills by induction of oxidative damage. PvD1, a defensin isolated from *Phaseolus vulgaris* cv. *Perola* seeds, is a 6-kDa peptide that exhibits a wide range of anti-yeast activity towards *Candida* spp., *Kluyveromyces marxianus* and *S. cerevisiae* (Games et al. 2008; Salas et al. 2015). In addition to membrane permeabilization, PvD1 can inhibit the glucose-stimulated acidification of the medium by yeasts and induce the formation of ROS and nitric oxide (NO) in yeast cells (Mello et al. 2011). Some plant defensins possess intracellular targets, such as PsD1, whose antifungal activity is based on the halt of fungal cell cycle.

Due to multiple mechanisms and/or interactions with important fungal membrane structures, the frequency of resistant mutants is likely to develop more slowly than antifungal molecules interacting with a single site (Mccoll et al. 2018). However, mutants of *S. cerevisiae* show resistance to the defensin HsAFP1. The action of this peptide requires a properly working yeast respiratory chain (Karin Thevissen et al. 2007; Aerts et al. 2011) and *S. cerevisiae* can generate spontaneous respiration-deficient mutants.

Plant defensins can generally maintain their activity in extreme temperatures, pH or oxidative conditions. However, the effect of cations appears to be peptide- and fungus-dependent. For example, Rs-AFP2 retains its activity against *F. graminearum* in presence of KCl or $CaCl_2$, whereas the inhibitory effect of Ms-Def1 is reduced in presence of Ca^{2+} ions.

Lipid transfer proteins

As their name suggests, the primary role of the small proteins, named **lipid transfer proteins (LTPs)**, is to transfer lipids within the plant tissues. All land plants, such as radish, barley or onion contain LTPs, in most tissues, either in seeds or leaves (Nawrot et al. 2014). They are also important allergens in fruits or nuts (Egger et al. 2010). In addition to their lipid carrier function, LTPs display antifungal activity. The 2 subfamilies of LTPs, LTP1s and LTP2s have respectively a molecular mass of 9 kDa and 7 kDa. These proteins are made of 4 to 5 helices stabilized by 4 disulfide bonds with a strong hydrophobic feature. LTPs are thought to permeabilize the fungal membrane, as highlighted by the mechanistic study of the *Helianthus annuus* peptide Ha-AP10. However, the mechanism is not fully identified. Cc-LTP1, isolated from coffee seeds (*Coffea canephora),* exhibits antifungal activity against *C. tropicalis* and to a lesser extent *S. cerevisiae*. Cc-LTP1 permeabilizes yeast plasma membrane and is able to interfere with the glucose-stimulated H^+-ATPase-dependent acidification of the growth medium (Zottich et al. 2011). Ca-LTP1, isolated from chili peppers, inhibits the growth of *F. oxysporum* (100 µg/ml), *C. tropicalis* and *S. cerevisiae* (8 µg/ml) (Diz et al. 2011; Cruz et al. 2010). Ca-LTP1 has been shown to use different mechanisms, such as disruption of membrane of *C. tropicalis*, and the ability to inhibit of growth medium acidification by *S. cerevisiae* (Diz et al. 2011). The antifungal activity of several LTPs has been reported to be thermo-, pH-, and proteolytically-stable (Peng Lin et al. 2007).

Snakins

Snakins were originally isolated from potato, but have recently been found in other species, such as tomato (*Solanum lycopersicum*). These 7 kDa-peptides contain a stable two α-helical structure stabilized by 6 disulfide bonds, with an essential C-terminus. This conserved C-terminus ensures biological activity and stability of the peptide. Upon tissue degradation or invasion, potato (*Solanum tuberosum* cv *Jaerla*) tubers express a peptide termed snakin-2 (StSN2). StSN2 shows activity towards several phytopathogens such as *Fusarium* spp. and *A. niger* (IC_{50}= 1–20 µM). Other snakins, such as a 64-residue homolog of StSN2 identified in French bean or snakin Z isolated from Jujuba fruits, are active against *A. niger* (Daneshmand, Zare-Zardini, and Ebrahimi 2013). Another 66-residue snakin-2 (Sn2) found in tomato shows a broad-

spectrum activity, including against *S. cerevisiae* (MIC = 4.25 µM). The mechanism of Sn2 includes its agglomeration into single yeast cells prior to killing (Herbel, Schäfer, and Wink 2015) and the formation of pores in the membrane of target cells. However, snakins are not predicted to act via membrane permeabilization, since they were inefficient on artificial liposomes. There is still a lack of knowledge about their mode of action (Berrocal-Lobo et al. 2002). The major hurdles for food application is the alteration of their activity in the presence of cations, as shown with Sn2 against *S. cerevisiae*. Moreover, Sn2 shows 20% hemolytic activity at 17 µM and 100% at 35 µM.

Glycine-rich peptides

Common guava (*Psidium guajava*) and coffee (*Coffea canephora)* seeds contain storage peptides with antifungal potential, termed **glycine-rich peptides**. They have an abundant content of Gly (more than 60%) and a specific hydrophobic character. The structure of these peptides consists of α-helices at C- and N-termini, with the presence of Arg residues at their extremities. These peptides do not form disulfide bonds, due to the absence of Cys residues. The antifungal activity of Gly-rich peptides appears to be species-dependent. A *coffee* seeds Gly-rich peptide, *Cc*-GRP, (Zottich et al. 2013) inhibits *F. oxysporum* and yeasts (Mousavi and Hotta 2005). It also alters the permeability of *F. oxysporum* hyphal membrane (Zottich et al. 2013) and prevents colony formation by yeasts (Mangeon, Junqueira, and Sachetto-Martins 2010). This peptide seems to inhibit *F. oxysporum* with several mechanisms, as revealed by its ability to target fungal cell wall or nucleus. However, these peptides are generally poorly studied with regard to their antifungal activity or their stability.

2S albumins

2S albumins are storage proteins used by seeds as a source of nitrogen during germination. 2S albumins play a protective role in plants, exhibiting antifungal activity (Agizzio et al. 2003, 2006), as shown with 2S albumins isolated from *Malva parviflora* or radish (*Raphanus sativus*) seeds. They are synthesised as a polypeptide of 18-21 kDa in response to pathogens, and cleaved into two peptides as a post-translational modification (PTM). The two peptide subunits of small and large chain are around 4–9 kDa. They present similar characteristics to AFPs, such as richness in cationic

residues and presence of disulfide bonds. The 2S albumins Pf2 and Pe-AFP1, identified in seeds of *Passiflora edulis f. flavicarpa,* show activity against *Aspergillus fumigatus, F. oxysporum,* and the yeast *S. cerevisiae* (P. B. Pelegrini et al. 2006; Meneguetti et al. 2017).

These 2S albumins permeabilize the fungal membrane, leading to an impairment of the H^+/ATPase and inhibition of the glucose-stimulated acidification of the media. This mechanism is also found for the 2S albumin, Ca-Alb, isolated from *Capsicum annuum* (Suzanna F F Ribeiro et al. 2012). Cells treated with the 2S albumin Pf-Alb exhibit an apoptotic-like behaviour, including shrinking cytosol and cell wall deformation. In addition, the growth of yeasts *K. marxianus* and *C. parapsilosis* is inhibited via NO induction in presence of Ca-Alb. Unfortunately, 2S albumins are also considered to be food allergens (F. J. Moreno and Clemente 2008).

The presence of 8 Cys residues and several α-helices ensures a high stability of the protein structure (Ullah et al. 2015). However, the sensitivity of 2S albumins to high concentrations of salt and the concentration required to induce significant inhibition represent crucial hurdles to their potential use as food preservatives (Suzanna F F Ribeiro et al. 2012). Among the 2S albumin family, napins show high water solubility, an interesting property for food applications. Napins have been first isolated from *Brassica napus* (rapeseed) and several napin-like peptides have been identified (Ngai and Ng 2004).

Puroindolines

Puroindolines have been isolated from wheat and barley and are characterized by five disulfide bridges with a similar structure to LTPs. These 13 kDa-proteins contain a unique amphiphilic Trp-rich domain. Seed puroindolines are divided into two variants, puroindoline-a (PIN-a) and puroindoline-b (PIN-b), with distinctive sequences. The antifungal activity of purodindolines has been reported against *S. cerevisiae, F. graminearum* and *F. oxysporum*. The Trp-rich domain is involved in the antifungal activity via membrane-binding and the creation of ion channels (Charnet et al. 2003). The stability and, more generally, the role of puroindolines as antifungal agents remains poorly studied.

7S globulins

7S globulins (vicilin-like) belong to another class of storage proteins that could represent up to 80% of total seed proteins. Cysteine-rich peptides from 7S globulins are capable of inhibiting potential pathogens around the germinating seed (Marcus et al. 1999). MiAMP2c, a precursor protein homologous to 7S globulins, inhibits the growth of *S. cerevisiae* at a concentration of 50 $\mu g.mL^{-1}$ (Marcus et al. 1999). Another antifungal vicilin-like peptide from *Capsicum baccatum* (melon fruit) seeds also inhibits *S. cerevisiae*. The chitin-binding ability of 7S globulins and subsequent membrane and H^+-pumps degradations are associated with their antifungal properties (S. F F Ribeiro et al. 2007; Bard et al. 2014). However, some 7S globulins can inhibit the growth of *S. cerevisiae*, indicating that these proteins could associate with other cell surface components (S. F F Ribeiro et al. 2007).

Hevein-like peptides

Hevein-like peptides are named after the peptide **hevein**, due to amino acid sequence and structure similarities. Isolated from the rubber tree *Hevea brasiliensis*, **hevein** is a small 4.7 kDa protein. It is characterized by a high content of Cys residues and chitin-binding abilities (Van Parijs et al. 1991). **Hevein-like peptides** are 29 to 45 residues long and have been identified in several plants. All hevein-like peptides have a cystine-knot motif with coil and β-structures. The distinction in 3 subfamilies is based on the number of Cys residues (6, 8 or 10), with 8 Cys being the most common form. Besides the fact that the typical chitin-binding motif contains usually several Cys and Gly residues, there are Pro-rich hevein-like peptides, found in *Ginkgo* species, the so-called ginkgotides (Wong et al. 2016). Hevein-like peptides are thought to bind chitin and subsequently inhibit the cell wall biosynthesis (Beintema 1994). The presence of aromatic residues in the peptide sequence plays a major role in the interaction between the hydrophobic groups of chitin and the π-electron systems. Other mechanisms, such as permeabilization of the fungal membrane and alkalinization of the media, have been shown for the peptide GAFP. Following interaction with chitin, this peptide, isolated from the leaves of *Ginkgo biloba*, inhibits several *Fusarium* species, namely *F. graminearum*, *Fusarium moniliforme* (X. Huang, Xie, and Gong 2000). The peptides WAMP-1a and WAMP-1b from wheat *Triticum kiharae* seeds show a strong inhibition of *Fusarium spp.*, with an IC_{50} of 5-30 µg/ml (Odintsova et al. 2009).

In contrast to most hevein-like peptides, a Japanese morning glory (*Pharbitis nil*) peptide Pn-AMP1, does not require the typical binding of chitin. It is active via actin depolarization leading to growth arrest. Pn-AMP1 shows interesting properties such as high basicicty (pI 12.02) and heat-stability. Moreover, the hevein-like peptides, vaccatides, from *Vaccaria hispanica*, keep their antifungal activity after heat-treatment and in acidic (pH 2.0) conditions (Wong et al. 2017). However, the antifungal activity of several hevein-like peptides, namely EAFP1 and EAFP2, from *Eucommia ulmoides Oliv*., is antagonized by calcium ions (R. H. Huang et al. 2002).

Plant knottins

Plant knottins are part of a superfamily of peptides of around 30 residues. Knottins, most of which are linear, typically contained 3 conserved disulfide bonds, which forms a cystine knot. However, their Cys motifs, and associated secondary and tertiary structures, differ amongst subfamilies. First discovered as protease inhibitors (PIs), knottins and PIs share a characteristic cystine-knot (CCK) motif. The CCK pattern is thought to give the peptides good stability and make the overall structure amphipathic (Grage et al. 2017). This pattern is found in knottin-type AFPs isolated from plants, such as Mj-AMP1 from the Peruvian *Mirabilis jalapa* L. and the pokeweed (*Phytolacca americana)* PAFP-S (Gao et al. 2001; Cammue et al. 1992). PAFP-S is made of an anti-parallel β-sheet (of 3 strands) and a long loop region. The γ-core of the peptide (a largely conserved structure among AMPs and, in most cases, essential for their activity) forms a β-hairpin in the C-terminal domain.

Plant knottins are characterized by a high stability to heat, chemical or enzymatic treatment (Heitz et al. 2008). The peptides of the **cyclotide** subfamily are among the most stable knottin-type peptides. These circular peptides of 28 to 37 residues have been identifed in bacteria, plants, and animals. A head-to-tail cyclical backbone and 3 intramolecular disulfide bonds confer them a supplementary stability. Cyclotides are classified into several subfamilies: Moebius cyclotides, which contain a 180° backbone twist, the bracelets cyclotides and hybrid Moebius cyclotides, named kalata, first isolated from *Oldenlandia affinis* (Patrícia B. Pelegrini, Quirino, and Franco 2007). Coffee plants contain f*our cyclotides* (kalata, circulin A and B, and cyclopsychotride) with activity against *Candida kefyr* (MIC 14.0 - 29.0 μM) and *C. tropicalis* (MIC 21.4 -56.5 μM) (J P Tam et al. 1999). Membrane interactions between

cyclotides and phospholipids, and a cell penetrating behaviour, may be involved in the various biological activities (Greenwood et al. 2007), but their mechanism of action remains unknown. The tricystine-stabilized α-stranded structure of cyclotides makes them stable under high salt conditions. Thus, this type of constraint is envisioned in the design of novel peptides to minimize conformational changes and salt sensitivity.

α-hairpinins

Peptides with a characteristic cysteine arrangement ($C(1)X_3C(2)X_nC(3)X_3C(4)$) and an helix-loop-helix structure stabilized by disulfide bonds are generally classified as **α-hairpinins.** A few numbers of these Lys/Arg-rich peptides displaying antifungal activity have been reported (James P. Tam et al. 2015). Among these, Sm-AMP-X1-2 from *Stellaria media* and Tk-AMP-X1/X2 extracted from *T. kiharae* display antifungal activity against phytopathogens such as *A. niger* or *F. oxysporum* (Slavokhotova et al. 2014; Odintsova et al. 2009). Antifungal activity of a barnyard grass *Echinochloa crus-gali* peptide, termed Ec-AMP1, has been demonstrated against several phytopathogens (IC_{50} = 1–10 μM) after binding to the conidial surface and internalization (Utkina et al. 2013). Interestingly, the α-hairpinin scaffold has a similar structure to potassium channel blockers, which could indicate a related mechanism of action (Berkut et al. 2014). Interestingly, heat treatment leads only to a partial degradation of α-hairpinins.

Protease inhibitors

Protease inhibitors (PI) are found in a variety of plants, although plants of the *Solanaceae* family, such as *Solanum tuberosum* show particularly high levels. They are considered as storage proteins and defensive compounds. They generally have a higher molecular weight than other AFPs and contain a unique disulfide-linked, nine-residue loop. Among these PIs, the most studied are soybean trypsin inhibitors (Kunitz) and soybean proteinase inhibitors (Bowman-Birk, BBI) (J. Y. Kim et al. 2009; Pesoti et al. 2015). BBIs are cysteine-rich proteins of 1.5 - 20 kDa, with up to 7 disulfide bonds, that possess inhibitory activities against proteases (Fernandez et al. 2007). Kunitz-type inhibitors, on the other hand, are 18 - 24 kDa peptides with 2 SS bonds. *F. oxysporum* has been reported to be sensitive to BBI from broad beans and a

Kunitz-type trypsin inhibitor from the roots of ginseng (H. X. Wang and Ng 2006). These PIs are commonly believed to be stable in a broad range of pH and at a temperature of 100°C (Losso 2008), although some studies show conflicting results (Dipietro and Liener 1989).

Unclassified peptides

Other peptides have not been classified into the previous groups, due to a specific sequence or structural profiles. The rose balsam *Impatiens balsamina* produces small AFPs made of 20 residues, named **Ib-AMPs**. These peptides contain a loop structure stabilized by two disulfide bonds and a distinctive Glu residue at the N-terminus. However, these bonds are thought to be unessential in the antimicrobial activity. Ib-AMPs are active against a broad range of fungal phytopathogens, including the mould *F. culmorum* (IC_{50} = 1 - 2 µg/ml) and the yeasts *Pichia pastoris* and *S. cerevisiae e*(Karin Thevissen et al. 2005). It is noteworthy that they are still active in 1 mM $CaCl_2$ and 50 mM KCl (Peng Wang et al. 2009), despite a strong reduction of activity. Filamentous fungi are more sensitive to Ib-AMPs than yeasts. A possible explanation is the binding properties of Ib-AMPs to chitin, a component found in higher concentration in filamentous fungi cell wall than in yeasts (van der Weerden and Anderson 2013). However, the mechanism of action of Ib-AMPs is not clearly identified but involves binding to the cell wall.

Shepherins are plant AFPs with a high basic character (net charge +8). Due to the presence of multiple histidine residues (C. J. Park et al. 2000) and the repetition of a Gly-Gly-His sequence motif, sheperins have unique properties. The shepherd's purse, *Capsella bursa-pastoris* shepherins, Shepherin 1 and Shepherin 2, are active against *C. tropicalis* and *S. cerevisiae* but inactive against filamentous fungi (Remuzgo et al. 2014; C. J. Park et al. 2000). The mechanism of shepherin's yeast inhibitory activity has not been identified. Other AFPs include peptides isolated from the Japanese bamboo, *Phyllostachys pubescens* (Pp-AMP) (Fujimura et al. 2005), or the basic 38 to 44 residues ToAMPs, identified from *Taraxacum officinale* flowers (Astafieva et al. 2013). TnAFP1, an interesting 1.2 kDa peptide isolated from water chestnut, or MiAMP1, from the nut kernel of *Macadamia integrifolia* (Manners 2009) belong to the class of AFPs. Interestingly, MiAMP1 is a 76-amino acids basic peptide, which has a particular Greek key motif that has also been found in yeast killer toxin.

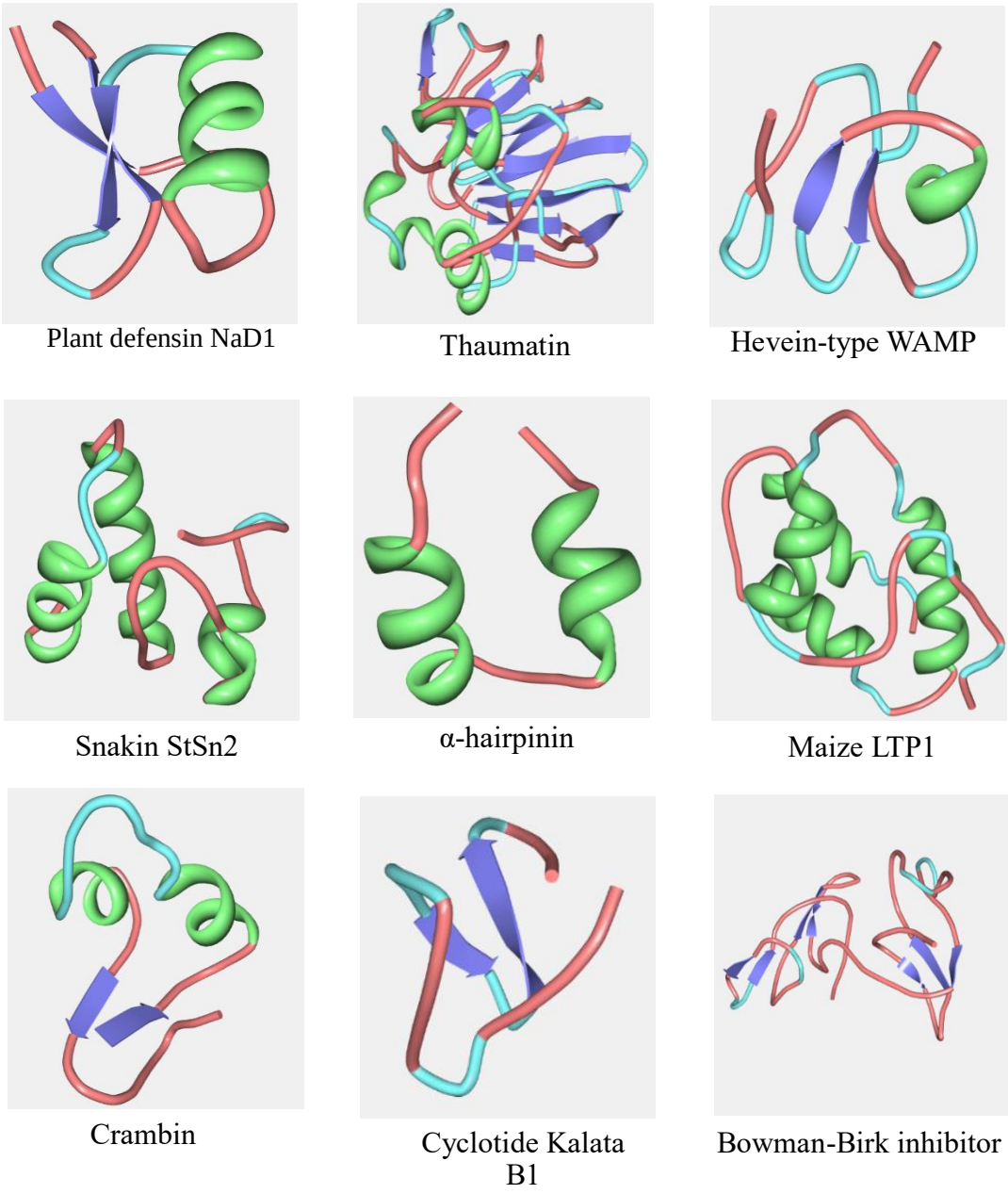


Figure 2.1. Structural diversity of plant antifungal peptides/proteins. Secondary structures are represented by green α-helices, purple arrows as β-strands, red random coil and blue turns. The 3D representations were from RCSB (http://www.rcsb.org/) and modified via Protein Works.

Table 2.3. Families and sequences of antifungal peptides/proteins isolated from plant sources (obtained from apd3) and discussed here

Family	Peptides	Sequence
Thaumatin-like proteins	Thaumatin	ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTKGGKIWARTDCYF
	Osmotin	ATIEVRNNCPYTVWAASTPI-----GGGRRLDRGQTWVINAPPGTKMARVWGRTDCNF
Thionins	Viscotoxin A3	KSCCPNTTGRNIYNACRLTGAP-RPTCAKLSGCKIISGSTCPSDYPK
	Crambin	TTCCPSIVARSNFNVCRLPGTP-EALCATYTGCIIIPGATCPGDYAN
	Pth-St1	RNCESL—SHRFK---GPCTRDSNC
	α-purothionin	KSCCRTTLGRNCYNLCRSRG-A--GKLCSTVCRCKLTSFLSCPKGFPK
Plant defensins	NaD1	---REC-KTESNTFPGICITKPPCRKACIS---EKFTDG-HCSKI—LRRCLCTKPC
	Rs-AFP2	--QKLC-QRPSGTWSGVCGNNNACKNQCIRLE—KARHG-SCNYVFPAHKCICYFPC
	MsDef1	---RTC-ENLADKYRGPCFSG--CDTHCTTKE--NAVSG-RCRDD---FRCWCTKRC
Lipid transfer proteins	Mung bean	-MTCGQVQGNLAQCIG-FLQK-GGVVPPSCCTGVKNILNSSRTTADRRAVCSCLKAAAGAVRGINPNNAEALPGKCGVNIPYKISTSTBCNSIN
	Ca-LTP1	-VTCGQIQVGVVNCLP-YLQN--RGPLGRCCGVIKDLLKLCKTPHERRKSCRCVKTAANTIKGIDFGKTAGLSGVCGVKIPSEISPSVDCSKVK
	Bn-LTP1	ALSCGTVSGNLAACIG-YLTQ—GPLPRGCCTGVTNLNNMARTTPDRQQACRCLVGAANAFPTLNAARAAGLPKACGVNIPYKISKSTNCNSVR
Snakins	StSN2	YKKIDCGGACAARCRLSSRPRLCNRACGTCCARCNCVPPGTSGNTETCPCYASLTTHGNKRKCP
	Snakin-like	LQQIDCNGACGARCRLSSRPNLCKRACGTCCQRCNCVPPGTSGNQEMCPCYASLTTRGGKRKCP
2S albumins*	*C. annuum*	P-QCPRCSQQFQQAKQLRCSCQS
	B. napus (napin)	PFRIPKCRKEFQQAQHLR-ACQQ
	R. sativus	P-R-QRCQKEFQQSQHLR-ACQR
	Pf2	-PSERCRRQMQGQDFS - - ----
	Pe-AFP1	QSERFEQQMQGQDF-SHDERFLSQAA PFRIKKRQGPFERP

Table 2.3. *contd*

Family	Peptides	Sequence
Puroindolines*	PIN-a	AQQCPVETKLNS-CRNYLLDRCSTMKDFPVTWRWWKWWKGGCQELLGE-CCSRLGQMPPQCRCNIIQGSIQGDLGGIFGFQRDRASKVIQEAKNLPPRCNQGPPCNI
	PIN-b	SQQCPQERPKLSSCKDYVMERCFTMKDFPVTWPT-KWWKGGCEHEVREKCCKQLSQIAPQCRCDSIRRVIQGRLGGFLGIWRGEVFKQLQRAQSLPSKCNMGADCKF
Hevein-like peptides	Hevein	EQCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQSN-CKD–
	Pn-AMP1	QQCGRQASGRLCGNRLCCSQWGYCGSTASYCG--AGCQSQ-CRS–
	WAMP-1	QRCGDQARGAKCPNCLCCGKYGFCGSGDAYCG-AGSCQSQ-CRGC
	vH1	FQCGRQAGGARCSNGLCCSQFGYCGSTPPYCG-AGQCQSQ-CA---
	GAFP	PTCSVLGDFK-CNPGRCCSKINYCGACYQWRFGLTPAR
	EAFP-1	QTCASRCPR-PCNAGLCCSIYGYCGSGNAYCG-AGNCRCQ-CRG--
Knottins	PAFP-S	AGCIKNGGRCNASA-GPPYCCSSY-CFQIAGQSYGVCKNR
	Mj-AMP1	-QCIGNGGRCNENV-GPPYCCSGF-CLRQPGQGYGYCKNR
Cyclotides	Kalata B1	GLPVCGETCVGGTC-NT--PGCTCSWPVCTRN
	Circulin A	G-IPCGESCVWIPC-ISAALGCSCKNKVCYRN
α-hairpinins	SmAMP-X2	---VDPDVRAYCKHQC-MSTRGDQ--ARKICESVCMRQD------
	ECAMP1	-----GSGRGSCRSQC-MRRHEDEPWRVQECVSQCRRRRGGGD-
Impatiens balsamina peptides	Ib-AMP1	QWGRRCCGWGPGRRYCVRWC
	Ib-AMP3	-YRHRCCAWGPGRKYCKRWC

*: only large subunit was represented without disulphide bonds

Table 2.4. Antifungal peptides/proteins isolated from plant sources

Peptide group	Peptides	Source	Antifungal activity	References
Plant defensins	TPP3	Tomato, *Solanum lycopersicum*	*Candida* spp.	(Baxter et al. 2015)
	Rs-AFP2	Radish, *Raphanus sativus*	*Aspergillus* spp., *S. cerevisiae*	(Franky R G Terras et al. 1992; Karin Thevissen, Terras, and Broekaert 1999)
	MsDef1	Alfalfa, *Medicago sativa*	*Fusarium* spp.	(Sagaram et al. 2011)
Thaumatin-like	Osmotin	Tobacco, *Nicotiana tabacum*	*S. cerevisiae, F. solani*	(Abad et al. 1996)
Thionins	Pth-St1	Potato, *Solanum tuberosum*	*Fusarium solani*	(M. Moreno, Segura, and Garcia-olmedo 1994)
	Viscotoxin A3 and B	Mistletoe, *Viscum album*	*F. solani*	(Giudici et al. 2006)
LTPs	Ca-LTP1	Chili Pepper, *Capsicum annuum*	*F. oxysporum, C. tropicalis*	(Diz et al. 2011)
	Bc-nsLTP	Mustard, *Brassica campestris*	*F. oxysporum*	(Peng Lin et al. 2007)
2S albumin	Pf2, Pef	Passion fruit, *Passiflora edulis*	*F. oxysporum*	(P. B. Pelegrini et al. 2006)
	Bn-2S	*Brassica napus*	*F. culmorum, F. oxysporum*	(Ngai and Ng 2004)
Ib-AMPs	Ib-AMP1-4	*Impatiens balsamina*	*F. culmorum, P. digitatun*	(Peng Wang et al. 2009; Karin Thevissen et al. 2005)
Snakins	StSN1-2	Potato, *Solanum tuberosum*	*Fusarium* spp., *A. flavus*	(Berrocal-Lobo et al. 2002)
	Snakin Z	Jujuba fruits	*A. niger*	(Daneshmand, Zare-Zardini, and Ebrahimi 2013)
Cyclotides	Kalata B1	*Oldenlandia affinis*	*C. tropicalis, C. kefyr*	(J P Tam et al. 1999)
Knottins	Mj AMP2	Marvel-of-peru, *Mirabilis jalapa*	*F. oxysporum, B, cinerea*	(Cammue et al. 1992; Gao et al. 2001)
	PAFPs	American pokeweed	*F. oxysporum, F. graminearum*	

Table 2.4. *contd*

Peptide group	Peptides	Source	Antifungal activity	References
Hairpinins	Sm-AMP-X2	Common chickweed, *Stellaria media*	*A. niger, F. oxysporum*	(Slavokhotova et al. 2014) (Utkina et al. 2013)
	EcAMP-1-3	Barnyard grass, *Echinochloa crus-galli*	*Alternaria alternata, A. niger*	
Hevein-like peptides	GAFP	*Ginkgo, Ginkgo biloba*	*F. graminearum, F. moniliforme*	(Wong et al. 2017, 2016)
	WAMP-1a and –b	Wheat, *Triticum aestivum*		
	Vaccatides	*Vaccaria hispanica*		
Puroindolines	Puroindoline A	Wheat, *Triticum aestivum*	*S. cerevisiae, F. culmorum, F. graminearum*	(Charnet et al. 2003)
	Puroindoline B	Barley, *Hordeum vulgare*		
Gly-rich	Cc-GRP	Coffee, *Coffea canephora*	*F. oxysporum*	(Zottich et al. 2013)
Protease inhibitors	Kunitz –type	Potato *Solanum tuberosum*	*S. cerevisiae*	(J. Y. Kim et al. 2009; Pesoti et al. 2015)
	Bowban-birk			

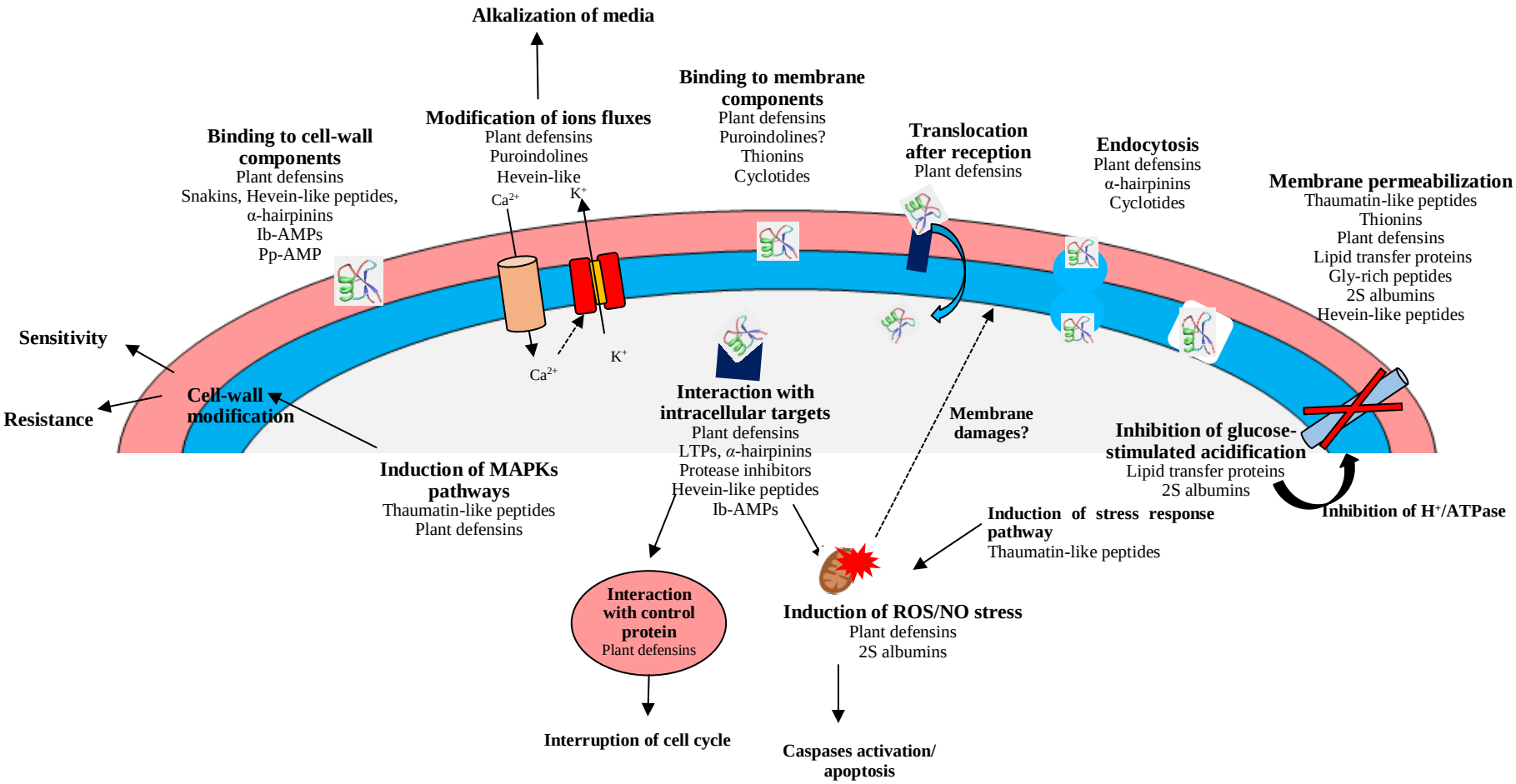


Figure 2.2. Diversity of mechanisms used by natural antifungal peptides isolated from plants. This representation is a modification of the figure (and described mechanisms) presented in the review by Van der Weerden et al. (2013)

2.3 Other sources

Plants have developed a specific defence system including a large number of AFPs in order to respond to fungal invasion. The particular characteristics of AFPs, such as basic and hydrophobic residues and conformations often stabilised by Cys residues, are also found in peptides produced by organisms from the other kingdoms. Vertebrates, invertebrates and microbes are not immune to fungal threats and respond to attacks with a large range of AFPs. However, these peptides are not considered in detail in this review, due to generally less convenient extraction processes, a limited "natural" aspect for consumers and potential toxicity. For a more complete description of these peptides, the reader is referred to previous reviews (Lohner and Leber 2016; Faruck, Yusof, and Chowdhury 2016; Matejuk et al. 2011; Van Der Weerden, Bleackley, and Anderson 2013; Håvard Jenssen, Hamill, and Hancock 2006).

Vertebrate sources

Vertebrates have developed a complex immune system with innate and adaptive components. Despite its efficacy, the slower response of the adaptive immune system requires a quick action of the innate system. This system includes a rich composition of AFPs with some of these being active against food-related microorganisms. A non-exhaustive list of peptides/proteins from vertebrates is presented in Table 2.3.

Lactoferrin is a multifunctional mammalian, iron-binding, antimicrobial glycoprotein (78 kDa), which is present in neutrophil granules, breast milk, and on many mucosal surfaces. Several mechanisms are involved in the antifungal activity of this protein. It causes a slight K^+ efflux and depolarizes the fungal membrane, inducing cytoplasmic acidification. Cellular respiration has been shown to be necessary for its activity, suggesting that lactoferrin does not disrupt the membrane but targets intracellular components. Enzymatic degradation of lactoferrin produces several antifungal peptides (lactoferricin H, B) with greater antifungal activity than the parental protein (Kanyshkova, Buneva, and Nevinsky 2001; Fernandes and Carter 2017) against *S. cerevisiae* and *P. digitatum*. Bovine and human lactoferricin adopt different folded structures, stabilized by disulfide bonds. The difference in the content in α-helix and antiparallel β-sheet structures contributes to different efficacies.

Cathelicidin peptides belong to a family of peptides of 12 to 80 residues found in mammals. They possess diverse structures, although most are linear. Indolicidin and tritrpticin are cathelicidin-related peptides, rich in Trp residues and showing antifungal activity against *Candida* spp. (MIC, 8–32 μg/ml) (D. G. Lee et al. 2003). *S*everal analogues of bovine cathelicidin-derived peptides, called BMAP, are effective inhibitors of *Candida* spp., *S. cerevisiae*, and the moulds *Aspergillus* spp. and *Penicillium* spp. (2-32 μM) (Benincasa et al. 2006). However, filamentous fungi show lower sensitivity than yeasts to cathelicidins, a difference attributed to the evolutionary response to the greater threat of yeast to mammals. Despite the structural diversity, all cathelicidins appear to perturbate the fungal membrane. Although their use is limited due to hemolytic activity at high concentrations, they are used as a lead structure for less hemolytic peptides, such as CP-11 (D. I. Chan, Prenner, and Vogel 2006).

Mammalian defensins are structurally similar to plant defensins, lacking only one disulfide bond. They are found in high concentrations in small intestine neutrophils and Paneth cells and are divided into 3 categories based on structural properties and cysteine spacing: α-, β- and θ-defensins (P. M. Silva, Gonçalves, and Santos 2014; Lehrer and Lu 2012). β-defensins *contain* 38 to 47 amino acids, with a disulfide linkage motif C1-C5, C2-C4, C3-C6. Human β-defensins are active against *Candida* spp., including *C. tropicalis* and *Candida krusei*, with a MIC between 2-15 μg/ml. The mechanisms involved in the antifungal activity of defensins remain to be elucidated. It is suggested this does not involve membrane disruption, as highlighted by calcein-release assays. Instead, the peptides are thought to bind cell-wall components and target intracellular components. The triggers of stress response pathways, such as the high-osmolarity glycerol (HOG) pathway, have been highlighted in *S. cerevisiae* after incubation with *β*-defensin. Increased concentrations of cations are known to reduce the antifungal activity of *β-defensins*, including the highly positively charged human *β*-defensin 3. However, a synthetic linear HBD-3 retains its activity against *F. culmorum* in presence of 100 mM NaCl, in addition to its heat stability (Thery et al. 2016). Unlike other members of the defensin family, the rhesus Θ-defensin, RTD-1, isolated from monkey leucocytes, is salt-insensitive.

Histatins are human saliva peptides with a specific activity towards fungi, especially against *Candida* species, including food-related species (Nikawa et al. 2001). These

linear His-rich peptides adopt α-helical structures in hydrophobic environments and possess a low amphipathicity. Their low amphipathicity is likely to be related to an internalization mechanism. After binding to the membrane protein Ssa2p, histatin 5 is internalized and alters the integrity of the mitochondrial membrane. It is also able to induce a release of ATP. The activity of histatins appears to be inhibited by the presence of Ca^{2+}, which limits the interaction and binding between the peptide and the heat-shock protein Ssa2p.

Several AFPs are also expressed in the liver and, hence, belong to the liver-expressed antimicrobial peptide (LEAP) family. Among these peptides, **hepcidins** are active against *A. niger* and *S. cerevisiae* (C. H. Park et al. 2001).

Frog skin contains an important arsenal of AMPs, including brevinins and temporins. **Brevinins** and **temporins** belong to the families of peptides identified in *Rana* frog species (Pál et al. 2006). The brevinin superfamily includes the brevinin-1 family, containing brevinin-1YB, a 24-residue peptide with antifungal activity against *Candida* spp. They are characterized by a looped C-terminal region comprising a single disulfide bond. The Cys residues are essential, since their substitution with Ser abolishes the antifungal activity. **Temporins** are small peptides (10 to 14 residues), isolated from the skin of the red frog species, including the red frog *Rana temporaria*. These C-amidated peptides show activity against several *Candida* species and *S. cerevisiae*, by presumably permeabilizing the cell membrane. However, the full mechanism underlying their antifungal activity remains unknown. Other frog species also possess a large range of AFPs such as magainins or dermaseptins. Magainins**,** secreted by the African frog *Xenopus laevis*, are a family of cationic amphipathic peptides that are between 21 to 26 amino acids in length and are enriched in Gly and Ser residues. Magainins have antifungal activity against *C. tropicalis* (MIC, 12.5 μg/ml), *C. krusei* (MIC, 12.5–25.0 μg/ml) and *S. cerevisiae* (Burrows et al. 2006; Morton et al. 2007). Dermaseptins **(S and B)** are Lys-rich peptides with a characteristic Trp at position 3. They have been identified in the skin of tree frogs of the genus *Phyllomedusa*. Dermaseptins are cidal to pathogenic fungi including yeasts and the mould *A. fumigatus* (Morton et al. 2007; De Lucca and Walsh 1999). Other AFPs from frog, active against Candida spp. include **bombinins**, **buforins**, **aureins**, **citropin 1.1.** (Souza et al. 2013; Kamysz et al. 2006; Simmaco, Kreil, and Barra 2009).

Table 2.5. Antifungal peptides/proteins isolated from vertebrate sources

Peptide group	Peptides	Source	Antifungal activity	References
Cathelicidin	BMAP Indolicidin	Human sweat Bovine neutrophils	*A. fumigatus* *A. niger*, *Penicillium* spp., *S. cerevisiae*	(Benincasa et al. 2006; D. G. Lee et al. 2003)
Histatins	Hst5	Human saliva	*C. tropicalis*, *C. krusei*, *S. cerevisiae*	(Nikawa et al. 2001)
Mucins	MUC7	Human saliva	*C. krusei*, *S. cerevisiae*	(Situ et al. 2003)
Dermaseptin	Dermaseptin S1-5	Arbroral frog	*A. niger*, *S. cerevisiae*	(Morton et al. 2007; De Lucca and Walsh 1999)
Lactoferrin-Lactoferrin analgoues	hlF (1-11) Lfcin B	Breast milk Bovine neutrophils	*A. fumigatus* *Penicillium* spp., *A. niger*, *A. fumigatus*	(Fernandes and Carter 2017; Kanyshkova, Buneva, and Nevinsky 2001)
Defensin	α-defensin β-defensin	Mammalian neutrophils, Paneth cells	*Candida spp* *Aspergillus* spp. *Fusarium* spp.	(Ganz 2003; P. M. Silva, Gonçalves, and Santos 2014)
Magainin	Magainin 2	African clawed frog	*C. krusei*, *C. tropicalis*, *S. cerevisiae*	(Morton et al. 2007; Burrows et al. 2006)
Frog skin active peptides	Brevinin-1 and -2 Temporin-Shf	Eurasian and North American frogs Sahara frog, *Pelophylax saharica*	*C. tropicalis*, *C. krusei*, *S. cerevisiae*	(Pál et al. 2006; André et al. 2015)
Pleurocidin	Pleurocidin	Winter flounder, *Pseudopleuronectes americanus*	*S. cerevisiae*, *F. culmorum*, *A. niger*	(Souza et al. 2013)
Other frog peptides	Citropin 1.1 Aurein 1.1	Blue mountains tree frog, *Litoria citropa* *Grass frog*, *Litoria raniformis* (grass frog)	*Candida* spp.	(Kamysz et al. 2006)
Bombinin and bombinin-like	Bombinins H1-7	Oriental fire-bellied toad/frog, *Bombina orientali*	*C. tropicalis*	(Simmaco, Kreil, and Barra 2009)
Hepcidin and hepcidin-like	Hepc20/Hepc25	Human liver	*A. niger*, *S. cerevisiae*	(C. H. Park et al. 2001)

Invertebrate sources

Due to the absence of adaptive immune system, invertebrates need a potent first line of defence and a great number of AFPs have been identified in their innate immune system. Insects, marine invertebrates, and spiders are, in particular, a rich source of natural AFPs. Many of these peptides are similar to AFPs from other species, such as insect defensins, or thanatin, a peptide that presents high similarity with the frog peptides brevinins (Table 2.4).

Thanatin is an inducible and non-hemolytic peptide of 21 residues isolated from the spined soldier bug *Podisus maculiventris.* This peptide has a single disulfide bond that forms a highly positively charged C-terminal loop. Thanatin is fungicidal against several species, including *F. culmorum*, at a concentration below μM level, and *A. fumigatus* (10–20 μM) (Fehlbaum et al. 1996; Koch et al. 2012). However, the effect of thanatin on hyphae or its mechanism of activity against fungi are not known.

Every insect that has been screened so far produced **insect defensins** but few show high homology with mammalian and plant defensins. *Drosomycin*, an insect defensin structurally related to the plant defensin Rs-ARF2, is a 44-residue peptide. Produced by the fruit fly (*Drosophila melanogaster)*, drosomycin has significant fungicidal activity against filamentous fungi (*e.g., F. oxysporum,* MIC<5 μM*)* and recent studies have revealed its anti-yeast activity (*S. cerevisiae*, MIC, 12 μM) (Zhang and Zhu 2009). Interestingly, like several plant defensins, drosomycin inhibits hyphal growth and can destroy spores in a dose-dependent manner. **Heliomicin**, isolated from the budworm *Heliothis virescens*, is active against moulds such as *Fusarium* spp. and *A. fumigatus*, and the yeast *P. pastoris* (Lamberty et al. 2001). Heliomicin's mechanism of activity is analogous to the defensin Rs-AFP2. **Termicin**, isolated from termite, is a Gly-rich peptide with a defensin-like motif that shows strong activity against *F. culmorum*. Interestingly, termicin is secreted together with glucanases that break down the fungal cell wall carbohydrates, an action that might facilitate the action of the peptide (Bulmer et al. 2009). Although their mechanism is thought to involve electrostatic interactions with target cell membrane, the characterization of insect defensins needs further investigation.

The most well studied insect AMPs are **cecropins**, isolated from silkworm moth. The majority of cecropins share two characteristics, a Trp in the first or second residue

position and an amidated C-terminal amino acid. Cecropin A (4 kDa) is able to kill *Aspergillus* spp. (MIC 25-100 µg/ml), *F. oxysporum* and *F. moniliforme* (MIC, 12.5 µg/ml). Cecropins do not show the same potency against conidia and hyphae, as revealed by the selective killing of only germinating *Aspergillus* by cecropin A. This selectivity relates to changes in the cell wall composition that occur at various stages of growth. Apart from the disruption of fungal membrane, the mechanisms used by cecropins remain to be elucidated. **Gomesin**, isolated from the tarantula spider hemolymph, show similarities with protegrins, Arg-rich peptides isolated from porcine leukocytes (P. I. Silva, Daffre, and Bulet 2000). Low concentrations of gomesin are effective against both filamentous fungi and yeasts. The structural analysis of this 18-mer peptide has revealed a two-stranded antiparallel β-sheet structure, comprising 2 disulfide bonds. Its amphipathic nature is correlated to its ability to disrupt lamellar vesicles and suggests membrane permeabilization as a mode of action. Gomesin and cecropins share significant homology with **tachyplesins. These chitin-binding peptides are** found specifically in small granules of the hemocytes of horseshoe crabs (Nakamura et al. 1988), an important source of marine antimicrobial peptides (Destoumieux-garzon et al. 2016). Among this family, tachyplesin II and polyphemusins I and II are know to inhibit *Fusarium* spp. or *Candida kefyr*, but the mechanism is still unknown. The presence of 2 disulfide bonds is thought to give resistance to low pH and high temperature.

Other marine invertebrates, the penaeid shrimps, also produce chitin-binding AFP, termed **penaeidins**. These 47- to 63-residue peptides do not have a well-defined skeleton, but possess a N-terminal domain rich in Pro and an α-helical C-terminal domain comprising three disulfide bonds. Penaiedins appear to increase the hyphal branching of *Aspergillus* spp. and *F. oxysporum* (Destoumieux et al. 2000) and inhibit sporulation of filamentous fungi.

Invertebrates' venom possess a high content of AFPs, such as **mastoparans** from wasps, non-disulfide bridged peptides (**NDBP**) isolated from scorpions' venom or **ponericin** from the ant *Pachycondyla goeldii* (Moerman et al. 2002; Orivel et al. 2001; Zare-Zardini et al. 2015; Zeng et al. 2012). Although these venom peptides are not highly hemolytic at concentrations required to inhibit fungi, their toxicity at higher concentrations may limit their use.

The termite *Pseudacanthoterme spiniger* stores in its haemocytes the antifungal peptide **spinigerin**. Unstructured in an aqueous environment, this 25-residue acquires an amphipathic α-helix in hydrophobic conditions. Due to similar structural characteristics with magainin 2, spinigerin is thought to also act via membrane permeabilization.

Tachystatins are 41- to 44- residue peptides and belong to the knottin-type family based on sequence and structural resemblances to plant knottin-type peptides. Like plant knottins, these peptides bind chitin and probably cause fungal membrane disruption (Nawrot et al. 2014).

Table 2.6. Antifungal peptides isolated from invertebrate sources

Peptide group	Peptides	Source	Antifungal activity	References
Tachyplesin/polyphemusin	Tachyplesin-1	Asian horseshoe crab, *Tachypleus tridentatus*	*C. kefyr, C. tropicalis* *F. culmorum, F. oxysporum, A. fumigatus*	(Nakamura et al. 1988; Rosa and Barracco 2010)
	Gomesin	Tarantula spider, *Acanthoscurria gomesiana*	*A. brassicola, A. fumigatus, Fusarium* spp., *S. cerevisiae*	(P. I. Silva, Daffre, and Bulet 2000)
Insect defensins	Drosomycin	Fruit fly, *Drosphiloa melanogater*	*Fusarium* spp., *Candida* spp., *Aspergillus* spp.	(Zhang and Zhu 2009) (Lamberty et al. 2001)
	Termicin / Spinigerin	Termite, *P. spiniger*	*F. culmorum, S. cerevisiae, F. oxysporym*	
Thanatin	Thanatin	Spiner soldier bug, *Podisus maculiventris*	*F. culmorum, F. oxysporum, A. fumigatus*	(Fehlbaum et al. 1996; Koch et al. 2012)
Cecropins	Cecropin A	Giant silk moth, *Hyalopora cecropia*	*F. oxysporum, F. solani*	(DeLucca et al. 1997)
Ponericin	Ponericin G1-6	Ant, Pachycondyla *goeldii*	*S. cerevisiae*	(Orivel et al. 2001)
Knottin-like	Tachystatin	Crab	*Pichia pastoris*	(Van Der Weerden, Bleackley, and Anderson 2013)
Insect venom	Mastoparan	Wasp, *Sphodromantis viridis*	*F. culmorum, S. cerevisiae*	(Moerman et al. 2002)
	Mastoparan-S		*A. niger, A. fumigatus*	(Zare-Zardini et al. 2015)
<u>Scorpion NDBP</u>				
Long chain peptide	Opistoporin-1	South-African scorpion *Opistophtalmuscarinatus*	*F. culmorum, S. cerevisiae*	(Moerman et al. 2002)
Short AMP	BmKbpp2	Chinese scorpion *M. martensii*	*F. culmorum*	(Zeng et al. 2012)

Microbial sources

Eukaryotic and prokaryotic microorganisms can produce AFPs, which are mostly known to play a competitive role in ecological niches (Table 2.5). Most of these peptides are isolated from fungal species but are not related to other AFPs, although fungal defensins have also been isolated. These latter include plectasin from *Pseudoplectania nigrella*, eurocin from *Eurotium amstelodami* (P. M. Silva, Gonçalves, and Santos 2014). The filamentous fungi *Aspergillus giganteus* and *Penicillium chrysogenum* are, respectively, able to secrete the proteins AFP and PAF (P. M. Silva, Gonçalves, and Santos 2014). These proteins are active against *Fusarium* spp. and *A. fumigatus* and are also able to inhibit the environmental fungal species *Zygomycetes* sp. The peptide, AnAFP, expressed by *A. niger*, also shows anti-yeast activity (P. M. Silva, Gonçalves, and Santos 2014). The antifungal mechanisms of AFPs from fungi are well characterized. These fungal AMPs act via interaction with receptors on the cell wall/membrane and induce signalling cascades, accompanied by Ca^{2+} influx. Fungi are also able to produce several types of lipopeptides, such as **echinocandins**, **pneumocandins**, **aculeacins**, or **iturins**, which are powerful antifungal agents. Despite their efficacy, their role in therapeutic of food applications is limited due to an extreme hemotoxicity. However, analogues of these peptides with low hemolytic activity have been created. The fungal lipopeptides, **a***ureobasidins*, produced by *Aureobasidium pullulans* are cyclic lipophilic depsipeptides composed of 8 residues and an α-hydroxyacid (Takesako et al. 1993). Several members of this family are active against *Candida* spp. (e.g., aureobasidin A with a MIC of 0.05 to 0.2 μg/ml).

Killer toxins are small protein-based toxins secreted by so-called "killer" yeasts and which display remarkable antifungal activity, mostly against species of the same genus (Lowes et al. 2000). The host genome does generally not encode the yeast killer toxin but the expression might occur upon infection by dsRNA viruses. The same virus dsRNA also encodes the genes involved in the immunity of the host cell, which provides protection to the host. Virally encoded proteins contain α- and β-domains linked by a disulfide bond and an additional γ-domain. The binding of the toxin to the yeast cell wall is recognized as the first step of the antifungal action. However, other mechanisms have been discovered, including perturbation of membrane integrity or cell functioning (Belda et al. 2017; Izgu and Altinbay 2004; Hatoum, Labrie, and Fliss 2012). A vast majority of killer toxins have a limited spectrum of activity, display poor stability and

are only active in a narrow range of pH (Santos et al. 2009; Soares and Sato 2000). There are a few exceptions of toxins with higher stability, such as the killer toxins of *Williopsis mrakii* (Yamamoto et al. 1988) and *Williopsis saturnus* (Buzzini et al. 2004). These toxins are able to withstand heat treatment and are active over a wide pH range. (Albergaria and Arneborg 2016). However, yeast killer toxins usually possess high MIC values (250-1000 μg/mL), which limits their use for food applications. Interestingly, a single filamentous fungus, *Ustilago maydis*, is able to produce a killer toxin, named KP4. Its fungistatic activity includes the blocking of Ca^{2+} channels, which leads to the inhibition of Ca^{2+}-regulated pathways.

Some bacteria are also a good source of AFPs. *Syringomycins* and related peptides are a group of cyclic lipopeptides, called lipodepsinonapeptides, produced by *Pseudomonas syringae*, and active against a number of isolates of *Candida* spp. *(*MIC, 2.5–25 μg/ml) and *A. fumigatus* (MIC, 5–40 μg/ml) (Sorensen, Kim, and Takemoto 1996). The bacterium *Streptomyces tendae* is also able to produce the chitin-synthesis inhibitors, nikkomycins (R. K. Li and Rinaldi 1999), or the non-bacteriocin protein, AFP1, which are active against *S. cerevisiae*. However, nikkomycins are not active against filamentous fungi (D. A. Freitas et al. 2005). Several bacterial species express specific peptides with antifungal activity, such as cepacidines or flagellin (Lim et al. 1994; X. Zhao et al. 2013).

Lactic acid bacteria (LAB), including strains approved for food application, are also able to produce peptides showing antifungal activity. *Lactobacillus plantarum* strain LR/14 produces a series of peptides active against *A. niger* or *Penicillium chrysogenum* (Gupta and Srivastava 2014). Interestingly, as highlighted for other AFPs, the sensitivity of fungi to these peptides is growth stage dependent, mostly due to structural changes that occur between the conidial and hyphal stages. *L. plantarum* IS10 is also able to secrete peptides active against strains of *Aspergillus* spp. and *Penicillium roqueforti*. Among these peptides, the peptide FPSHTGMSVPPP (1.2 kDa) displays the characteristics of an AFP, with a positive net charge +1, and 58% of hydrophobic residues. However, its activity only appears at high concentration (5 mg/ml) (Muhialdin et al. 2016). The bacteriocin F1 (2 kDa) produced by *Lactobacillus paracasei* subsp. *tolerans* is the first reported bacteriocin active against *Penicillium glaucum*, *A. niger* and *Aspergillus flavus* (Miao et al. 2014). Most of these peptides show strong heat stability, which is beneficial in food process, but are sensitive to proteolytic digestion.

The degradation of these peptides by proteases of the intestinal tract would limit their activity against intestinal flora. These interesting properties for food applications are, however, limited by a high MIC. LAB are also able to release antifungal peptides during fermentation. Thus, a sourdough extract produced with *Lactobacillus brevis* AM7 contains a group of 8 peptides which inhibit the bread spoilage fungus, *P. roqueforti*, with a MIC of 3.5 to 8.2 mg/ml (Coda et al. 2008). Although specific to sourdough bread, the synergistic antifungal effect obtained with the combination of sourdough extract and antifungal protein-rich plant extracts is a promising tool for preventing spoilage (Coda et al. 2008; Rizzello et al. 2011).

Table 2.7. Antifungal peptides/proteins isolated from microbial sources

Peptide/protein group	Peptides	Source	Antifungal activity	References
Bacterium AFP	AFP1	*Streptomyces tendae* Tu901	*A. fumigatus*	(D. A. Freitas et al. 2005)
Fungal antifungal protein	AnAFP	*A. niger*	*A. fumigatus, F. oxysporum, F. solani, S. cerevisiae*	(Hajji et al. 2010; Hegedüs and Marx 2013; P. M. Silva, Gonçalves, and Santos 2014)
	AcAFP	*Aspergillus clavatus*	*F. oxysporum, F. solani, A. niger*	
	PAF	*Penicillium chrysogenum*		
Syringomycin	Syringomycin E	*Pseudomonas syringae pv. syringae*	*Aspergillus* spp. *Candida* spp.	(Sorensen, Kim, and Takemoto 1996)
Nikkomycins	Nikkomycin Z	*Streptomyces tendae*	*Aspergillus* spp,. *Fusarium* spp., *Penicilium* spp., *Candida* spp.	(R. K. Li and Rinaldi 1999)
Other peptides	Cepacidines	*Burkholderia cepacia*	*A. niger*, *F. oxysporum*, *Candida* spp.	(Lim et al. 1994)
	Bacterial flagellin	*Bacillus amyloliquefaciens* BH072	*A. niger* *B. cinerea*	(X. Zhao et al. 2013)
Aureobasidin	Aureobasidin A	*Aureobasidium pullulans*	*Candida* spp., *Aspergillus* spp.	(Takesako et al. 1993)
Killer toxins	PMKT2	*Pichia membranifaciens*	*S. cerevisiae, K. lactis*	(Santos et al. 2009)

2.4 Mode of action

The internal environment of yeasts and filamentous fungi is protected by a cell wall and a lipid membrane, each of which has distinctive differences. The cell wall has a protective role by limiting access to the cell membrane, and a functional role for cells mechanisms such as adhesion or signalling. The outer layer is made of glycosylated proteins decorated by carbohydrates residues and connected via disulfide bonds, which limits the porosity of the structure. The yeast cell wall comprises mannose residues, whereas filamentous fungi can also contain glucose or galactose. The second layer is made of a β-glucan network, mostly made of (1,3)-β-glucans, although yeasts also contain (1,6)-β-glucans. This network is linked to the cell wall proteins. The closest element to the plasma membrane is a layer of chitin, involved in the recognition of some AFPs. The cell membrane of fungi contains a high proportion of negatively charged phospholipids (phosphatidylserine and phosphatidylinositol), unlike mammalian cells. This difference may contribute to the selectivity of AFPs. Another characteristic element is the presence of ergosterol, which to a certain extent, stabilizes the fungal membrane.

The antifungal action of AFPs can involve several mechanisms, such as perturbation of the fungal membrane integrity, translocation in order to reach intracellular targets, or alteration of fungal metabolism (De Coninck, Cammue, and Thevissen 2013; Rautenbach, Troskie, and Vosloo 2016; Hegedüs and Marx 2013; Van Der Weerden, Bleackley, and Anderson 2013). Regarding membrane permeabilization, several models are commonly proposed: the barrel-stave model, the toroidal pore model, and the carpet model, which are described in previous reviews (Bechinger and Gorr 2017; Sani and Separovic 2016; Nawrot et al. 2014). These models are generally based on the activity of AMPs against bacterial membranes, but similar mechanisms are thought to occur with fungal membranes. AFPs activity generally involves membrane-related mechanisms, but inhibition of proteins or of cell wall components synthesis has been shown. Moreover, some peptides are able to induce the formation of ROS (Karin Thevissen, Terras, and Broekaert 1999) and damage nucleic acids. The combination of several mechanisms is quite common. For example, magainin 2 kills fungi via cell membrane disruption and DNA damage. The diversity of mechanisms is well illustrated by AFPs of the histatins family. After binding to cell wall proteins, Hst-5

first internalizes and binds to a K^+ channel transporter. A subsequent mitochondrial membrane degradation and ATP release induces unchecked signalling cascades, leading to cell death. Figure 2.3 summarizes the diversity of receptors and mechanisms used by several AFPs to combat fungal pathogens. Antifungal activity is generally peptide- and species-dependant. In addition, structural differences between yeasts and filamentous fungi are usually cited as an explanation for differences in sensitivity to a particular peptide (Erwig and Gow 2016).

In comparison to traditional microbial inhibitor compounds, the plurality of mechanisms of action reduces the risk of development of microbial resistance (Prema and Pruthvi 2012). However, several fungi are able to withstand AFPs and develop resistance via the activation of signalling pathways related to cell wall modification or alkaline pH stress response (Kwon et al. 2016; Van Der Weerden, Bleackley, and Anderson 2013). Despite a low risk of resistance, the isolation and purification of natural AFPs generally involve time-consuming processes, ultimately with low yields, which is a disadvantage for food applications. This issue is one of several hurdles, amongst which poor solubility, poor resistance to environmental factors and potential toxicity are conspicuous. A better understanding of the requirements for antifungal activity should enable such issues to be overcome. In addition, the development of novel synthesis technologies facilitate the development of synthetic peptides, which could be designed for increased resistance to salt or serum and which can be produced in economically viable amounts.

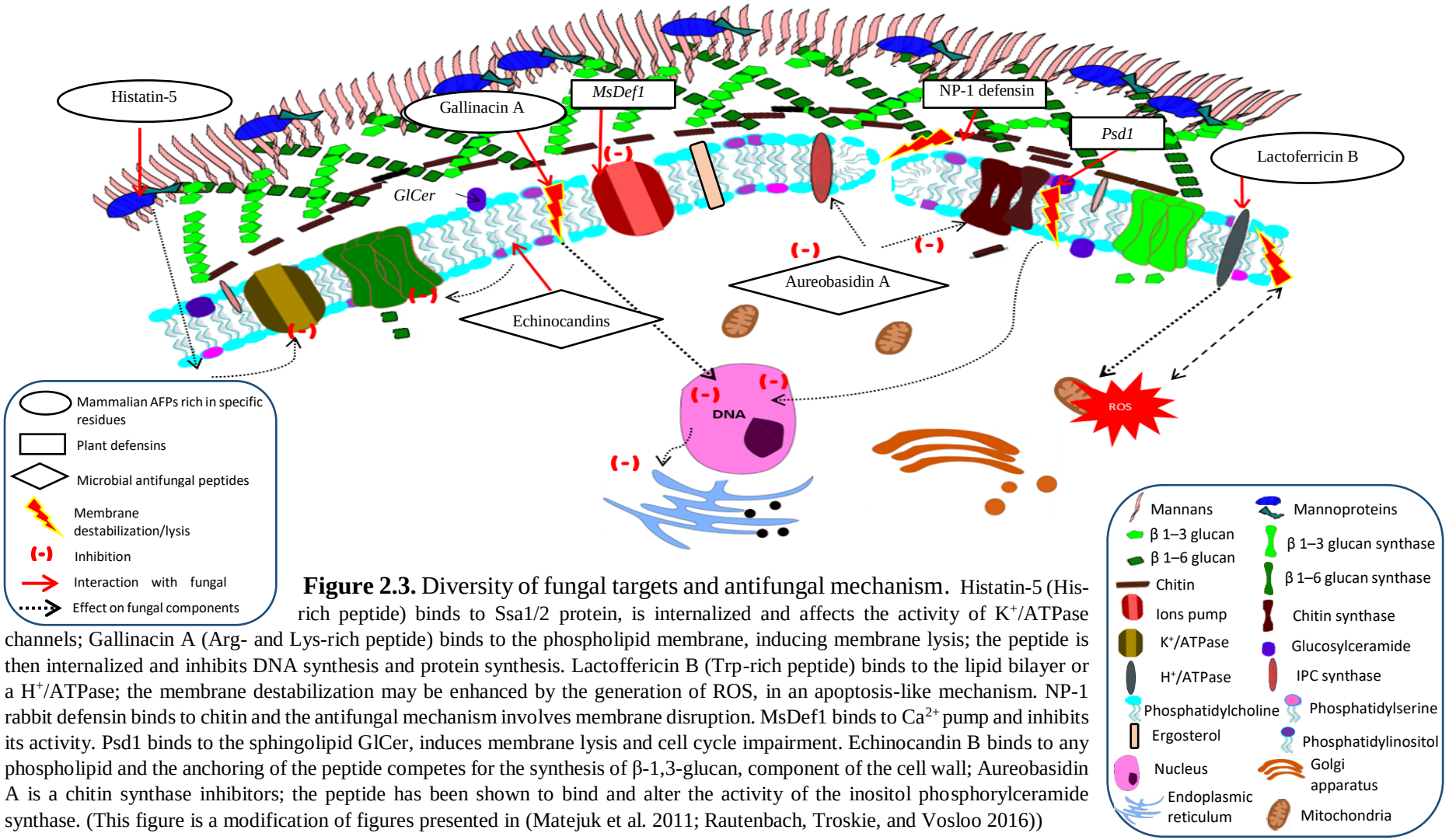


Figure 2.3. Diversity of fungal targets and antifungal mechanism. Histatin-5 (His-rich peptide) binds to Ssa1/2 protein, is internalized and affects the activity of K^+/ATPase channels; Gallinacin A (Arg- and Lys-rich peptide) binds to the phospholipid membrane, inducing membrane lysis; the peptide is then internalized and inhibits DNA synthesis and protein synthesis. Lactoffericin B (Trp-rich peptide) binds to the lipid bilayer or a H^+/ATPase; the membrane destabilization may be enhanced by the generation of ROS, in an apoptosis-like mechanism. NP-1 rabbit defensin binds to chitin and the antifungal mechanism involves membrane disruption. MsDef1 binds to Ca^{2+} pump and inhibits its activity. Psd1 binds to the sphingolipid GlCer, induces membrane lysis and cell cycle impairment. Echinocandin B binds to any phospholipid and the anchoring of the peptide competes for the synthesis of β-1,3-glucan, component of the cell wall; Aureobasidin A is a chitin synthase inhibitors; the peptide has been shown to bind and alter the activity of the inositol phosphorylceramide synthase. (This figure is a modification of figures presented in (Matejuk et al. 2011; Rautenbach, Troskie, and Vosloo 2016))

2.5 Unnatural peptides

These natural peptides are a source of inspiration for the design of novel synthetic powerful peptides, suitable for numerous applications (Bondaryk et al. 2017). In recent years, different studies have focused on the biological activity of peptide fragments derived of precursor physiological proteins. These peptides can be derived via natural cleavage or *in vitro* proteolysis, so called cryptides. Many peptides, derived from proteolytic cleavage of natural peptides, lactoferricin (Lfcin) being one of the most studied (Fernandes and Carter 2017), have been shown to display antifungal activities. Synthetic derivatives include, for instance, the hexapeptide (RRWQWR–NH_2) based on lactoferricin B. This peptide is active towards filamentous fungi such as *P. digitatum*, via membrane permeabilization.

Fragments of the antifungal human salivary protein mucin-7 (357 residues), comprising 12 residues (MUC7 12-mer) and 20 residues (MUC7 20-mer), also retain their activity against *C. krusei* and *S. cerevisiae*. Interestingly, despite a lower positive net charge, MUC7 12-mer is more active than MUC7 20-mer (Situ et al. 2003). Examples of derivatives are presented in Table 2.6, including peptides from the cathelicidin family (e.g., IB-367 (Barchiesi et al. 2007), omiganan KR-20 (G. Wang 2008; Wiesner and Vilcinskas 2010)), insect AFPs (e.g., D4E1, K18M) (De Lucca et al. 1998; Fehlbaum et al. 1996), and peptides with unnatural residues, such as Temporin-Shf derivatives (André et al. 2015).

The studies of these short fragments of natural AFPs provide a deeper knowledge of the key determinants of antifungal activity. In addition, their short size makes them good candidates for peptide technologies, including solid-phase peptide synthesis and engineering methods, in a cost-effective manner. The development of synthetic peptides has grown due to an interest in avoiding the common drawbacks associated with natural peptides, including toxicity or unstability.

In addition to these derivatives, many synthetic peptides have been constructed via *de novo* synthesis (Table 2.6). Among these, PAF26 is a 6-residue peptide with the sequence Ac-rkkwfw-NH_2, designed using a combinatorial library. Small letters indicate that the residue is in its D-form, as commonly used in peptide representation (this will be used in the rest of manuscript). This peptide is active against *A. niger* and

P. digitatum (MIC 4 μM) with interesting modifications such as N-acylation and C-amidation to improve its activity. One member of the synthetic AMPs kaxins, dF21-10K, possesses a hydrophobic core made of D-enantiomers (kkkkkkkkkkaafaawaafaa-NH_2), and showed a MIC between 16 and 64 μg/ml against *Candida* spp. (Burrows et al. 2006). The design which includes a high number of cationic and hydrophobic residues has been used in the construction of the Trp-rich peptide, Pac 525 (C. W. Wang et al. 2009), or (KW)n based-peptides (Gopal et al. 2012) which are active towards *Candida* spp and *S. cerevisiae*. An exhaustive list of antifungal cryptides and *de novo* synthetic peptides has been reviewed previously (Ciociola et al. 2016).

For larger peptides, the use of recombinant organisms is more appropriate, despite, generally, a low yield of production and the necessity of using strains which are resistant to the AFP being produced (Matejuk et al. 2011). Recombinant microorganisms are a cost-effective and time-efficient way to obtain peptides. Several organisms have been used to synthesize recombinant AFPs. Fusion proteins produced in *E. coli* are the most widely used in recombinant engineering, due to their low cost and the large body of knowledge on available strains and plasmid expression vectors. However, recombinant peptides produced with *E. coli* are often produced in low amount and lack PTMs, which are, in some cases, necessary for the antifungal activity. In this case, the use of eukaryotic resistant strains, such as *P. pastoris*, is required to express and produce the AFPs.

Several defensins, such as the plant defensin, Psd1, or the human defensin, HBD-3, have been expressed in *P. pastoris* (James et al. 2014). Although the yields are quite low, some expression systems can reach levels of 10 g/L. The overexpression of recombinant peptides is particularly used for biotechnological applications to provide protection to phytopathogens. For instance, a recombinant defensin from the leguminous weed *Tephrosia villosa*, active against *F. oxysporum* and *F. moniliforme* (Vijayan, Guruprasad, and Kirti 2008), has been overexpressed in tobacco. Several other defensins, expressed in recombinant plants and fruits, show similar activity to the native peptides (rPsd1, rTvD1), presenting PTMs, pH- and heat-stability (Larentis et al. 2004; Meneguetti et al. 2017).

Table 2.8. Examples of antifungal cryptides and *de novo* peptides

Peptide	Source	Antifungal activity	References
MUC-7 12-mer MUC-7 20-mer	MUC-7	*C. krusei* *S. cerevisiae*	(Situ et al. 2003)
P-113	Histatin-5 fragment	*C. kefyr, C. krusei, C. parapsilosis, C. tropicalis*	(Rothstein et al. 2001)
KR-20	Human cathelicidin LL-37	*A. niger*	(G. Wang 2008)
Temporin-SHf Analogues	Temporin-SHf: FFFLSRIF-NH_2	*C. tropicalis*	(André et al. 2015)
D4E1	Cecropin A: FKLRAKIKVRLRAKIKL	*Aspergillus* spp., *Fusarium* spp.	(De Lucca et al. 1998)
K18M	thanatin (8-21): KPVPIIYCNRRTGKCQRM	*F. culmorum*	(Fehlbaum et al. 1996)
IB-367	Synthetic protegrin-1 derivative	*C. krusei, C. tropicalis, C. parapsilosis*	(Barchiesi et al. 2007)
Omiganan CP10A/CP11	Synthetic indolicidin derivative	*C. krusei, C. parapsilosis, C. tropicalis*	(Rozek et al. 2003; Wiesner and Vilcinskas 2010)
(KW)n/RWn	Lys (or Arg)- and Trp-rich	*F. solani, F. oxysporum*	(Gopal et al. 2012)
D-V13K	*De novo* peptide	*Aspergillus* spp. *Rhizomucor*	*(Jiang et al. 2009)*
Pac525	Trp-rich peptide: Ac-KWRRWVRWI-NH_2	*C. krusei, C. parapsilosis, C. tropicalis, S. cerevisiae*	(C. W. Wang et al. 2009)
kaxins	dF21-10K: kkkkkkkkkkaafaawaafaa-NH_2	*C. krusei, C. parapsilosis, C. tropicalis*	(Burrows et al. 2006)
PAF26/PAF32	Ac–rkkwfw–NH_2	*Penicillium* spp., *F. oxysporum, S. cerevisiae*	(Muñoz et al. 2007; López-Garcia et al. 2000)

2.6 How to design synthetic antifungal peptides?

The synthesis of peptides has been greatly facilitated by the development of solid phase synthesis, reducing the time previously involved in the extraction of natural peptides. In addition to the high costs of synthesis, the solubility and toxicity of peptides are still significant hurdles. The comparison of different peptide sequences and structures has highlighted the key role of several parameters in the antifungal potency, such as the net charge, hydrophobicity and the distribution of the residues (Sagaram et al. 2011; Yount and Yeaman 2004). These properties can be modified in the design of optimized peptides (see Fig. 2.4), including shorter sequences and higher resistance.

The first barrier faced by AFPs is the fungal cell wall, with which AFPs either interact and/or are taken up to fungal membrane via transporters (Rautenbach, Troskie, and Vosloo 2016). A large amount of AFPs interacts with the different lipids constituting the membrane barrier, leading to membrane permeabilization/destabilization. The presence of negatively charged lipid heads at the external surface of the membrane is exploited by AFPs and their positively charged residues to secure the initial electrostatic interactions. This interaction is usually followed by a penetration of the hydrophobicity of the peptide into the bilayer. Helicity, amphipathicity and structure of the peptide are also clearly involved in the potency of AFPs (Liu, Fang, and Wu 2013). The following paragraphs will highlight the role of these parameters in the antifungal activity of peptides and how they can be used to design potent new AFPs.

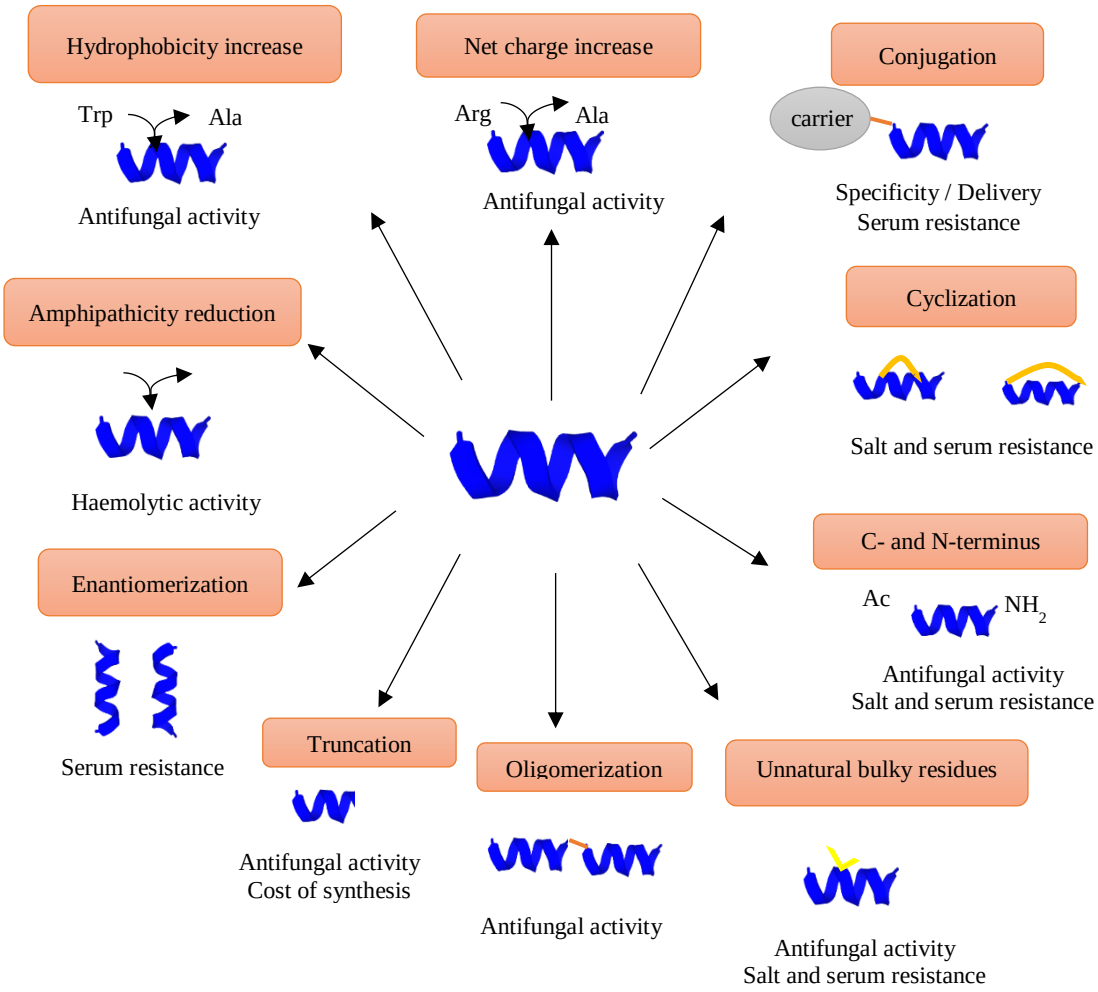


Figure 2.4. Common modifications used in peptide design and studied properties

Cationicity

The net charge of antifungal peptides categorizes them as cationic, neutral or anionic peptides. Around 87% of AMPs are positively charged, the positive charge explaining the attraction to negatively-charged membranes.

The increase in the number of basic residues in antifungal peptides commonly improves the antifungal activity (Jin et al. 2016; Lum et al. 2015; André et al. 2015). In fungal or yeasts cell walls/membranes, this interaction involves negatively charged components, such as glycophospholipids (GPLs), sphingolipids or mannoproteins (Fig. 2.3). Thus, the insertion of Arg residues into the d-hexapeptide, rktwfw-NH_2, increases the activity against *P. digitatum* (López-Garcia et al. 2000). A similar modification enhances the activity of temporin-Shf analogueagainst *S. cerevisiae* (André et al. 2015). On the contrary, the replacement of these basic residues in the

hexapeptide, PAF26 (Muñoz et al. 2007; De Brucker et al. 2014), has led to a loss of antifungal activity. In addition to an overall cationicity, positively charged residues are involved in covalent and non-covalent interactions with other residues or components of the membrane bilayer (Falla and Hancock 1997; Aliste, MacCallum, and Tieleman 2003). The replacement of Lys residues in the defensin TPP3 increases the solubility of the AFPs, but it also leads to a loss of tetramerization and antifungal activity (Baxter et al. 2015).

Although the presence of basic residues is important, the choice between Lys and Arg residues is potentially might be problematic (Guo et al. 2013; Gopal et al. 2012). Due to its more dispersed charge and associated stability than Lys, the HB interaction between Arg residues and the membrane surface is suggested to be favoured, facilitating entry into the hydrophobic environment (Gopal et al. 2009). Cation–π interactions also make the entry of Arg residues energetically more favourable. Thus, it is assumed that the presence of Arg, rather than Lys, improves the antifungal activity but also increases the hemotoxicity (Schibli et al. 2006).

It is evident that not only the overall charge, but also the location of the basic residues in the peptide is important. In α-helix peptides with strong hemolytic activity, the insertion of an Arg or Lys in the hydrophobic face of the helix decreases the hydrophobic moment, resulting in a lower hemolytic activity (Jin et al. 2016).

However, Lyu et al. have showed that the correlation between a net charge increase and the antimicrobial activity reached a threshold (Lyu et al. 2016). Thus, increasing cationicity or hydrophobicity of human cathelicin derivatives has not changed their antifungal activity, whereas increasing both parameters has improved the activity by 2-fold (Y. Park et al. 2003), demonstrating their complementarity.

The incorporation of the amino acid His in the peptide can also act as a positive-charge carrier. Due to the lower pI of His (=7.5), the net charge of His-containing peptides is only increased at pH < 5.5 (Sharma et al. 2010). His can be used as a more neutral residue to replace Arg and Lys, if the presence of these residues negatively affects the amphipathicity of a peptide (Rothstein et al. 2001).

Hydrophobicity

Hydrophobicity is recognised as another important parameter, as the AFP is not expected to bind to the membranes in absence of hydrophobic residues. Lee et al. have showed the positive correlation between the hydrophobicity of 1,4-β-helical peptides and their antifungal activity (M. R. Lee et al. 2014). Furthermore, Chen et al. (2007) demonstrated that this correlation is valid until a certain threshold of hydrophobicity, in gramicidin-derived peptides (Chen et al. 2007). The primary residues used to impart a hydrophobic character on the peptide or to increase resistance to salt and serum are Trp and Phe (Van Der Kraan et al. 2005; Sonesson et al. 2011; Muñoz et al. 2007). However, the loss of some hydrophobic residues through residue substitution in peptides does not imperatively induce a loss of activity, showing that hydrophobicity alone does not dictate the antifungal activity of these peptides.

As mentioned above, the addition of structural features induces changes in structure and ring size of the peptides, which modulates activity (Kondejewski et al. 1996). The hydrophobicity of non-polar faces generally correlates with higher helicity of α-helical peptides, enhancing penetration into a membrane.

Although the presence of hydrophobic residues is important for improving the antifungal activity, the position and nature of these residues in the peptide are crucial. The presence of Trp rather than Phe favours side chains interactions with basic residues and penetration into the membrane (Jin et al. 2016). The insertion of a Trp residue on the hydrophilic face of an helix modifies the amphipathicity of a peptide and reduces its activity, whereas the insertion of hydrophobic residues on the hydrophobic face of the His-rich peptide, P-113, does not affect its candidacidal activity (Rothstein et al. 2001). Beyond a certain value, a systematic increase in hydrophobicity (particularly on the non-polar face) has a negative impact, leading to self-association of the peptide (Jiang et al. 2009; López-Abarrategui et al. 2015). The lack of activity is also possibly explained by the resident time of the peptides in the membranes that impairs the membrane disruption. In addition, a hydrophobicity that is too high is generally correlated with increased hemolytic activity. Moreover, the replacement of hydrophobic residues (Gopal et al. 2013; Jiang et al. 2009) is sometimes used to improve the therapeutic index of a peptide. Thus, the replacement of Trp by the less hydrophobic Phe lowers the hemolytic activity of the Trp-rich Tritrpticin (Schibli et al. 2006).

An increase in hydrophobicity can also be achieved by substitutions involving Val, Ile and Leu residues, resulting in an increase of antifungal activity (H. Kim et al. 2014; Avrahami and Shai 2004**),** salt resistance (H. Kim et al. 2014), or a reduction of the hemolytic effect (Gopal et al. 2009).

Amphipathicity and structure

The hydrophobic moment (HM) is generally used to evaluate the amphipathicity of a peptide. This parameter, which describes the repartition of polar and non-polar residues, is also expressed as a polar angle for α-helices (Fernández-Vidal et al. 2008)**.** The amphipathicity and the helix formation of a peptide are affected by the presence and position of charged and aromatic residues. These residues are involved in salt bridges, cation-π interactions, and π-stacking between aromatic rings. Furthermore, these interactions also play a role the partition/location of the peptide in the microbial bilayer.

It appears that, together, hydrophobicity and amphipathicity regulate the antifungal and hemolytic activity of peptides. Hollman et al. (2016) designed two peptides with the same hydrophobicity, but with different HMs. The increase in HMs correlates with a strong hemolytic activity (Hollmann et al. 2016; Gautier et al. 2008) (Fig. 2.5). In cathelicidin-BF derivatives, an increase in hydrophobicity, accompanied by a strong decrease of HMs, increases the hemolytic activity (Jin et al. 2016). However, the increase of amphipathicity of the Trp-rich cathelicidin, indolicidin derivative results in a strong decrease of hydrophobicity and thereby a less hemolytic peptide (Falla and Hancock 1997). Thus, the desired properties of a peptide, namely strong antifungal activity and low hemolytic activity, are assumed to be correlated with a specific range of both hydrophobicity and amphipathicity.

A)

B)

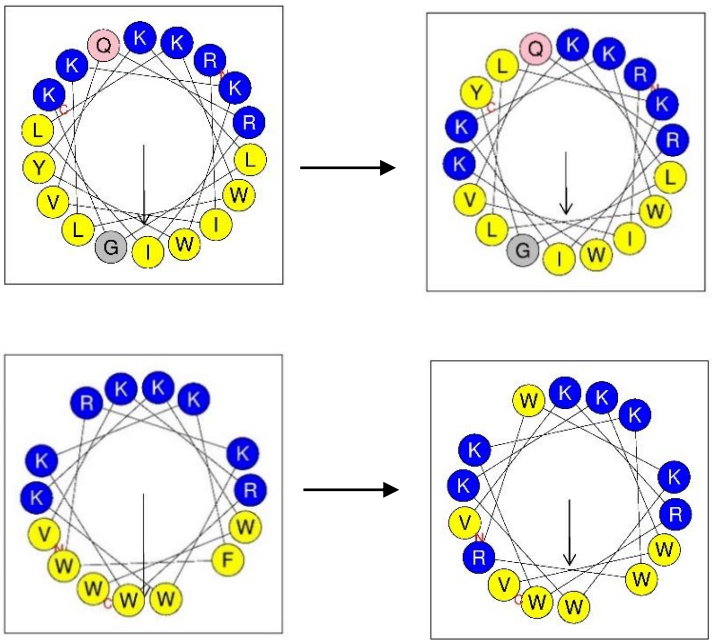


Figure 2.5. Helical wheels of novel peptides highlighting: A) the reduced amphipathiciity in the peptides reduces the HM, leading to a lower haemolytic activity. B) In cathelicidin-BF derivatives, the substitutions lead to a lower HM but higher hydrophobicity, making the peptide more haemolytic. Yellow circles represent hydrophobic residues; blue circles represent basic residues; other colours represent specific residues. The length of the arrow correlates with the hydrophobic moment. The creation of wheels is available at: http://heliquest.ipmc.cnrs.fr/ (Gautier et al. 2008)

In general, the positive correlation between HM and hemolysis is more evident with perfect α-helices. Although helicity and hydrophobicity dictate, together, the antifungal activity of several β-helical peptides (M. R. Lee et al. 2014), helicity is usually more associated with peptide toxicity than antimicrobial activity. The reduction of helicity of hemolytic peptides, via incorporation of D-amino acids lowers the hemolytic activity, while preserving the antimicrobial properties. The helix propensity of the individual residues directly correlates with the the peptide helicity. The residue Pro has a low helix-forming propensity and a structure-breaking ability, inducing "kinks" in the helical structure. Thus, its use in the HP (2-20) analogue has been shown to increase its antifungal activity 2-fold against several contaminants, such as S. cerevisiae (J. K. Lee et al. 2013), and to reduce its hemolytic activity. In addition, the flexibility of peptides, and their subsequent ability to change conformation, must be considered, due to its importance during the membrane insertion process.

Length

A peptide needs to be made of at least 7-8 amino acid residues in length to ensure a good amphipathicity. Furthermore, the length of a peptide needs to be considered based on the structure of the peptide. Thus, an efficient α-helical peptide needs at least 22 residues to be able to cross the plasma membrane. However, shortening peptides may be a means of increasing antifungal activity and reducing hemolytic activity. For example, truncated versions of several antifungal peptides, including LfcinB, display higher antifungal activity than the native peptide (Situ et al. 2003; Lyu et al. 2016). On the contrary, a too long chain can impart a loss of activity, due to the self-association of the peptides. Other potential adverse effects of a too long peptide are the development of hemolytic activity and poor solubility (Lyu et al. 2016; Gopal et al. 2013).

The length of a peptide illustrates the interaction between the factors that determine peptide activity. These factors need to be considered simultaneously when designing novel peptides. These properties are the primary considerations during peptide design, since the nature and position of the residues directly influence factors such as cationicity and hydrophobicity, and thus the activity of the peptide. Despite the identification of key elements, such as positive net charge and amphipathic structure, the efficiency of novel, synthetic AFPs remains unpredictable. However, modern techniques and a continuous increase in knowledge have highlighted other parameters that could be modulated to improve both the activity and the stability of newly designed peptides. The development of computer-based methods and tools for the prediction of activity have led to a faster identification of potent AFPs.

2.7 Computer-based methods

Predicting the activity of a novel peptide based solely on sequence similarities requires powerful tools due to the huge diversity of probable structures and characteristics. The use of computational techniques facilitates the screening of peptides, reducing the time and cost spent in designing novel, potent peptides. Three approaches are commonly used to design a peptide: template-based design, biophysical modelling and virtual screening studies. In template-based studies, a known AMP is used as a starting point

in the design of a new peptide. The biophysical method includes structural features of the peptides and atomic interactions. On the contrary, virtual screening is the most complex approach, but the most complete one. This approach quantifies the different descriptors of the peptide that are then numerically related to the biological activity of the peptide. This quantitative structure-activity-relationship (QSAR) method has greatly evolved since the 1980s. A brief history is presented in the review by Fjell et al. (2012).

In order to simultaneously screen thousands of peptides, a QSAR predicted peptide can be randomized to generate a positional scanning synthetic combinatorial library (PS-SCL). SCL enables the quick identification of active peptides without considering structural properties. Blondelle and Lohner (2000) generated thousands of peptides by randomization at defined positions of the peptide, YLK, which displays both antifungal and hemolytic activity. This method highlights the correlation between hydrophobicity and the hydrophobic moment and the activity of the peptides (Blondelle and Lohner 2000).

In addition to QSAR methods, advanced nonlinear and machine learning techniques, such as artificial neural networks (ANNs) or decision tree (DT) methods have been used to predict the activity of a peptide. These learning techniques are employed by AMP databases and AMP prediction tools. Fjell et al. (2009) used QSAR descriptors and ANN models with the activity of 1400 random peptides to screen a library of 100,000 theoretical peptide confirmations (Fjell et al. 2009). This model showed a 94% accuracy of prediction of highly active peptides. More generally, ANN models are able to predict accurately the antifungal activity of peptides.

These computational methods are now based on the various peptide databases available. A chronological list of the different databases, as well as the source of the peptides (e.g., PhytAMP focuses only on plants AMPs), are presented in the review by Wang (Wang, 2015). The most complete database, APD3, has been recently updated (access address: http://aps.unmc.edu/AP/main.php) (G. Wang, Li, and Wang 2016). This version includes PTMs, which are commonly used in peptide design (see section below).

Computer-based methods are powerful tools to predict the antifungal activity of a peptide. However, they present, in some cases, some limits, such as the inability to

identify similar sequence motifs (Yount and Yeaman 2004) or structural patterns (Ping Wang et al. 2011). Another limitation of the use of computational tools in the design of novel AMPs is their complexity to the extent that they are hardly understood by peptide microbiologists (H Jenssen 2011).

2.8 Use of PTMs in peptide design

Mimicing PTMs has several objectives, including reducing peptides' toxicity, increasing serum stability and their resistance to proteolytic digestion. Natural AMPs are processed via different PTMs and the use of PTMs for peptide design has been reviewed recently (G. Wang et al. 2015). Here, only the most common modifications used in the design of AFPs are discussed (Table 2.7).

Peptidomimetics

Peptidomimetics are compounds whose main elements mimic a peptide structure and which generally arise from the modification of an existing peptide. Residues of natural peptides are usually present in the L-enantiomeric form. D-isomers of residues can, however, be found in AFPs after PTM, such as in bacterial lipopeptides or bombinins (Simmaco, Kreil, and Barra 2009). D-amino acid residues have the advantage of not being degraded by proteases, which are in this case, unable to break the bond formed by D-amino acids, due to a change in spatial configuration (Makovitzki, Avrahami, and Shai 2006). In addition to protease stability, linear D-enantiomers of Ib-AMPs have been shown to be as active as native Ib-AMPs on fungi. Moreover, these D-enantiomers are 2- to 8-fold more active against yeasts, suggesting that their action does not require a specific receptor. In addition, they show more resistance to an increase in ionic strength of the medium (Karin Thevissen et al. 2005). A complete enantiomerization, which maintains the structure of the native peptide, is, however, required to avoid loss of activity, as shown for polybia-MPI (Y. Zhao et al. 2016). Another method to enhance stability is to modify the "common" backbone of peptides, via the design of oligomers or unnatural backbone, for instance. These modifications generally increase the therapeutic index and reduce the cytotoxic activity of peptides. These peptidomimetics include peptoids (N-susbtituted glycine oligomers), β-

peptides, acrylamide oligomers, phenylene ethynylenes and sulfono-γ-AA peptides. Their design has been reviewed recently (Bolarinwa et al. 2017; She et al. 2017; Mojsoska and Jenssen 2015; Galetti et al. 2012).

These non-peptidic analogues present several advantages over native peptides. Their small size, for instance, increases the stability of some of them (Hua et al. 2010). Ornithine (Orn) is an unnatural residue commonly used due to its positive charge and its higher resistance to protease activity (Bisht et al. 2007; Sharma et al. 2010). The use of unnatural residues can also improve antifungal activity (Grieco et al. 2013; Guo et al. 2013) or selectivity against fungal cells (Chongsiriwatana et al. 2011). Replacing Trp residues with bulkier aromatic residues, such as β-didehydrophenylalanine (ΔPhe), allows the stabilization of secondary structures and increases antimicrobial properties (Maurya et al. 2013). The replacement of Trp or His residues with analogues drastically reduces their sensitivity to salt and increased the proteolytic stability by minimizing the interaction with proteolytic enzymes (Chu et al. 2013).

Cyclization

Three patterns of cyclization are found in natural AFPs, which are cyclization by disulfide bonds, head-to-tail cyclization between residues at the N- and C-termini, or internal bonds between the side chains. The synthesis of disulfide bridges generally makes the development of synthetic peptides more complex.

In several cases, the antifungal activity is maitained in spite of the absence presence of cysteine residues and disulfide bonds. Thus, the substitution of Cys residues with Ala in the mucin 7 derivative, MUC7 12-mer, does not alter the antifungal activity against *Candida* spp. (Situ et al. 2003). Similarly, the linearization of the spider crab Pro-rich peptide, arasin-1, or the human defensin, HBD-3, does not reduce their activity against *S. cerevisiae* or filamentous fungi, respectively (Paulsen et al. 2013; Thery et al. 2016). Interestingly, linearization of arasin-1 inhibits its antibacterial activity (Paulsen et al. 2013). Cyclization can sometimes be detrimental for the antifungal activity of peptides (Delattin et al. 2014; Sharma et al. 2010). The structural modification implied by the absence of disulfide bonds modifies characteristics, such as plasticity in the protegrin-1 analogue(Rodziewicz-Motowidło et al. 2010) or hydrophobicity of HBD-1 (Schroeder et al. 2011), favouring their antifungal potency.

The effect of cyclization on the toxicity of a peptide appears to be peptide-dependent. Indeed, the replacement of Cys by Ser in granulysin lowers the hemolytic activity of the peptide (Q. Li et al. 2005). Cyclization increases the haemolytic activity of the linear peptide RRWWRF whereas it decreases the hemolytic activity of magainin 2 (Unger, Oren, and Shai 2001). A partial maintenance of the structure of the peptide after deletion is, however, necessary to retain the antifungal activity. Moreover, cyclization improves the proteolytic stability of the peptide indolicidin with a slight reduction of its antimicrobial activity (Friedrich et al. 2001; Rozek et al. 2003),
Similarly, the resistance and the increase of the antifungal activity of members of the Histatin family are greatly increased after cyclization that induces a more compact folded structure (Oudhoff et al. 2009). The linear version of peptide KKLKKFKKLQ adopts a β-sheet structure after cyclization, enhancing its penetration into the membrane (Mika et al. 2011). Although not a determinant of their antifungal activity, backbone cyclization and disulfide bridging increase the serum stability of lactoferrin analogues (Nguyen et al. 2010), and the salt resistance of protegrins. It appears that the effect of cyclization depends on the peptide and on the cyclization method used.

Capping

Capping, which consists in the addition of specific motifs at the N- or C-termini of a peptide sequence, is generally used to impart a sequence with more characteristics of native peptides or proteins. Amidation of the C-terminus and acetylation with acetic anhydride at the N-terminus are the most commonly used capping PTMs applied in peptide design. These two characteristics have been identified in several AFPs (Mura et al. 2016). Amidation of a peptide at its C-terminus has become of increasing interest, due to the removal of the negative charge, increasing antifungal activity and supporting resistance against proteases (G. Wang 2014; Torrent et al. 2011). Thus, the anti-candida activity of P-113, a 12-amino acid fragment of Hst, is increased after amidation (Rothstein et al. 2001). On the contrary, acetylation of the N-terminus decreases the net positive charge, but, on the other hand, provides resistance to proteolytic digestion. The acetylation with acetyl or succinyl groups drastically reduces the antifungal activity of lactoferrin against *S. cerevisiae* (Pan et al. 2007). Interestingly, the protection of both ends of Lfcin analogues provides them with greater stability to serum than the individual capping (Nguyen et al. 2010).

Capping at the N-terminal end is also achieved with lipophilic moieties and is known to increase the α-helix content of peptides. A list of fatty acids, and their role as antifungal agents, have been reviewed (Carballeira 2008). The increased structural complexity makes the peptide more resistant to proteolytic breakdown. The nature of the lipid moiety affects the change of structure and generally determines the antifungal activity (Avrahami and Shai 2002). However, a chain tail that is too long increases the hydrophobicity of the peptide and is commonly associated with an increase in hemolytic activity and cytotoxicity (Laverty et al. 2010; Thery et al. 2018).

Conjugation

Conjugation of peptides has been the target of many researches in recent years, to generate more active, stable and/or peptides with increased selectivity. These advances have been recently reviewed by Reinhardt & Neundorf (2016). The repetition of the PAF motif (rkkwfw), arranged in a repeating polymer, increased the antifungal potency compared to the monomer (López-García et al. 2015). However, the solubility and the proteolytic resistance of the peptide are lowered by oligomerization. This issue has been resolved through the incorporation of the AGPA sequence "hinge" between the repeats. The creation of hybrids of different antifungal peptides, e.g, HP(2-9)-magainin 2(1-12), also increases the potency compared to the single peptides alone (Y. Park, Lee, and Hahm 2004).

Table 2.9. Effect of modifications in peptide design

Modifications	Native peptide	Effect	References
Arg increase	Temporin-Shf	Higher activity	(André et al. 2015)
Arg/Lys→Ala	PAF26	Lower activity	(Muñoz et al. 2007)
Lys6 → Ala6	TPP3	Loss of tetramerization Loss of activity	(Baxter et al. 2015)
Arg → Lys	Tritrpticin	Lower hemolytic activity	(Schibli et al. 2006)
Arg→Lys	granulysin	Lower hemolytic activity	(Q. Li et al. 2005)
Arg/Lys on hydrophobic face	Cathelicidin-BF derivatives	Disruption of α-helix Lower hemolytic activity	(Jin et al. 2016)
Leu11/Tyr18 ←→ Lys7/Lys14	RIVQRIKKWLLKWKKLGY	Increased hydrophobic moment Higher hemolytic activity	(Jin et al. 2016)
Asn → Lys Val→Lys (non-polar face)	KU2: GIWKKWIKKWLNVLKNLF-NH_2	Lower hydrophobicity Lower antifungal and hemolytic activity	(Lum et al. 2015)
Lys → His	DDA-DL6K6	Higher activity at pH < 5.5	(Sharma et al. 2010)
Insertion of Trp	(rD5) of HMWK	Higher activity Increased salt resistance	(Sonesson et al. 2011)
Trp/Phe → Ala	PAF26	Lower activity	(Muñoz et al. 2007)
Addition of hydrophobic residues	PAF26	Higher activity	(Muñoz et al. 2007)
Asn → Trp	HP (2-20): AKKVFKRLEKLFSKIQNDK-NH_2	Higher activity	(J. K. Lee et al. 2013)
Val10 → Trp10	KU1	Higher activity	(Lum et al. 2015)
Trp→ Phe	Tritrpticin	Lower hemolytic activity	(Schibli et al. 2006)
Ala → Leu	DV13-K	Increased hydrophobicity Higher activity against *Ascomycetes* only Higher hemolytic activity	(Jiang et al. 2009)
Val → Ile/Leu	GNU5	Salt resistance Higher activity	(H. Kim et al. 2014)
Glu → Pro	Anal3(HP(2-20)	Kink in helical structure Higher activity	(J. K. Lee et al. 2013)

Table 2.9. *contd*

Modifications	Native peptide	Effect	References
Truncation	PMAP-36	Lower hydrophobicity Lower hemolytic activity	(Lyu et al. 2016)
KW repetition	(KW)n motif	Increased cationicity and hydrophobicity Higher activity	(Gopal et al. 2013)
D-enantiomers	Kaxin F17-6K	Similar or higher activity	(Burrows et al. 2006)
D-enantiomers	Polybia MPI	Increased stability Similar activity	(Y. Zhao et al. 2016)
D-enantiomers	Ac-RKTWFW-NH_2	Acquisition of antifungal activity	(López-Garcia et al. 2000)
D-enantiomers	Ib-AMPs	Higher activity	(Karin Thevissen et al. 2005)
Gly→Aic or Cha	Dnal	Acquisition of candidacidal activity	(Grieco et al. 2013)
Addition of bulky β-naphthylalanine	Ac-KKWRKWLA KK-NH_2	Improved salt resistance	(Chu et al. 2013)
Linearization	Arasin-1	Similar antifungal activity	(Paulsen et al. 2013)
Cyclization	RRWQWR	Serum stability	(Nguyen et al. 2010; Rozek et al. 2003)
Cyclization	Indolicidin	Similar antifungal activity Higher hemolytic activity	(Falla and Hancock 1997)
Cyclization	granulysin	Lower hemolytic activity	(Q. Li et al. 2005)
Cyclization	Magainin-2	Lower hemolytic activity	(Unger, Oren, and Shai 2001)
Acetylatyion	Lactoferrin	Lower activity	(Pan et al. 2007)
Acetylation	RRWQWR (Lfcin derivative)	Slight serum resistance	(Pan et al. 2007)
Amidation	P-113	Higher activity	(Rothstein et al. 2001)
Amidation	Trp and Arg-rich analogs	Decreased salt resistance	(D. I. Chan, Prenner, and Vogel 2006)
Acetylation and amidation	RRWQWR	Higher serum resistance	(Pan et al. 2007)
Lauric acid	O3TR	Higher activity Hemolytic activity	(Thery et al. 2018)
Double repetition	PAF26	Higher activity Lower solubility and proteolytic resistance	(López-García et al. 2015)
AGPA hinge for concatemerization	PAF26	Higher activity Maintain of resistance	(López-García et al. 2015)
Conjugation	Hp-magainin hybrid	Higher activity	(Y. Park, Lee, and Hahm 2004)

2.9 Spectrum of activity

Many AFPs presented in this review possess a broad spectrum of antifungal activity, against both moulds and yeasts. Natural plant AFPs are particularly effective against fungal phytopathogens. Nonetheless, the screening of the activity of AFPs is most studied against human pathogens, especially *Candida albicans*. Thus, studies against a broader spectrum of microorganisms are necessary to evaluate the potential application of a larger number of natural AFPs in foods.

Furthermore, the fungal target and the fungus-peptide interaction will have a direct influence on the antifungal potency of natural AFPs (Carvalho and Gomes 2009). Thus, although it shows strong effect on the growth of certain fungi, the radish defensin, Rs-AFP2 is inactive at 100 μg/mL against other species (Urdangarín et al. 2000).

2.10 Production of AFP

The lack of an optimized process and the limited studies on the effects of natural AFPs in food restrict their application (Tajkarimi and Ibrahim 2012). Seeds are a convenient source that is commonly used for isolation of AFPs (Table 2.2). However, the yield of plant AFPs from seeds is generally low, at 14-30 mg/kg (De Zélicourt et al. 2007; Franky R G Terras et al. 1992). Extraction processes using seeds can be optimized in various ways, through induced expression of the plant AFPs in seedlings in response to several biotic and abiotic stimuli (Stotz, Thomson, and Wang 2009). An increase of the level of AMPs has been observed in plant species during development (Kovaleva et al. 2011; Grosbois, Guerbette, and Kader 1989; F. R. Terras et al. 1995), after wounding, or in presence of signalling molecules (Thomma et al. 1998; Vriens, Cammue, and Thevissen 2014). Nevertheless, in spite of relatively cheap raw materials, the extraction and purification of natural AFPs remains an expensive process. Since the production of synthetic peptides is becoming cheaper and cheaper, it might be, in the future, economically more interesting compared to natural extraction or production of recombinant (natural) peptides (see section “unnatural residues”).

2.11 Effect of salt

As a food preservative, an efficient peptide must retain at least partial inhibitory activity in presence of salt. The antifungal activity of a large amount of AFPs, including defensins and snakins (Berrocal-Lobo et al. 2002), is decreased at high salt concentrations and/or in presence of cations. On the contrary, several natural peptides, such as cecropin A or the fungal defensin, AcAFP (Skouri-Gargouri, Jellouli-Chaker, and Gargouri 2010), have been shown to maintain their activity in the presence of highly concentrated salt solutions. The relationship between peptide activity and high salt concentration has been explained by the basic character of peptides. In addition, cations might affect peptide organization, in terms of secondary structure, or induce peptide aggregation, limiting its activity. However, certain peptides with the same net charge do not display the same salt-resistance. Hence, conformational rigidity and good amphipathicity are thought to maintain the activity under high salt conditions.

A decreased activity in the presence of high salt concentrations has also been found for the synthetic peptide $[RLLR]_5$, which has been explained by the disruption of the α-helix pattern (I. Y. Park et al. 2004). Several strategies can be used to improve the salt resistance of peptides. The structure of $[RLLR]_5$ has been stabilized by stapling with helix capping motifs, resulting in a peptide with antifungal activity, even at high salt concentrations (I. Y. Park et al. 2004). The replacement of Trp or His residues with bulky amino acids, such as β-naphthylalanine in the peptide P-113, makes the peptide resistant to up to 150 mM NaCl (H. Y. Yu et al. 2011). Other methods, such as end-to-end cyclization (Q. Yu, Lehrer, and Tam 2000), creation of dimers, or hybridization of peptides can also improve the salt resistance (Han et al. 2016).

2.12 Effect of temperature, pH and proteolysis

Heat treatment has always been a process employed to prevent food contamination (e.g. pasteurisation). However, in the case of low temperature treatment or a faulty process, the survival of fungal species, particularly fungal spores may occur. Due to multiple disulfide bonds, most AFPs are structurally compact and hence possess high thermal (in many cases pH-dependent), chemical, and enzymatic stability.

Many peptides have been shown to be stable and retain their antifungal activity over a broad range of pH (Y. S. Chan et al. 2012). Due to their basic nature, AFPs have generally an enhanced activity in low pH. In the case of His-containing peptides, the enhancement occurs due to an increase in peptide net charge and a facilitated translocation (Kacprzyk et al. 2007). This hypothesis is well illustrated in recent studies showing that high pH inhibited the antifungal activity of synthetic peptides (Walkenhorst et al. 2013), although some peptides display better activity at higher pH (Hajji et al. 2010). In order to provide a larger pH range, dimers (P-113) can be created, although a deeper understanding of the effect on peptide antifungal activity is needed.

Due to their compact structure, defensin-like peptides and many others, such as LTPs (Peng Lin et al. 2007), are also resistant to high temperature, although a slight loss of activity can occur at temperatures higher than 80-90°C, when the structure is irreversibly changed (Gallarate et al. 2011). Killer toxins produced by yeasts are usually active within a narrow range of pH and rapidly inactivated at temperatures higher than 40-50°C (Soares and Sato 2000; Wilson and Whittaker 1989). However, yeast killer toxins produced by halotolerant yeast can present higher pH stability and an enhanced activity at higher salt concentrations. Among these, the killer toxins PMKT and PMKT2, produced by *Pichia membranifaciens*, represent promising protective agents in food and beverage production (Belda et al. 2017). Studies of the effect of low temperature on the activity of peptides, especially against fungi, are quite limited and this effect seems to be peptide-dependent.

The structure of AFPs provides a certain resistance to protease degradation. Although several AFPs maintain their activity after treatment with trypsin or chymotrypsin (P. Lin and Ng 2009; X. Zhao et al. 2013; H. Kim et al. 2014), the proteolytic resistance of AFPs is complex, and structurally stable peptides can also be sensitive (Hajji et al.

2010). Indeed, pepsin or proteinase K digestion up to 9 hours does not affect the antifungal activity of PAF, but the peptide is sensitive to a longer exposure. The development of synthetic peptides can improve the proteolytic resistance, with the use of oligomers, D-enantiomers (López-García et al. 2015), or cyclization (Nguyen et al. 2010).

The requirement for thermal, pH and enzymatic stability is mainly based on the assumption that these peptides would be applied to food products as ingredients, therefore passing through all the processing stages. Alternatively, AMPs can be incorporated in the food matrix through dipping or spraying treatments, or even through the more recent approach of active packaging (AP) (Corbo et al. 2009).

2.13 Cytotoxicity

Most plant AFPs are recognized as being non-toxic to mammalian cells due to their preferential interaction with microbe-specific targets, such as ergosterol or GlCer, that are absent on mammalian cells (Lohner and Leber 2016). Moreover, differences in the composition of membrane lipids between mammalian and fungal cells are an important factor, in the absence of toxic effects due to human consumption (Vriens et al. 2016). For example, the higher content of cholesterol in their membrane confers mammalian cells a protection against the toxicity of AFPs (Boesze-Battaglia and Schimmel 1997; Matsuzaki 2009). The insertion of PTMs, the modification of peptide backbone or disruption of α-helical structures are common methods employed to decrease the toxicity of AFPs.

2.14 Food applications

The interest in AMPs is increasing for both pharmaceutical and biotechnological applications, due to their multiple modes of action, which limits the risk of the development of resistance. The non-native expression of several plant defensins in plants conferred resistance towards phytopathogenic fungi. Thus, Rs-AFP2 and the hevein Pn-AMP were expressed in tobacco or a TL from *Ocimum basilicum* in

Arabidopsis. AMPs appear to be more active than weak acids commonly used as food preservatives, which are active at concentrations of 1-10 mM against yeasts (Bell, Etchells, and Borg 1959), but also inhibited filamentous fungi (18-236 µg/ml). In addition, these acids are highly active in a narrower range of pH (Razavi-Rohani and Griffiths 1999), generally up to pH 5. However, an advantage of these acids is that their use is unaffected by high salt concentrations (Razavi-Rohani and Griffiths 1999).

Despite a growing number of reports of food applications of antimicrobial compounds, studies on application of AMPs to foods are still quite limited (Lucera et al. 2012). The existing reports of food applications of AMPs mostly deal with the antibacterial peptide, nisin, a lantibiotic mostly active against Gram-positive bacteria and, to a much lower extent, against the food-borne human pathogens, such as *E. coli* or *Salmonella* (Rai et al. 2016). The proof of concept of the efficacy of antimicrobial compounds in food systems is necessary, since the peptide activity may be altered by factors such as the food matrix and composition of ingredients, or any processing steps. Indeed, a decrease in the antimicrobial potency of several compounds in presence of food components, such as sugars, lipids or proteins is common. For instance, the activity of dermaseptin S4 derivatives was reduced by 2-8 fold in apple juice, compared to the activity in microbiological medium (Rydlo et al., 2006).

However, synthetic peptides have been shown to be active in food matrixes, such as 6K8L in apple juice and O3TR in several beverages (Thery et al. 2018). Studies also reported that recombinant peptides can protect food or ingredients from fungal degradation, such as TrxAq-AMP in oranges (Paola DiazDellavalle 2014) and rHBD-3 in bread (Thery et al. 2016).

Several AFPs have been approved for food applications as preservatives, such as lactoferrin certified by the European Food Safety Authority (EFSA) since 2012. In addition to lactofferin, several AFPs have been allowed in other applications, such as the soy protein BBI used as a drug (Losso 2008), or thaumatin for use as a sweetener. The high solubility of thaumatin, its stability, over a pH range of 2.7-6.0 and under pasteurization conditions, makes it an interesting candidate for other food applications. It is however, more heat-labile at higher pH (Kaneko and Kitabatake 2001). Brazzein is a 54-amino-acid defensin-like protein, first isolated from the fruit of *Pentadiplandra brazzeana*, and 500 times sweeter than sucrose. Its small size (6 kDa) and extreme stability over a wide range of pH and temperatures, due to the presence of four

disulfide bridges, makes it a good candidate for food applications. Furthermore, recombinant brazzein production through secretory expression in *Kluyveromyces lactis* has been reported (C. R. Yun et al. 2016). So far, brazzein is still being discussed with respect to FDA approval.

In addition to their incorporation as ingredients, AFPs are interesting candidates for AP, as demonstrated for several bacteriocins (Malhotra, Keshwani, and Kharkwal 2015; Rydlo, Miltz, and Mor 2006).

2.15 Conclusion

Commonly used natural food preservatives, such as enzymes or bacteriocins, present several limitations, such as poor stability or narrow spectrum of activity. Organic acids are generally used to fight fungal spoilage, but their activity is limited to a narrow pH range and their use as synthetic agents is seen as a concern by consumers. On the contrary, plants and their essential oils represent promising natural antifungal agents and they are gaining interest for potential application in food preservation since most of them possess GRAS status. However, the essential oil constituents are generally not effective as single components and they can modify the organoleptic properties of food products. Intensive research on new ways to fight human and plant fungal pathogens has highlighted the potential of AFPs in the immune system of organisms, including plants. This review, and numerous reports, highlight the promising activity of these plant AFPs against food-related fungi. However, the necessary time-consuming extraction process, a limited stability and the requirement for a high amount can limit the direct application of many AFPs in food products.

Despite the remaining gaps in knowledge, especially regarding their mechanism of action, an understanding of key determinants of the activity of AFPs is a solid foundation for the synthesis of many novel, potent synthetic peptides that have low cost, low toxicity and high stability. The increasing use of computational based methods has streamlined the design and optimization of novel peptides, which explains the increasing number of peptides with biotechnology applications. Despite promising results, the use of these synthetic peptides as food preservatives must face the

increasing consumers' demand for green-labelled, natural preservatives and the challenges associated with obtaining regulatory approval. Although it does not represent the most efficient solution for the prevention of fungal spoilage in food, the application of isolated compounds (*e.g.*, lactoferrin), or plant extracts containing highly stable peptides, such as brazzein, appears to be the best compromise.

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2.17 Author contributions

Thibaut Thery was responsible for the compilation of the data presented in the manuscript, the writing of the manuscript and the design of figures. Kieran Lynch critically reviewed and edited the manuscript. Elke Arendt was responsible for the structure and the content of the manuscript, the correction and the final approval for the version to be submitted.

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Chapter 3
Antifungal activity of a synthetic human β-defensin 3 and potential applications in cereal-based products

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Abstract

Defensins are cysteine-rich antimicrobial peptides of the innate immune system involved in constitutive and adaptive immunities. The aim of this work was to characterize the antifungal activity of a linear human β-defensin-3 (HBD-3) and test this peptide as a potential food preservative. Antifungal activity of the synthetic HBD-3 was demonstrated against *Fusarium culmorum, Penicillium expansum* and *Aspergillus niger*. To determine potential baking applications with the defensin, a parental wild yeast *Saccharomyces pastorianus* strain, called CMBS-33, was transgenically modified. The novel lager yeast strain, called CM-INT-51, was able to express the gene encoding HBD-3. The use of CM-INT-51 to ferment the dough extended the shelf life of bread by three days in comparison to CMBS-33. Furthermore, synthetic HBD-3 (80 $\mu g.ml^{-1}$) delayed fungal growth on bread for more than 13 days. These results revealed that both the synthetic peptide and the yeast containing HBD-3 have the potential to increase the shelf life of baked goods.

3.1 Introduction

The fungal spoilage of cereal-based products is a source of economic losses (~€500 millions/year) and has the potential to cause serious health problems (Legan 1993, Pitt & Hocking 2012). The main fungal contaminants of bakery products belong to *Penicillium* genus. In the baking industry, heat treatment reduces the risk of microbial contamination but fungal spores survive the baking process, including spores from wheat contaminants such as *Fusarium* spp. or *Aspergillus* spp.. The resulting mould growth is responsible for the formation of unpleasant flavours, reduction of the shelf life and has the potential to produce mycotoxins and allergens (Nielsen & Rios 2000).

A consumer study published by Van Sint Fiet (2015) highlights that the appearance of mould is the first reason for people to discard bread. To satisfy consumers' demands, bread should be a high quality product with extended shelf life and a limited proportion of chemical preservatives. Before the advent of cultured yeast in baking industry, sourdough used to be the standard method for bread leavening. The development of gluten-free bread and the research for a healthy bread lead to a revival of sourdough. Among the alternative methods to current preservatives (e.g., calcium propionate E282), sourdough is the most commonly used and has been proven to be efficient in preserving bread (Giuseppe Rizzello et al. 2009). However, the control of the different parameters of the production of bread with sourdough, like the pH or the starter cultures, can be a challenge for large-scale bread production (Denkova et al. 2014).

Antimicrobial peptides are usually small peptides, between 12-57 amino acid residues, with cationic amphipathic properties. Those AMPs are members of the innate immune system of many living organisms. The expression of these antimicrobial peptides can be constitutive or can be inducible by infectious and/or inflammatory stimuli (Jenssen et al. 2006). Natural antimicrobial peptides are found or used in food products, mainly bacteriocins such as nisin produced by certain strains of *Lactococcus lactis* (Cleveland et al. 2001) or lactoferrin, a glycoprotein of milk (Keymanesh et al. 2009).

Among these AMPs, defensins represent the largest family. These are basic and small peptides (between 45-54 amino acids residues), with a high proportion of cysteine residues. They have been identified as host defence members of various species of invertebrates, plants and vertebrates. The mammalian defensins are divided into three

classes based on disulfide connectivity and their three-dimensional structure. The α-defensins are found only in neutrophils and Paneth cells of humans (Cunliffe 2003). The θ-defensins are only found in old world monkeys and possess a cyclic structure (Lehrer & Lu 2012). Human β-defensins (HBD) are expressed in different tissues of the human system (Dhople et al. 2006) and display antimicrobial activity against human pathogens such as *Escherichia coli*, *Staphylococcus aureus* (Harder et al. 2001, Hoover et al. 2003) or *Candida albicans* (Vylkova et al. 2007). Different models have been proposed to explain the mode of action of these peptides. In these models, it is proposed that due to their positive net charge and their amphiphatic nature, the defensins are capable of interacting with the microbial cell wall (Sahl et al. 2005). The ensuing antimicrobial activity would be the result of several processes, *e.g.*, cell leakage, production of reactive oxygen species or induction of cell apoptosis (Guilhelmelli et al. 2013).

Apart their antimicrobial activities, HBDs possess immunomodulatory activity. There is some evidence that these defensins play a role in certain genetic diseases, such as cystic fibrosis, and cancer (Droin et al. 2009, Pazgier et al. 2006). To date, among the six characterized human β-defensins, HBD-3 is the defensin with the highest net charge (+11 at pH 7). HBD-3 is a peptide of 45 amino acid residues, whose native form includes 6 cysteine residues, bound as C1-C5; C2-C4; C3-C6. Although these defensins represent an interesting candidate to fight microbial spoilage, no application has been so far conducted in food products (Davidson et al. 2005). However, a recombinant HBD-3 has been proven to inhibit the growth of spoilage bacteria in beer (James et al. 2014). In addition, Liu et al. (2008) showed that the linear analogue of HBD-3 has no hemolysis activity towards rabbits erythrocytes nor cytotoxicity on human conjunctival epithelial cells. Furthermore, to our knowledge, HBD-3, or any analogue, have never been shown to display allergenic activity. We report here the activity of synthetic HBD-3 as well as the activity of a *S. pastorianus* strain expressing HBD-3 against fungal contaminants isolated from cereal-based products. Weak organic acids are common preservatives used in baking industry (see Chapter 2). The potential application of HBD-3 as a preservative agent for bread was also studied. However, given the limited *in vivo* research on defensins and more generally AMPs, no defensin has been approved so far, by safety authorities, for food application (Hintz et al. 2015).

3.2 Material and methods

Defensins

A synthetic linear analogue of the human β-defensin 3 (sHBD-3, 5.1 kDa, purity >70%) was purchased from GLBiochem, Shanghai, China. A crude peptide was synthesized by solid phase peptide synthesis using Fmoc chemistry on a solid support of rink amide MBHA resin. Following a final deprotection with 20% piperidine in dimethylformamide (DMF) and cleavage with a mixture of TFA/water (90:10), crude peptide was purified using reversed phase HPLC on a Gemini-NX 5 µm C18 column (4.6 x 250 mm, 110 Å). The lyophilized peptide was reconstituted in 0.1% acetic acid (pH 3.5) as described by the manufacturer and stored in vials of different concentrations at -20°C.

Fungal strains

The filamentous fungi used in this study represent some of the main contaminants of the cereal-based products (Beuchat 1987). They were *Fusarium culmorum*, *Aspergillus niger* and *Penicillium expansum*. The fungal strains *F. culmorum* FST 4.05, *A. niger* FST4.21, *P. expansum* FST 4.22 originated from the culture collection of the School of Food and Nutritional Sciences, University College Cork (Cork, Ireland).

Antifungal activity assay

The antifungal activity of sHBD-3 was determined using a microtiter plate assay as described previously (Finkina et al. 2008, Li et al. 2013) with some modifications. Briefly, fungal spores were collected from mature colonies grown for 72 h on PDA (Sigma-Aldrich) plates at 28°C, counted on a Neubauer chamber haemocytometer and inoculated in synthetic nutrient-poor broth. Fungal spores were then inoculated in half-strength potato broth (½ PDB) (Sigma-Aldrich) to obtain a final concentration of 10^3-10^4 spores.ml^{-1}. Different concentrations of sHBD-3, ranging from 5 µg.ml^{-1} to 150 µg.ml^{-1} (final concentration), were added into the aliquot of fungal cells and the growth inhibition was assessed by measuring the solution absorbance at 620 nm for 96 h at 28°C on a 96-well microplate reader (Multiskan TM, Thermo Scientific). As negative

control, 0.1% acetic acid in ½ PDB (pH=5.0) was used instead of sHBD-3. The inhibition of the fungal growth by the presence of sHBD-3 was then examined on PDA plates. The same solutions were prepared as described above and after 24 h of incubation at 28°C, 50 µl were spot-inoculated on PDA plates. Pictures of the plates were taken every 24 h for a period of three days.

The inhibition of *F. culmorum* by sHBD-3 peptide was observed using a phase contrast microscope. An aliquot of ½ PDB inoculated with a concentration of 10^4 spores.ml^{-1} of *F. culmorum* and 10 µg.ml^{-1} of sHBD-3 was incubated at 28ºC, and observed over a 24-h incubation time. The experiments were performed in triplicate.

MIC/IC_{50} determination

After 96 h of incubation at 28ºC, the MIC was read as the lowest concentration of sHBD-3 resulting in complete inhibition of fungal growth (Fig. 3.1). The required concentration to inhibit 50% of the fungal growth (IC_{50}) was determined by non-linear regression, using the software Graph PRISM (GraphPad Software, Inc., La Jolla, CA) with these data.

Effect of NaCl on antifungal activity of sHBD-3

HDB-3 has already been reported to be the least sensitive defensin to salt solutions (Jenssen et al. 2006). Indeed, the peptide retains its antimicrobial activity against *E. coli* and *S. aureus* in solutions containing 100 mM NaCl (Hoover et al. 2003). Here, the activity of sHBD-3 was studied against *F. culmorum*. Fungal spores (10^3-10^4 cells.ml^{-1}) were inoculated into ½ PDB solutions containing either 50 mM or 150 mM of NaCl. Reconstituted sHBD-3 (final concentration = 15 µg.ml^{-1}) was added into the two spore solutions and the antifungal activity was determined as described above. The effect of NaCl on the antifungal activity of sHBD-3 was measured as the difference of percentage of inhibition of the fungal growth between the non-salted and the salted solutions, based on the microtiter plate results.

Thermal stability of sHBD-3

Recombinant porcine β-defensins have been reported to be stable after different heat treatments (Li et al. 2013). In order to investigate the thermal stability of sHBD-3, a peptide solution (in 0.1% acetic acid) was heated at 100°C for 14 min (baking time used in this study). After cooling, the activity of sHBD-3 (final concentration = 15 $\mu g.ml^{-1}$) was measured against *F. culmorum* FST 4.05 in a 96-well microtiter assay as described above.

Yeast strains

Two *Saccharomyces pastorianus* strains were used in this study. The parental strain *S. pastorianus* CMBS-33 is a bottom-fermenting lager yeast strain. CMBS-33 was used to create a novel strain, by integrating the gene coding for HBD-3 into its genome. The novel strain, called CM-INT-51, secreted recombinant HBD-3 (rHBD-3) into its growth medium (James et al. 2014)(see Appendix I for construct). *S. pastorianus* CMBS-33 and CM-INT-51 were from the Moyne Institute culture collection, Trinity College Dublin. A commercial *Saccharomyces cerevisiae* strain (Pante instant dry yeast, Puratos, Belgium) was used as a control for the baking process.

Preparation and growth of cells

The *S. pastorianus* strains from a glycerol stock were first grown on yeast peptone sucrose (YPS) agar plates (0.3% (w/v) yeast extract, 1% peptone, 1% sucrose, and 2% agar) at 30ºC. For the selection of the recombinant CM-INT-51, the yeasts were grown on YPS agar containing geneticin (200 $\mu g.ml^{-1}$) (Sigma Aldrich, St. Louis, MO) as described by James et al. (2014). After 72 h, the yeasts colonies were harvested and inoculated in YPS medium at 30°C and the growth was monitored by measuring the optical density (OD 620 nm) and counting the cells using a Neubauer chamber haemocytometer every 6 h. After 48 h of growth (between the diauxic shift and post-diauxic shift phase) which corresponds to the best phase to obtain superior bread characteristics according to Rezaei et al.,(2014), the yeasts were harvested as described by Rezaei et al. (2014). The yeast culture was centrifuged at 870 *g* for 3 min in an Eppendorf centrifuge 5417D (Eppendorf AG, Hamburg, Germany). Cultures were stored at 4ºC to be used in baking trials. Since a part of rHBD-3 has been shown to be

expressed in the growth medium of CM-INT-51 (3-10 $ng.ml^{-1}$) (James et al. 2014), the supernatant was kept at -20ºC for analysis or freeze-dried for further use in bread preparation.

Quantification of metabolites in the fermentation medium

The concentrations of different sugars (sucrose, glucose and fructose) and organic acids (citric, succinic and acetic acids) in the supernatant were determined as described by Rezaei et al. (2014). Briefly, the supernatant collected previously was centrifuged at 11,000 *g* for 3 min and filter-sterilized with 0.20 µm filters (Macherey-Nagel, GmbH & Co., Germany). Twenty µl of each sample were injected into a HPLC system (Agilent technologies, 1260 Affinity) equipped with a refractive index detector (RID) and an ultraviolet-diode array detector (UV/DAD) to quantify carbohydrates and organic acids. The analyses were performed twice on two different fermented growth media The standard calibrations curves were determined at the beginning of the set measurement with four different concentrations of acids (10-100 mM) and sugars (0.5-5mM). The separation of sugars was carried out using Hi-Plex H column (300 × 7.7 mm, 8 µm, Agilent, Cork, Ireland), equipped with a guard column (50 × 7.7 mm, 8 µm, Agilent, Cork, Ireland), using water at a flow rate of 0.6 mL/min at 25°C. The organic acids were quantified using the same column, after elution with 5 mM H_2SO_4.

Quantification of rHBD-3

rHBD-3 levels expressed by CM-INT-51 were quantified by capture enzyme-linked immunosorbent assay (capture ELISA) kit (Peprotech Co., Rocky Hill, NJ, USA) as described by James et al. (2014) with some modifications. The levels of rHBD-3 produced by CM-INT-51 in the supernatant or in the cell lysate were determined. Briefly, after 48 h of fermentation, the yeasts cells and the supernatant were separated by centrifugation at 870 *g* for 3 min. The supernatant was filtered, purified on Sep-Pak C18 cartridge (Merck Millipore, Cork, Ireland) and freeze-dried. Yeast cell pellets (2.5-ml packed cell volume) of CM-INT-51 were washed twice in 20 volumes of phosphate-buffered saline (PBS). Total cell lysates were prepared as using CelLytic-Y (Yeast Cell Lysis/Extraction Reagent; Sigma-Aldrich) as described by James et al.

(2014). Wells of a Nunc Maxisorb (96-well) plate (Thermofisher, Loughborough, UK) were coated overnight at RT with 100 µl of polyclonal anti-HBD-3 capture antibody in PBS (1 $\mu g.ml^{-1}$). The wells were washed four times with 0.05% Tween 20 (PBS-Tween). The wells were then blocked for 2 h with 1% bovine serum albumin (BSA) in PBS, washed and incubated with 100 µl of different concentrations of sHBD-3 (as standard), previously diluted in 0.05% Tween-20, 0.1% BSA, PBS or samples.

Following incubation overnight at 4ºC, the wells were washed and incubated for 2 h with biotinylated polyclonal anti-HBD-3 antibody (0.1 $\mu g.ml^{-1}$) and washed as before. The wells were incubated for 30 min with 100 µl of streptavidin-HRP (1:2000 in 0.05% Tween 20, 0.1% BSA, PBS) and then washed with PBS-Tween. Antibody-binding was detected with 3,3′,5,5′-Tetramethylbenzidine (TMB, T8665; Sigma-Aldrich) and the colour development was monitored for 20 min. Following incubation, sulphuric acid (to 0.1 M) was added and the absorbance measured at 450 nm with a wavelength correction at 650 nm.

Bread preparation

The dough was prepared according to a UCC standard recipe, based on flour weight (baker's flour, Odlums, Ireland) with some modifications: 3% (w/w) of commercial fat, 2% (w/w) of sugar from Siucra (Ireland), 0.3% (w/w) of sodium chloride from Glacia British Salt Limited (UK), 0.5% of sodium stearoyl lactate (SSL) (Danisco, Copenhagen, Denmark), 0.1% of ascorbic acid (AllinAll Ingredients, Dublin, Ireland). The yeast strains were *S.pastorianus* CMBS-33, *S. pastorianus* CM-INT-51 and commercial *S. cerevisiae* dry yeast (Pante instant yeast, Puratos, Belgium). Tap water (37%, v/w, for the rehydration of Puratos strain) or fermentation medium (YPS) for the *S.pastorianus* strains was used. The yeast concentration was $6 * 10^{10}$cells/100 g of flour. Each dough of 50 g was fermented in a proofer (KOMA Sunriser, Roermond, Netherlands) for 1 h at 30ºC and 85% RH, and then baked for 14 min at 210ºC in a pre-steamed deck oven (MIWE, Arnstein, Germany). Finally, the breads were left for cooling for 2 h at room temperature before analysis. Three batches were prepared for each yeast strain.

Another baking trial was conducted with the Pante yeast strain, in which the tap water used in the recipe was supplemented with sHBD-3 (concentration = 80 $\mu g.ml^{-1}$). A

trial was performed with 0.3% (w/w, based on flour weight) calcium propionate (Sigma-Aldrich) as preservative agent commonly used in industry.

Bread characteristics

The breads were characterized as previously described by Hager et al. (2012). Specific volume was measured using a Volscan profiler (Stable Micro Systems, UK). After cutting the breads into 10-mm slices, three slices from the centre of each loaf were used for further analysis. Firstly, the crumb colour was analysed, using a chroma meter CR- 400 (Konica Minolta Inc., Tokyo, Japan) with CIE standard illuminant D65. The L*- value, as calculated by the software, was used to evaluate the crumb lightness. C-cell Bread Imaging System (Calibre Control International Ltd., UK) was used to characterize the crumb structure. The wall thickness and the average diameter of the cells were evaluated. The crumb texture was determined by texture profile analysis (TPA), using a TA- XT2i texture analyser (Stable Micro Systems, Surrey, UK), equipped with a 25-kg load cell and a 10-mm aluminum cylindrical probe. A speed of 5 mm/s and a force of 0.98 N were applied to compress the middle of the crumb to 50% of its original height. Three slices per batch were analyzed on the baking day.

Analysis of metabolites in breads

The concentrations of sugars and acids in the different breads were measured by HPLC as described by Rezaei et al. (2014). Briefly, slices of 10 mm of loaf were blended (Kenwood chef classic) for 30 s in two volumes of MilliQ water. After centrifugation at 11,000 *g* for 3 min, the supernatant was filter-sterilized with 0.20 µm filters and analyzed as described for the fermentation medium.

Quantification of rHBD-3 in the bread

To determine the concentration of rHBD-3 in the bread, 10 g of bread were soaked into 100 mL of water overnight at room temperature. After centrifugation, the supernatant of bread/water mix was filtered and purified on Sep-Pak C18 cartridge and freeze-dried. The lyophilisate was resuspensed in 0.5-1.0 ml of PBS and the concentration of rHBD-3 was determined as described above.

Shelf life

The study of the effect of the yeast strain CMBS-33 and the novel strain CM-INT-51 on the shelf life of bread was made as described by Dal Bello et al. (2007) with some modifications. Loaves were cut transversely in a sterile manner to obtain uniform slices of 10-mm thickness and left 5 min on each side exposed to the ambient air. Then, the slices were packed in heat-sealed plastics bags. Filter pipette tips were inserted in small slots on each side of the bag and the bags were incubated at room temperature. The slices were examined for mould growth over 13 days and the shelf life was determined by the day of appearance of the first fungal colony. The slices were sorted into five categories based on the percentage of contamination as described by Axel et al. (2015).

Statistical analysis

The results in the tables are presented as means ± SDs. A Kruskal–Wallis Test was performed with XLSTAT Version 2015.5.01. (Adinsoft Inc, New York, USA) to compare the metabolite concentrations in the growth media of the *S.pastorianus* strains, and to analyse the textural properties of breads and metabolites concentrations in the breads. Values of $p < 0.05$ were considered to be statistically significant.

3.3 Results and discussion

Antifungal assays

The growth curves of the three studied fungi (*P. expansum, A. niger* and *F. culmorum*,) are presented in Figure 3.1. sHBD-3 showed the highest antifungal activity against *F. culmorum* with an IC_{50} value of 11 µg.ml^{-1} and a MIC range of 11-15 µg.ml^{-1}. Higher concentrations of sHBD-3 were required to inhibit *A. niger* and *P. expansum* (respectively IC_{50} of 36.4 µg.ml^{-1} and 44.3 µg.ml^{-1}) (Table 3.1). The inhibition of the fungal strains was confirmed on PDA plates after 96 h (Fig 3.2). The small difference in sHBD-3 concentration between IC_{50} and MIC could indicate a concentration threshold before antifungal activity. On the other hand, for *P. expansum* and *A. niger*, a dose-response activity is likely to occur.

Table 3.1. Inhibition of fungal growth by a synthetic human β-defensin 3

Fungal species	IC_{50} (µg.ml^{-1})	MIC (µg.ml^{-1})
Fusarium culmorum FST 4.05	11.0	11-15.0
Penicillium expansum FST4.21	44.3	>150
Aspergillus niger FST 4.22	36.4	>150

>150: no total inhibition in the range of concentration and time used in this study

Fig. 3.1a

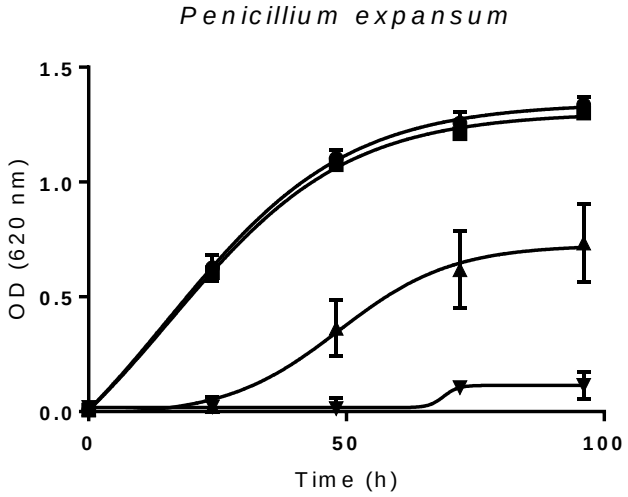


Fig. 3.1b

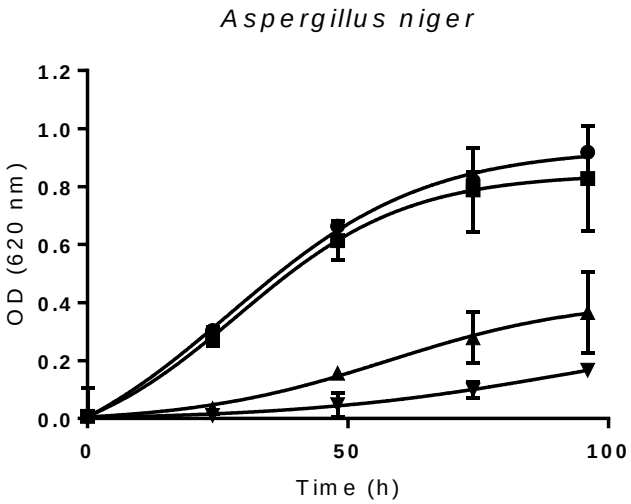


Fig. 3.1c

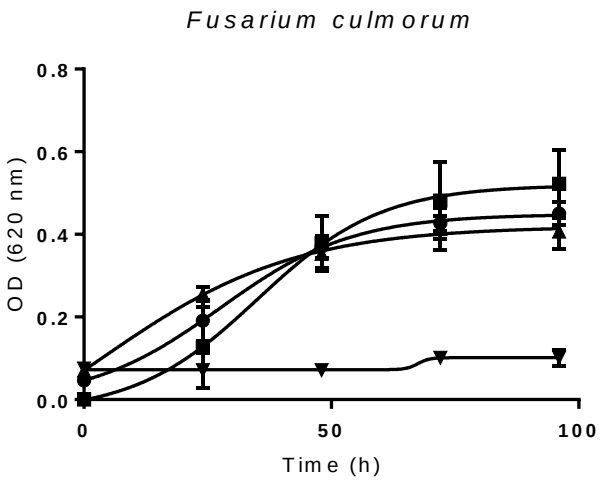


Figure 3.1. Fungal growth inhibition of sHBD-3**.** (a) *Penicillium expansum* FST4.21, (b) *Aspergillus niger* FST 4.22, (c) *Fusarium culmorum* FST 4.05. **Fig. 3.1a** and **b:** circles: 0 µg.ml$^{-1,}$ squares: 5 µg.ml^{-1}; triangles: 50 µg.ml^{-1}; reversed triangles: 150 µg.ml^{-1}; **Fig. 3.1c**: circles: 0 µg.ml$^{-1,}$ squares: 5 µg.ml^{-1}; triangles: 10 µg.ml^{-1}; reversed triangles: 15 µg.ml^{-1}

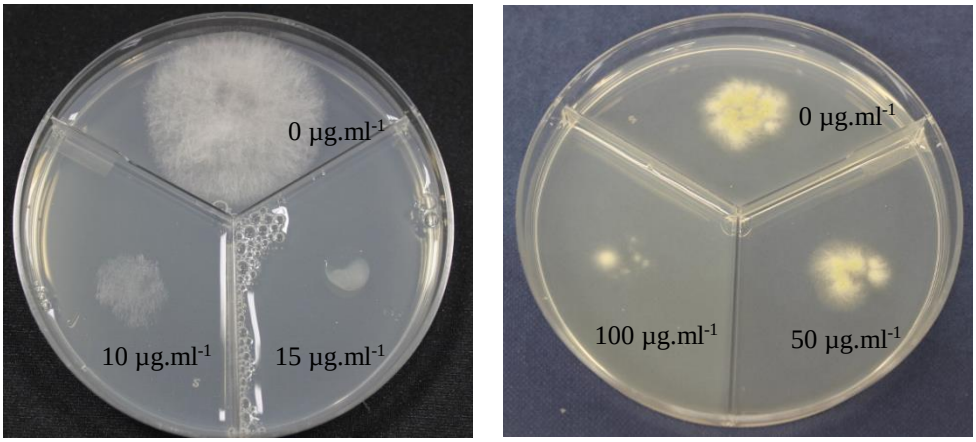


Figure 3.2. Examination of fungal inhibition on agar plates after 96 h of incubation in ½ PDB with different concentrations of sHBD-3. Left: *F. culmorum*; Right: *A. niger*. The concentrations of sHBD-3 in the broth are indicated in the different compartments.

Apart from its typical CSαβ motif, the configuration of human defensins is characterized by the presence of three disulfide bonds between the cysteine residues. Unlike native HBD-3, a synthetic HBD-3 does not have disulfide bonds. Despite this, sHBD-3 is able to inhibit fungal growth, which supports the idea that disulfide bonds are not essential for the antimicrobial activity (Hoover et al. 2003). However, the presence of disulfide bonds in the peptide has been shown to improve the antibacterial activity and has been proven to be involved in the chemotactic action of HBD-3 (Wu et al., 2003).

The mechanism behind the inhibition of *F. culmorum* by sHBD-3 was analysed by using phase contrast microscopy. As seen in Figure 3.3, after 24 h of incubation, the spores in the control medium have germinated. With 10 µg.ml^{-1} of sHBD-3, the conidia remained in the same state. Moreover, the intracellular content of the *Fusarium* spores appears to be released in the medium after disruption of the membrane.

Two major models are used to explain the mechanism of action of the defensins: the carpet and the pore-forming model. In both models, it is proposed that defensins interact with the negatively charged molecules of the microbial cell membrane, leading to an increase of permeabilization and cell leakage. However, for a given peptide and microbial genera, the mechanism may depend on the species. It has also been suggested that the native peptides and their linear analogues can use different mechanisms of action (Wu et al., 2003).

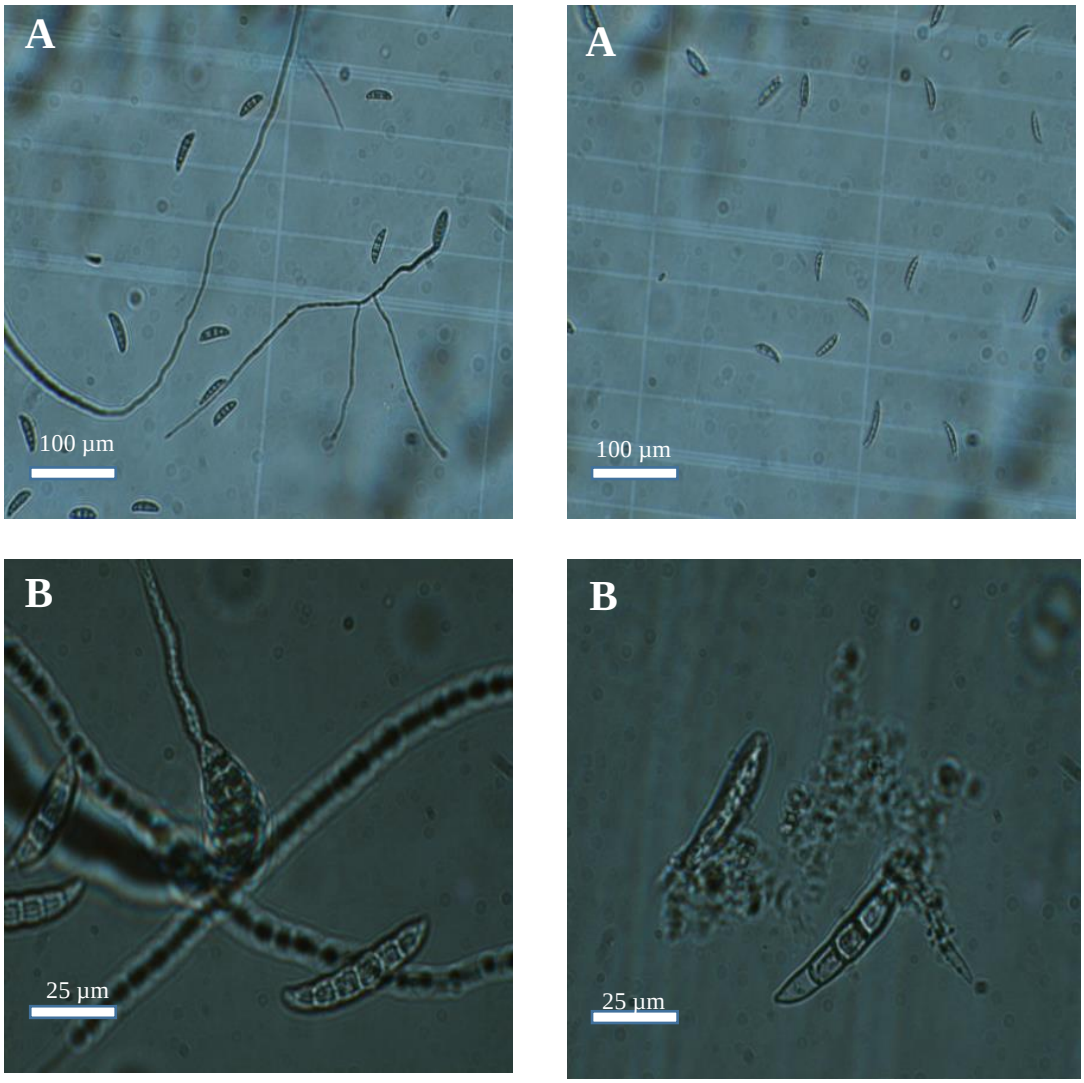


Figure 3.3. Phase contrast microscopy of *Fusarium culmorum* (10^3-10^4 spores/ml) after 24 h of fungi growth in the presence of control medium (left) or medium supplemented with synthetic HBD-3 (10 µg.ml^{-1}) (right). The content of the magnification bars = 100 µm (**A**), 25 µm (**B**)

NaCl effect on HBD-3 inactivation of *F. culmorum*

Sodium chloride is an important ingredient of baking process and its effect on antimicrobial peptides has already been studied. It has been reported by Wu et al. (2003) that the presence of salt tends to decrease the antimicrobial activity of this type of peptides. This effect is due to the weakening of electrostatic interactions between the cationic peptides and the negatively charged membrane of microbial cells (Wu et al., 2003). Here, the effect of salt on the activity of sHBD-3 against *F. culmorum* was studied.

The activity of sHBD-3 (15 µg.ml^{-1}) against *F. culmorum* was tested in solutions with 50 mM NaCl and 150 mM NaCl. The percentage of inhibition of *F. culmorum* is presented in Figure 3.4a. In the salt solutions containing up to 50 mM NaCl, sHBD-3 revealed antifungal activity (~90% of the control activity after 96 h) against *F. culmorum*. However, under physiological salt concentrations (150 mM NaCl), the activity of sHBD-3 was reduced by ~90% after 96 h. These results revealed that the activity of sHBD-3 against *F. culmorum* was reduced by the addition of salt.

Native HBD-3 has previously been reported to be salt-insensitive (up to a level of 100 mM NaCl) in its antibacterial activity (Hoover et al. 2003). Its ability to form oligomers has been proposed to explain its antibacterial activity against Gram-positive bacteria at physiological salt concentrations (Dhople et al. 2006). However, only linear HBD-3 holds its activity against *E. coli* at concentrations up to a level of 150 mM NaCl (Hoover et al. 2003). The high cationicity of the peptide (Sahl et al. 2005) is essential for its antibacterial activity and the upholding of this latter in salt solutions. It has been shown that the antifungal activity of HBD-3 is much more salt sensitive than the antibacterial activity (Vylkova et al. 2007). The mechanisms of action of HBD-3 against fungi and bacteria may be behind the difference in sensitivity. Indeed, the net charge and the secondary structure of the peptide do not appear to be critical for the inactivation of *C. albicans* (Dhople et al. 2006, Vylkova et al. 2007).

Thermal stability

A solution of synthetic HBD-3 (15 µg.ml^{-1}) in 0.1% acetic acid was treated at 100ºC for 14 min. After cooling at RT, the antifungal activity was determined against *F. culmorum.* As seen in Figure 3.4b, the antifungal activity of sHBD-3 was maintained after heat treatment. The heat stability of different recombinant or native defensins has been studied by several authors (Lacerda et al. 2014, Liu et al. 2008) and explained by the resistance of the secondary structure to heat denaturation. In the case of a synthetic HBD-3, the disulfide bonds are absent and the secondary structure of the peptide may be modified. However, the high flexibility of the linear forms of defensins (Hoover et al. 2003) could confer different configurations to the peptide. Many linear AMPs, including LL-37 and CP-29, are unstructured in an aqueous environment, and adopt an α-helical structure when they come into contact with microbial membrane (Wu &

Hancock, 1999). This α-helical structure is usually amphipathic, allowing the peptide to interact with phospholipid bilayers, key to membrane disruption and translocation into membranes. In addition to this, it has been proven that the secondary structure of HBD-3 has a little role in the antifungal activity of the peptide (Vylkova et al. 2007). It is generally accepted that heat treatment can enhance surface hydrophobicity of protein. Now, the hydrophobicity has been proven to play a role in the antimicrobial activity of cationic antimicrobial peptides (Laverty & Gilmore 2014). Specifically, the hydrophobicity of HBD-3 is important for its activity against Gram-positive bacteria and *C.albicans* (Hoover et al. 2003, Lee et al. 2014). Thus, the maintaining of the antifungal activity of HBD-3 after heat treatment could be attributed to the flexibility and the hydrophobicity of the linear peptide.

Fig. 3.4a

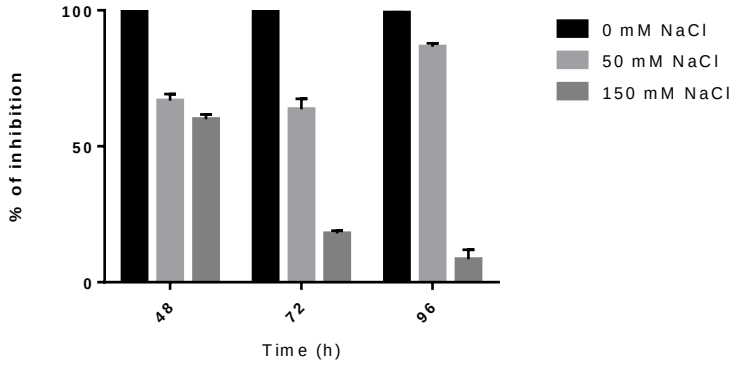


Fig. 3.4b

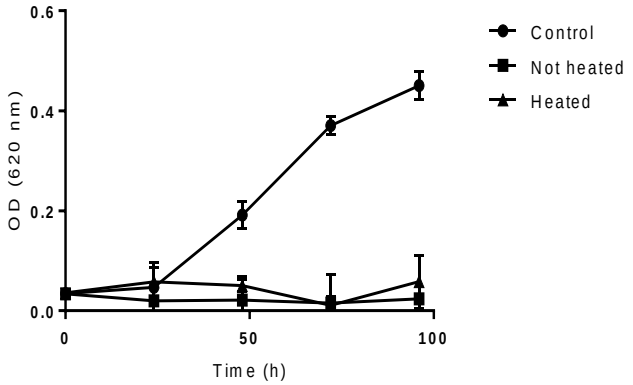


Figure 3.4. Antifungal activity stability of a synthetic HBD-3. The synthetic peptide (final concentration = 15 µg.ml^{-1}) was tested against *F. culmorum* (10^3-10^4 spores/ml). **Fig. 3.4a**: Effect of NaCl concentration on sHBD-3 antifungal activity. **Fig. 3.4b**: Effect of heat treatment (100ºC/14 min) on sHBD-3. circles: control (sHBD-3 = 0 µg.ml^{-1}); squares: sHBD-3= 15 µg.ml^{-1} without heating treatment; triangles: sHBD-3= 15 µg.ml^{-1} with heating treatment.

Quantification of rHBD-3

The concentration of rHBD-3 expressed by the yeasts was determined by ELISA assay. After 48 h of fermentation, the level of rHBD-3 in the supernatant was 8.5 ± 1.2 ng/10^8 cells. It appears that an important part of rHBD-3 remained bound to the yeast cell or is present into the cell, since the concentration in the total lysate is 13 ± 3 ng/10^8 cells. The presence of rHBD-3 was not detected in the bread. The expression of rHBD-3 extracellularly and intracellularly has already been reported by James et al. (2014).

Although the heterologous expression of peptide in yeast is more complex than in *E. coli*, the recombinant peptide expressed in yeast has been proven to be more efficient (Kant et al. 2009). The use of yeast to produce a recombinant defensin did not affect the peptide conformational features and allows post-translational modifications, such as the creation of disulfide bonds (Lacerda et al. 2014). Besides, recombinant human defensins have been proven to maintain antimicrobial activities (Pazgier et al. 2006).

Evaluation of the effect of defensins on bread characteristics

Three different yeast strains were used for the fermentation of the dough, namely *S. pastorianus* CMBS-33, a recombinant *S. pastorianus* CM-INT-51 and a commercial *S. cerevisiae* strain (Pante dry yeast) from Puratos. Doughs of 50 g were fermented for 1 h and baked at 210ºC for 14 min. The pictures of the bread are presented in Figure 3.5.

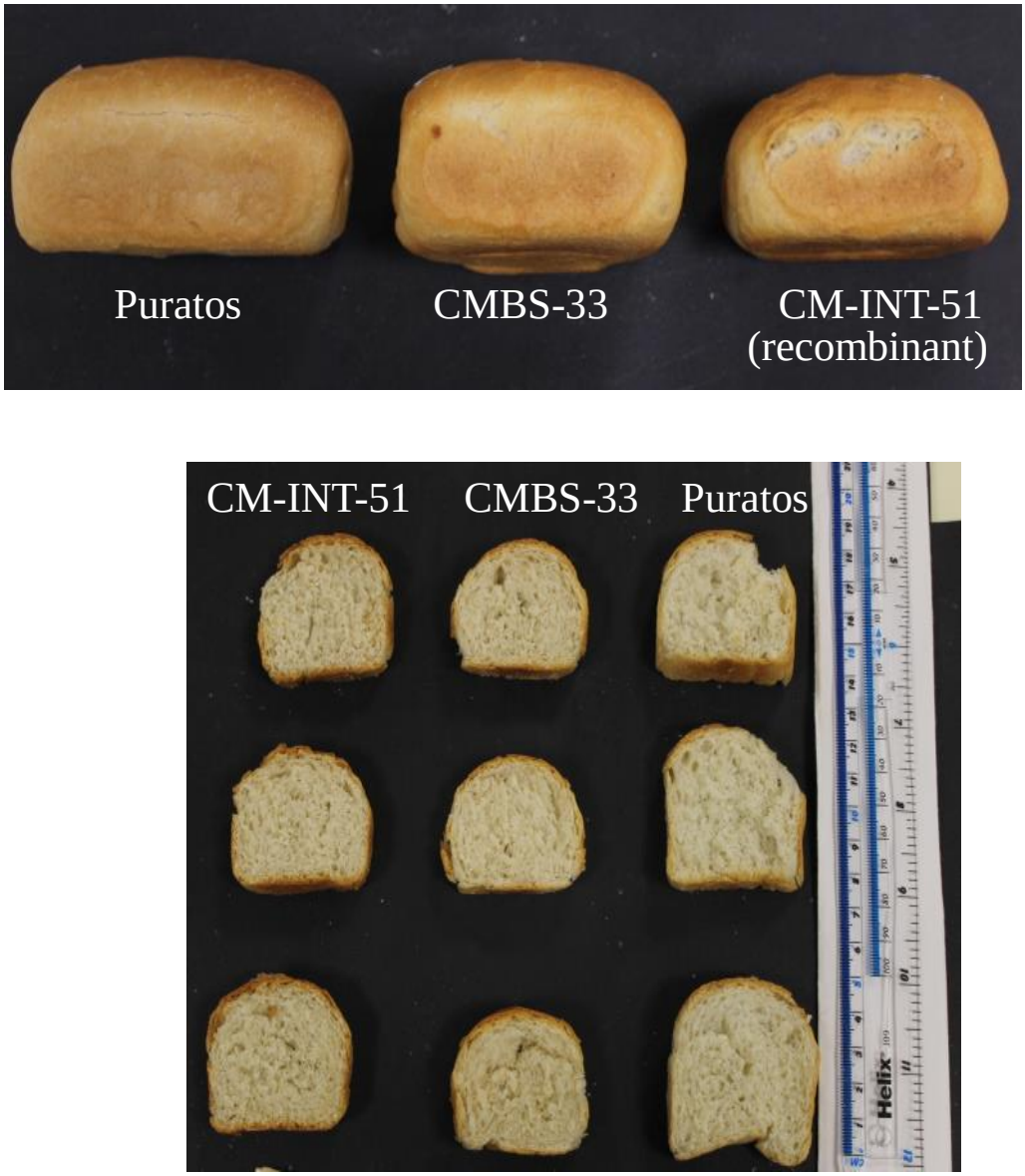


Figure 3.5. Pictures of breads made with the three different yeast strains. The doughs fermented with the fresh *S. pastorianus* strains led to smaller breads, which can be explained by the difference of ability of fermentation between *S. cerevisiae* and *S. pastorianus* species.

Characterization of the breads

The effect of the different yeast strains on different rheological characteristics of the bread was evaluated and is presented in Table 3.2. The different types of bread presented overall similar characteristics. The differences in loaf specific volume and hardness were not significant (p=0.65 and 0.25 respectively) between the breads made the three yeast strains. The breads made with the commercial yeast strain appeared to have a higher specific volume (~ 4.9 mL/g) and this can be attributed to the specific adaptation of this strain to the bread environment.

Another batch was made from the commercial yeast strain and the dough was supplemented with sHBD-3. The low sensitivity of S. cerevisiae to human defensins has been exploited to perform recombinant expression of HBD-1 (Cipakova & Hostinová 2005). The fermentation of the dough by the commercial yeast strain was not affected by the presence of sHBD-3, the supplemented bread showing similar characteristics to the control (Table 3.2). However, significant differences, such as crust colour, were observed. This difference could be attributed to the pre-harvest fermentation step and different fermentation abilities between the recombinant and wild strains.

Table 3.2. Structural characteristics of breads

Characteristic	Yeast strains				
	Pante (*S.cerevisiae*)	**Pante + sHBD-3**	***S.pastorianus* CMBS-33**	***S.pastorianus* CM-INT-51**	***p*-value**
Loaf specific volume (mL/g)	4.9 ± 1.8^{a}	4.22 ± 0.14^{a}	4.31 ± 1.3^{a}	3.84 ± 0.5^{a}	0.65
Crust colour	52 ± 2.7^{b}	50.5 ± 1.6^{b}	52.6 ± 2.0^{b}	46.5 ± 1.9^{a}	0.003
Hardness (g)	327 ± 60^{a}	257 ± 50^{a}	362 ± 62^{a}	273 ± 39^{a}	0.25
Springiness (%)	0.97 ± 0.01^{b}	0.94 ± 0.07^{ab}	0.94 ± 0.02^{ab}	0.91 ± 0.02^{a}	0.04
Cohesiveness	0.58 ± 0.05^{a}	0.69 ± 0.06^{ab}	0.68 ± 0.01^{b}	0.68 ± 0.02^{b}	0.06
Chewiness (g)	187 ± 44^{a}	165 ± 29^{a}	230 ± 39^{a}	170 ± 27^{a}	0.2
Cell diameter (mm)	2.13 ± 0.24^{a}	1.74 ± 0.24^{a}	2.7 ± 1.1^{a}	3.9 ± 1.7^{a}	0.33
Wall thickness (mm)	0.44 ± 0.01^{a}	0.42 ± 0.01^{a}	0.49 ± 0.04^{a}	0.49 ± 0.04^{a}	0.77

[1] Mean ± SDs calculated with at least three slices of two bread batches
Values with different letters in a row differ statistically (Kruskal-Wallis test, $p < 0.05$)

Analysis of metabolites

The concentrations of the different sugars (sucrose, glucose and fructose) and organic acids (citric, succinic, acetic) in the supernatant of the fermentation media of the three yeast strains is presented in Table 3.3. As nutrients, the concentration of sugars in

bread influences the development of fungal colonies. Organic acids, particularly succinic acid, are usual metabolites of the baking process (Rezaei et al. 2014, Yoshida & Yokoyama 2012). The presence of organic acids produced during the fermentation plays a role as well in fungal growth. Indeed, these acids have been shown to have antifungal activity (Shokri 2011).

Table 3.3. Acids and sugars concentrations of fermentation medium (YPS) with the yeast strains CMBS-33 and CM-INT-51

	Yeast strains		
	Pante *(S.cerevisiae)*	***S.pastorianus* CMBS-33**	***S.pastorianus* CM-INT-51**
Sugars (mM)			
Sucrose	$< 0.01^{a}$	1.30 ± 0.94^{a}	2.36 ± 1.38^{a}
Glucose	$< 0.01^{a}$	$< 0.01^{a}$	$< 0.01^{a}$
Fructose	$< 0.01^{a}$	0.05 ± 0.02^{a}	0.04 ± 0.03^{a}
Acids(mM)			
Citric	$< 0.01^{a}$	1.7 ± 1.47^{a}	$< 0.01^{a}$
Succinic	$< 0.01^{a}$	$< 0.01^{a}$	$< 0.01^{a}$
Acetic	4.13 ± 0.48^{b}	2.52 ± 0.08^{a}	1.31 ± 1.10^{a}

[1] Mean ± SDs calculated with duplicates of two growth media
Values with different letters in a row differ statistically (Kruskal-Wallis test, $p<0.05$)

After 48 h of growth, the concentration of the different metabolites was not significantly different between the media fermented with the strains CMBS-33 and CM-INT-51. The Pante strain appeared to ferment more efficiently sucrose (<0.01 mM) and to produce more acetic acid (4.13 mM) than CMBS-33 and CM-INT-51 (2.52 and 1.31 mM respectively). The differences can be attributed to the ability of fermentation at 30ºC between the two species. The concentrations of metabolites in the breads highlight the better ability of the commercial yeast strain (Pante) to ferment dough at 30ºC (Table 3.4).

The concentration of sugars and acids were slightly different between the breads made with CMBS-33 and CM-INT-51. The insertion of a gene expressing HBD-3 in the yeast genome could slightly affect the yeast performance (Nevoigt 2008).

Table 3.4. Sugars and acids concentrations in bread with the three yeast strains

Yeast strains	Pante (*S.cerevisiae*)	*S.pastorianus* CMBS-33	*S.pastorianus* CM-INT-51
Sugars (mM)			
Sucrose	7.35 ± 0.00^{a}	7.91 ± 0.01^{b}	9.61 ± 0.11^{c}
Glucose	0.24 ± 0.00^{a}	2.85 ± 0.02^{c}	1.53 ± 0.37^{b}
Fructose	3.93 ± 0.03^{a}	7.79 ± 0.04^{c}	5.88 ± 0.04^{b}
Acids (mM)			
Citric	$< 0.01^{a}$	$< 0.01^{a}$	1.71 ± 1.63^{a}
Succinic	10.7 ± 1.98^{a}	13.24 ± 0.11^{a}	7.12 ± 2.30^{a}
Acetic	0.56 ± 0.01^{c}	0.17 ± 0.01^{a}	0.3 ± 0.02^{b}

[1] Mean ± SDs calculated with duplicates of three batches
Values with different letters in a row differ statistically (Kruskal-Wallis test, $p<0.05$)

Shelf life

The breads produced with the different yeast strains were sliced and the appearance of moulds was monitored over a 13-day period. Each slice was classified among one of the five categories (based on the percentage of contamination) presented in Figure 3.6.

The use of the fresh *S. pastorianus* yeast strains delayed the appearance of the fungal colonies, in comparison with the commercial Pante strain. Some differences in the composition of the fermentation medium could explain this delay.

In addition to this, it appeared that the appearance of fungal colonies on the bread made with the recombinant yeast strain CM-INT-51 is delayed. Indeed, compared to the breads made with the strain CMBS-33, the first fungal colonies appeared 3 days later. It has been shown by James et al. (2014) that the yeast strain CM-INT-51 expresses rHBD-3 in its growth medium. We can assume that the presence of rHBD-3 plays a major role in the decreasing of the fungal growth. This is supported by the fact that the breads prepared with a supplement of sHBD-3 (80 $\mu g.ml^{-1}$) did not present any fungal contamination over 13 days (data not shown). Furthermore, the breads made

with CM-INT-51 presented the same extension of shelf life as “industrial” breads formulated with 0.3% calcium propionate (w/w) (Fig. 3.6D).

Besides, during baking, yeast cells are susceptible to release their intracellular content (Boekhout & Robert 2003), including rHBD-3. Surprisingly, rHBD-3 was not detected in the bread made with the yeast strain CM-INT-51. Interactions with other ingredients could impair the water solubility and subsequent extraction of the recombinant peptide. Further studies are thus necessary to highlight the potential interactions between HBD-3 and the bread matrix and to improve the method of extraction.

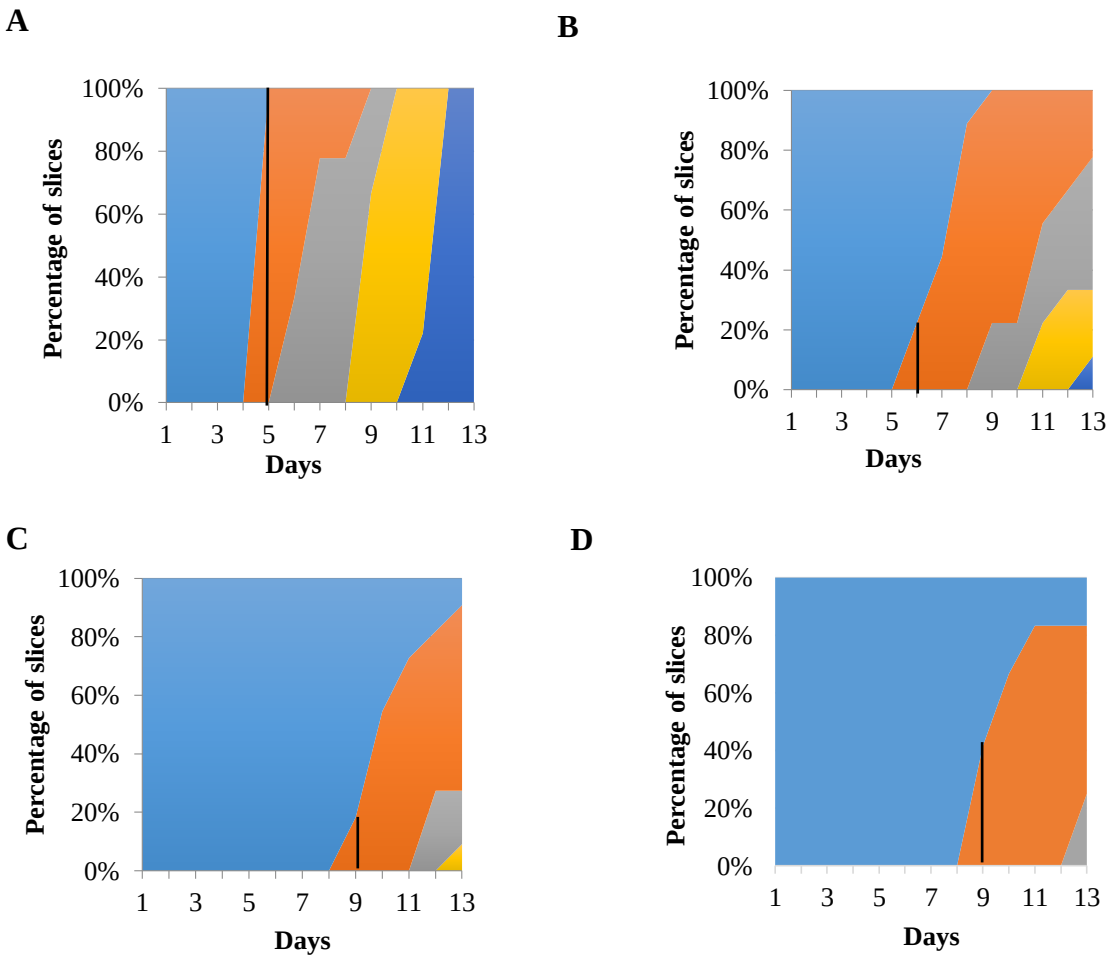


Figure 3.6. Shelf life of the four types of breads (A: Puratos strain; B: CMBS-33; C: recombinant CM-INT-51; D: Puratos strain with 0.3% calcium propionate). The slices are categorized depending on the percentage of contamination:

Mould free <10% 10-24% 25-49% >50%

The black line indicates the first day of appearance of fungal colony.

3.4 Conclusion

In conclusion, it is shown that a synthetic HBD-3 has a deleterious effect on the growth of fungal contaminants. This reinforces the potential of defensins to fight microbial spoilage of cereal-based products. Since the antifungal activity of sHBD-3 solution is both relatively heat- and salt- resistant, this peptide is a good model candidate for baking applications. A *S.pastorianus* strain expressing the defensin gene was used in baking process. Despite slight differences in the breads made with this strain and the bread made with a commercial yeast strain, the "modified" bread had an extended shelf life with a decrease of the fungal growth. Due to the lack of knowledge on the safety of sHBD-3 and the yeast strain CM-INT-51, no sensory analysis was performed. The use of HBD-3, and more generally of defensins, in synthetic form or produced by modified yeasts, may be a novel alternative to common preservatives in bread protection.

3.5 Acknowledgements

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Appendix: Construct of recombinant *S. pastorianus* strain CM-INT-51 (James et al. 2014, Jansen et al. 2005)

The amino acid sequence of human β-defensin-3 (HBD-3) (Table S1) was reverse translated into the corresponding nucleotide sequence, with codons optimized for expression in yeast. A pBlueScript SK vector was used to insert at the EcoR1 site a DNA fragment comprising a HBD-3 sequence and a *S. cerevisiae* α-factor secretory signal peptide (GenScript Inc., Piscataway, NJ, USA). A PCR amplification, using the primers Rec1 and Rec2 shown in Table S1, was subsequently performed. The primers were chosen due to high homology to sequences either upstream or downstream of the insertion site in the yeast shuttle vector, pGREG505 (Jansen et al. 2005), one of the pGREG series yeast shuttle vectors. The PCR products were cloned into the plasmid by *in vivo* homologous recombination in the *S. cerevisiae* strain S150-2B to generate the plasmid pGREGHBD3-505 in which HBD-3 gene construct was under the control of the GAL promoter and upstream of the plasmid-encoded 3′ CYC termination sequences. The pGREGHBD3-505 was used as a template for amplification of the HBD3 gene cassette for integration into the brewery yeast CMBS-33 at YPR159C-A on the right arm of the chromosome XVI. As part of the integration step, the GAL promoter was replaced with the constitutive phosphoglycerate kinase (PGK) promoter from *Saccharomyces bayanus*. The general Available pGREG vectors selectable markers as shown on Fig. S1. Additional restriction sites, a HIS3 fragment for the identification of positive *in vivo* recombination events and G418 resistance marker (kanMX) for geneticin were also included.

Table 3.S1. Primers used for PCR amplification

Primer	DNA sequence 5'-3'	Location (nts)
Rec1	GAATTCGATATCAAGCTTATCGATACCGTCGACAATGAGATTCCCATCTATTTT (F)	1065–1086*
Rec2	GCGTGACATAACTAATTACATGACTCGAGGTTATTTAGAAGTGGCGCGCCTCA (R)	1513–1535*

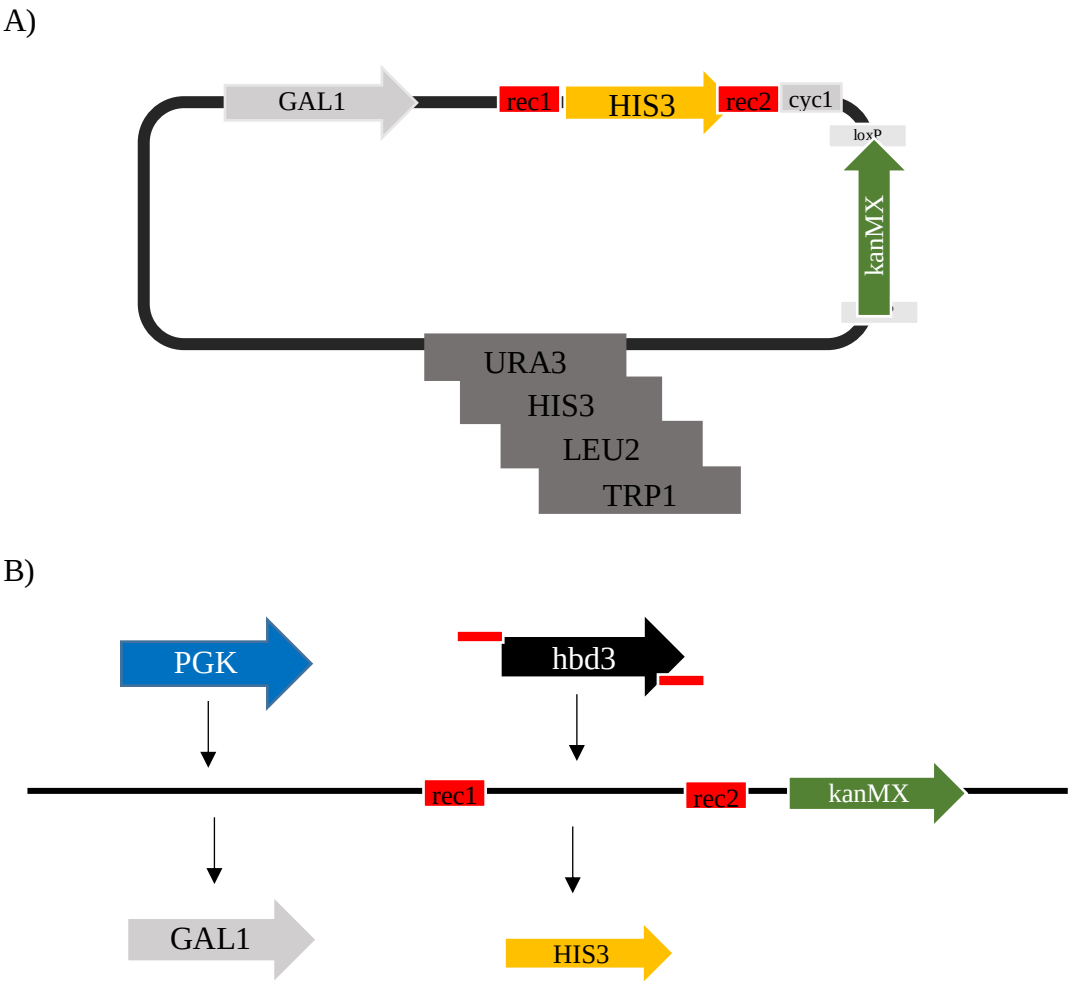


Fig. 3.S1. Schematic representation of PGREG-505 vector. (A) GAL1 promoter controls the expression of tags or generated fusion proteins. Downstream of the tags a HIS3 stuffer fragment is located flanked by specific sites for recombination, rec1 and rec2. PGREG-505 contains one of the selectable markers (URA3, LEU2, TRP1 and HIS3) as well as an additional KanMX cassette flanked by loxP sites. Sequences of rec1 and rec2 used for the targeted *in vivo* recombination of DNA fragments into the vector (B) HBD-3, flanked by Rec1 and Rec2 replaces the HIS3 stuffer fragment by successful gene integration, and a screen for loss of the selection marker identifies potential recombinants. Subsequent replacement of the GAL1 promoter by PGK promoter during integration.

Chapter 4
Antifungal activity of synthetic cowpea defensin Cp-thionin II and its application in dough

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Abstract

Plant defensins are small, cysteine-rich antimicrobial peptides of the immune system found in several organs during plant development. A synthetic peptide, KT43C, a linear analogue of the native Cp-thionin II found in cowpea seeds, was evaluated for its antifungal potential. It was found that KT43C displayed antifungal activity against *Fusarium culmorum*, *Penicillium expansum* and *Aspergillus niger*. Like native plant defensins, KT43C showed thermostability up to 100°C and cation sensitivity. The synthetic peptide decreased the fungal growth without inducing hyperbranching of fungal hyphae. Non-inhibitory concentrations of the peptide induced permeabilization of the fungal membrane. In addition, high concentrations of KT43C enhanced the formation of reactive oxygen species in the granulated cytoplasm. To investigate potential applications, the peptide was used as an additive in the preparation of dough that did not contain yeast. This peptide delayed the development of fungal growth in the dough by 2 days. Furthermore, KT43C did not induce red blood cell lysis up to a concentration of 200 $\mu g.ml^{-1}$. These results highlight the potential for the use of synthetic antimicrobial defensins for shelf life extension of food products.

4.1 Introduction

Plants have developed an ancient and complex defence strategy through their immune system to combat pathogens and abiotic stresses (de Beer & Vivier 2011, Lacerda et al. 2014). Among their many defence systems, the production of cationic antimicrobial peptides (cAMPs) is a major contributor to plant resistance to phytopathogens, due to their broad spectrum of activity (Stotz et al. 2009). Plant AMPs have been divided into several categories based on their amino acid structure, sequence identity or tertiary structure (Nawrot et al. 2014). Among these AMPs, plant defensins were first discovered in the seeds of wheat and barley (Colilla et al. 1990, Mendez et al. 1990). Plant defensins can be divided into three groups: defensins leading to morphogenic changes in the fungal hyphae, defensins causing reduction of hyphae without morphogenic changes, and defensins without antifungal activity (Broekaert et al. 1995). The expression of plant defensin genes has been reported to be increased in response to pathogens, which supports the idea that these peptides constitute a major defence mechanism (Garcia-Olmedo et al. 1998). In addition, the localization of the plant defensins in different plant organs and tissues, with a preferential cell-wall location in epidermal cells (Lacerda et al. 2014), is highly consistent with a defensive role. The defensins also play a role in the protection against insects, abiotic stress and metal tolerance (Carvalho & Gomes 2009).

Plant defensins are cysteine-rich cationic peptides of 5-8 kDa whose mature domain comprises 45 to 54 amino acid residues, with an isoelectric point around 9. The global fold of plant defensins consists of a cysteine-stabilized motif (CSα/β) made up of an α-helix and a triple-stranded β-sheet stabilized by four disulfide bridges (C1-C8/C2-C5/C3-C6/C4-C7) (Lay et al. 2003). In addition to this motif, Yount and Yeaman (Yount & Yeaman 2004) reported the importance of an additional conserved motif, named γ-core, for the antimicrobial activity of the peptide.

The mode of action of several plant defensins has been extensively studied and different mechanistic steps have been identified but are yet not completely understood (Thevissen et al. 2004, Thomma et al. 2002, Vriens et al. 2014). In many cases, it involves the recognition and binding to a cell wall (*e.g.*, glucosylceramide) or membrane (*e.g.*, phosphatidic acid) receptor. Then, plant defensins are either internalized into the fungal cell or stay at the surface of the membrane and induce

several mechanisms: activation of enzyme pathways, modification of ion fluxes, inhibition of conidial germination or induction of apoptosis. In contrast to mammalian and insect defensins, the insertion of plant defensins in the fungal membrane via pore-formation is uncommon and the mechanism of translocation of the peptides has not yet been identified.

Plant defensins are generally non-cytotoxic to mammalian and plant cells (Thevissen et al. 2004). However, some plant defensins have been reported to display Ca^{2+} channel inhibition, anticancer activity and mitogenic activity towards mammalian cell lines (Guzman-Rodriguez et al. 2015). The toxicity of defensins and cAMPs in general has been attributed to the presence of disulfide bonds, their high hydrophobicity and amphipathicity (Hollmann et al. 2016, Jenssen et al. 2006). Consequently, the design of linear analogues of AMPs and defensins showing antimicrobial properties has gained strong interest. For example, a linear variant of human β-defensin 3 has been shown to partially retain its antimicrobial activity, without any toxicity towards human erythrocytes and conjunctiva epithelial cells (Liu et al. 2008).

Fungal spoilage is a major issue in cereal-based foods and in the wider food industry. In addition to economic losses due to unpleasant flavours and appearance, mould has the potential to cause health issues due to the production of mycotoxins (Pitt & Hocking 2012). Among the different cereal-based products, chilled dough is recognized as a convenient solution to the consumer's desire for oven-fresh baked products. Despite the chilled conditions, bacterial and fungal growth can appear during the storage of this dough. In order to prevent spoilage, a range of preservatives is used, but the overuse of these artificial preservatives has a negative image with the consumer. Alternative solutions to such preservatives, including natural antimicrobial peptides such as nisin, are already in use but their application is still challenging, mainly due to their narrow spectrum of activity (Dielbandhoesing et al. 1998). In contrast, due to their natural role in plant protection, defensins present promising properties, such as inhibition of a broad range of microorganisms and this can be exploited in food applications.

The defensin Cp-thionin II has been extracted from cowpea seeds and displayed antimicrobial activity against Gram-positive and Gram-negative bacteria (Franco et al. 2006). The objective of this study was to characterize the antifungal activity of a linear analogue of Cp-thionin II, KT43C (Table 4.1). The application of this synthetic peptide

or the prevention of fungal growth in dough was also studied. In addition, the effect of the peptide on red blood cell integrity was examined to determine its toxicity.

4.2 Material and Methods

Defensin

A synthetic peptide (purity >70%, as indicated by the supplier), named KT43C (5.2 kDa), a linear analogue of the plant defensin Cp-thionin II, was synthesized by and purchased from GLBiochem, Shanghai, China. Synthesis was made by solid-phase method using Fmoc chemistry, as described by the manufacturer. Fmoc group was removed by treating the resin with a mix of piperidine/DMF (20/80). A MALDI-TOF mass spectrometry (SHIMADZU AXIMA Assurance) was performed by GLBiochem to confirm the stability of the peptide after synthesis (see Supplementary information, Appendix III). The lyophilized peptide was reconstituted in 0.1% acetic acid (pH 3.5) and stored in vials at different concentrations at -20°C.

Table 4.1. Characteristics of the linear peptide KT43C and comparison with plant defensins

Name	Source	Sequence	Identity	Activity	Function	Reference
Linear peptide KT43C (synthetic Cp-thionin II)	Synthetic peptide	KTCMTKKEGWGRCLIDTTCAHSCR KYGYMGGKCQGITRRCYCLLNC	-	*F. culmorum* *A. niger* *P. expansum*	-	This study
Cp-thionin II	*Vigna unguiculata*	KTCMTKKEGWGRCLIDTTCAHSCR KYGYMGGKCQGITRRCYCLLNC	100%	Gram-positive *S. aureus* Gram-negative *E. coli* *P. syringae*	γ-thionin	(Franco et al. 2006)
Cp-thionin	*Vigna unguiculata*	RVCESQSHGFKGACTGDHNCALVC RNEGFSGGNCRGFRRRCFCTLKC	42%	Unknown	Trypsin inhibitor	(Melo et al. 2002)
VrD1	*Vigna angularis*	RTCMIKKEGWGKCLIDTTCAHSCKN RGYIGGNCKGMTRTCYCLVNC	73%	Insecticidal	α-amylase inhibitor	(Liu et al. 2006)

Underlined cysteine residues are involved in disulfide bonds (C1-C8/C2-C7/C3-5/C4-C6)

Fungal strains

The filamentous fungi used in this study represent some of the main contaminants of cereal-based products (Beuchat 1987) including *Fusarium culmorum*, *Aspergillus niger* and *Penicillium expansum*. The fungal strains *F. culmorum* FST 4.05, *A. niger* FST4.21 and *P. expansum* FST 4.22 originated from the culture collection of the School of Food and Nutritional Sciences, University College Cork (Cork, Ireland).

Fungal growth inhibition assay

To determine the antifungal activity of the synthetic peptide, the fungal growth was monitored in a microtiter plate assay as described previously by Van der Weerden et al. (Van Der Weerden et al. 2008) with some modifications. Briefly, fungal spores were collected from mature colonies grown for 72 h on potato dextrose agar (PDA) (Sigma-Aldrich) plates at 28°C, counted on a haemocytometer and inoculated in synthetic nutrient-poor broth. Fungal spores were then diluted in half-strength potato broth (½ PDB) (Sigma-Aldrich) to obtain a final concentration of $1x10^4$ spores.ml^{-1}. Spore suspensions (80 µl) were added to the wells of a 96-well microtiter plate. Filter-sterilized synthetic peptides (20 µl) at concentrations ranging from 5 µg.ml^{-1} to 200 µg.ml^{-1} (final concentration) were then added to the fungal cells. Fungal growth was assessed by measuring the absorbance at 595 nm for 96 h at 28°C (Multiskan TM, Thermo Scientific). Cell suspensions in 0.1% acetic acid (in ½ PDB, pH=5.0) were used as negative controls. Each test was performed in triplicate.

The inhibition of fungal growth by KT43C was then examined on PDA plates. The same solutions were prepared as described above and after 24 h incubation at 28°C, 50 µl were spot-inoculated on PDA plates. Pictures of the plates were taken at 24 h intervals for a period of three days.

MIC/IC_{50} determination

After 96 h of incubation at 28ºC, the MIC was read as the lowest concentration of KT43C resulting in complete inhibition of fungal growth. The required concentration to inhibit 50% of the growth (IC_{50}) was determined by non-linear regression, using the software GraphPad PRISM (GraphPad Software, Inc., La Jolla, CA) with the microplate reader data.

Thermal stability of KT43C

In order to investigate the thermal stability of KT43C, a peptide solution was heated at 100°C for 15 min. After cooling at RT, the activity of KT43C (MIC = 20 µg.ml^{-1}) was measured against *F. culmorum* in a 96-well microtiter assay as described above.

Effect of cations on the antifungal activity of KT43C

To determine the effect of cations on KT43C activity, an antifungal assay was performed in different salt solutions. Fungal spores (1x10^4 spores.ml^{-1}) were inoculated in medium containing either 100 mM NaCl, 50 mM KCl, 5 mM $CaCl_2$ or 5 mM $MgCl_2$, as described previously (Terras et al. 1992). After the addition of the peptide, the inhibitory activity of KT43C (concentration = 20 µg.ml^{-1}) was measured against *F. culmorum* in a 96-well microtiter assay as described above.

Characterization of the antifungal activity

To visualize the action of KT43C against *F. culmorum* spores, the peptide, at concentrations of 5 and 20 µg.ml^{-1}, was added to ½ PDB inoculated with fungal spores. After 24 h, 500 µl of each solution was freeze-dried for further analysis. Five hundred µl of 70% glycerol was added to the solutions to avoid damage due to freeze drying. The lyophilized samples were then analysed using a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan), as described by Oliveira et al. (Oliveira et al. 2012), with some modifications. Briefly, fungal mycelium was mounted onto plain aluminium stubs using carbon double surface adhesive and coated with a 7 nm gold layer using a Gold Sputter Coater (BIO-RAD Polaron Division, SEM coating system, England) and observed under a constant accelerating voltage of 5 kV.

Membrane permeabilization assay

To characterize the mode of action of the synthetic peptides against *F. culmorum*, a SYTOX® Green (Molecular Probes) uptake assay was performed as described by Van der Weerden et al. (Van Der Weerden et al. 2008), with some modifications.

F. culmorum hyphae were grown in ½ PDB from a suspension of 1x10^4 spores.ml^{-1} for 18 h at 28ºC. The hyphae were then incubated with 10, 20 and 50 µg. ml^{-1} of KT43C

at room temperature with gentle agitation. SYTOX® Green was added (final concentration of 0.5 µM) and the hyphae were allowed to stand for 10 min. Fluorescence of hyphae was measured using a fluospectrophotometer (Varioskan® LUX reader) for 2 h with excitation and emission wavelengths of 488 and 538 nm, respectively, or visualized using a fluorescence microscope (Olympus Fluoview) (excitation wavelength 460-490 nm). Solutions of hyphae without peptides or with 1% Triton X-100 (Sigma-Aldrich) were used as negative and positive control, respectively. For each concentration of KT43C, the experiment was performed in triplicate.

Induction of reactive oxygen species (ROS) assay

The detection of ROS was performed as described by Van der Weerden et al. (Van Der Weerden et al. 2008) with some modifications. *F. culmorum* hyphae were grown as described above and then treated with KT43C or water for 12 h. Following the incubation, the hyphae was incubated with 10 µg.ml^{-1} dihydrorhodamine 123 (Sigma-Aldrich) for 2 h followed by extensive washing with 0.6 M KCl. Fluorescence of hyphae was measured using a fluospectrophotometer with excitation and emission wavelengths of 488 and 538 nm, respectively, or visualized using a fluorescence microscope (Olympus) (excitation wavelength 460-490 nm). Solutions of hyphae without peptides or with H_2O_2 (0.5 mM, Sigma-Aldrich) were used as negative and positive controls, respectively. For each concentration of KT43C, the experiment was performed in triplicate.

Circular dichroism

The analysis of secondary conformers of KT43C in different solutions was observed by using circular dichroism (CD) spectroscopy as described by Liu et al. (Liu et al. 2008). Peptide (1 mg.ml^{-1}) was diluted into deionized water or 20 mM sodium dodecyl sulfate (SDS). CD measurements were performed by using a Chirascan^{TM} CD Spectrometer (Applied Photophysics), at 27ºC within a wavelength range of 180-260 nm. Measurements were made in triplicate and the solvent CD were subtracted from the sample CD.

Chilled dough shelf life

Dough of 10 g was prepared according to a standard recipe (without yeast) consisting of 63% (w/w) flour, 1.5% (w/w) sugar, 3% (w/w) fat and 0.3% (w/w) salt. A solution of 20 µg.ml^{-1} KT43C in sterile distilled water was inoculated with 1x10^{2} spores.ml^{-1} (final concentration) of *F. culmorum* and added to the mix. Water, without peptide, was used as control. After preparation, the dough was packed into sealed plastic bags and filter pipette tips were inserted into the bag to allow gas exchange. Dough was stored at 4°C. The shelf life of the dough was monitored over 14 days, as described by Dal Bello et al. (Dal Bello et al. 2007).

Fungal contamination in the dough

Fungal contamination of dough was estimated by measuring the ergosterol concentration as described by Jedličková et al. (Jedlickova et al. 2008) with some modifications. Briefly, 10 g of ground dough was treated with 50 mL of methanol in a closed vessel and was shaken for 30 min. Then, 25 mL of cleaned extract was placed into a test tube and 3 g of KOH was added and stirred until dissolution of KOH. Ten mL of *n*-hexane was then added and the solution was incubated at 65°C in a water bath for 30 min. After cooling at RT, 5 ml of distilled water were added and the hexane layer was placed into a beaker. The extraction process using hexane was repeated three times using 10 mL of the solvent. The extracts were then pooled and evaporated until dry in a vacuum dryer (Scanvac, Labogene) and redissolved in 5 mL of methanol. The extracted samples were analyzed using HPLC with a DAD. The mobile phase was methanol, and 1.0 mL/min was the optimal flow rate. The column temperature was held at 25°C. A control was made with the addition of standard ergosterol (Sigma-Aldrich) into fresh dough. A 95% recovery was obtained with the standard ergosterol.

Haemolysis assay

KT43C was assayed for its ability to induce haemoglobin release from fresh defibrinated sheep erythrocytes as described by Laverty et al. (Laverty et al. 2010). Fresh sheep red blood cells (Thermo Fisher Scientific) were washed three times with equal volumes of PBS. After centrifugation for 15 min at 900 *g*, erythrocytes were resuspended to 4% (v/v) in PBS. Eythrocyte suspension (80 µL) was added to the wells

of a 96-well microtiter plate, followed by the addition of 20 µl of KT43C in PBS at different concentrations. After incubation at 37°C for 1 h, the suspension was clarified by centrifugation at 1,000 *g* for 10 min. Supernatant was transferred to a fresh 96-well microtiter plate and haemoglobin release was measured spectrophotometrically at 405 nm. Erythrocytes were treated with 0.1% Triton X-100 (in PBS) and PBS alone as positive and negative control, respectively. The percentage haemolysis was calculated as described by Laverty et al. (Laverty et al. 2010):

$$\% \text{ Haemolysis} = \frac{(Abs405\ peptide\ treatment)-(Abs405\ PBS)}{(Abs405\ 0.1\%\ Triton\ X-100)-(Abs405\ PBS)}$$

The release of haemoglobin was determined for six replicates.

Statistical analysis

Statistical analysis was carried out using Microsoft XLSTAT Version 2015.5.01. (Adinsoft Inc, New York, USA). Standard deviations were calculated for absorbance values at each concentration of KT43C based on triplicates, except for the haemolysis assay for which six replicates were used.

The effect of the different salts on the antifungal activity of KT43C was analysed with a one-way ANOVA followed by a Tukey-Kramer HSD test to identify differences relative to the positive control (KT43C in 0.1% acetic acid). In all cases, a probability of $p<0.05$ denoted significance. The same statistical analysis was carried out to determine individual differences in haemolytic activity for each concentration of KT43C in comparison to the negative control.

4.3 Results

Antifungal activity of KT43C

The growth of spores of *F. culmorum*, *P. expansum* and *A. niger* was followed in presence of different concentrations of the peptide KT43C, in a microtiter plate assay. After 96 h, KT43C showed the highest antifungal activity against *F. culmorum* with an IC_{50} of 12.5 µg.ml^{-1} and a MIC of 20 µg.ml^{-1} (Fig. 4.1a). Growth of *A. niger* was delayed but the MIC, after 96 h, was not apparent within the concentration range of the peptide used in the study (up to 200 µg.ml^{-1}) (Fig. 4.1b). The growth of *P. expansum* was only inhibited with a peptide concentration of 200 µg.ml^{-1} (Fig. 4.1c). The inhibition of fungal growth was controled on PDA plates (Fig. 4.2). KT43C inhibited fungal growth without induction of hyperbranching in *F. culmorum* hyphae, *e.g.*, branching, as observed with scanning electron microscopy (Fig. 4.3).

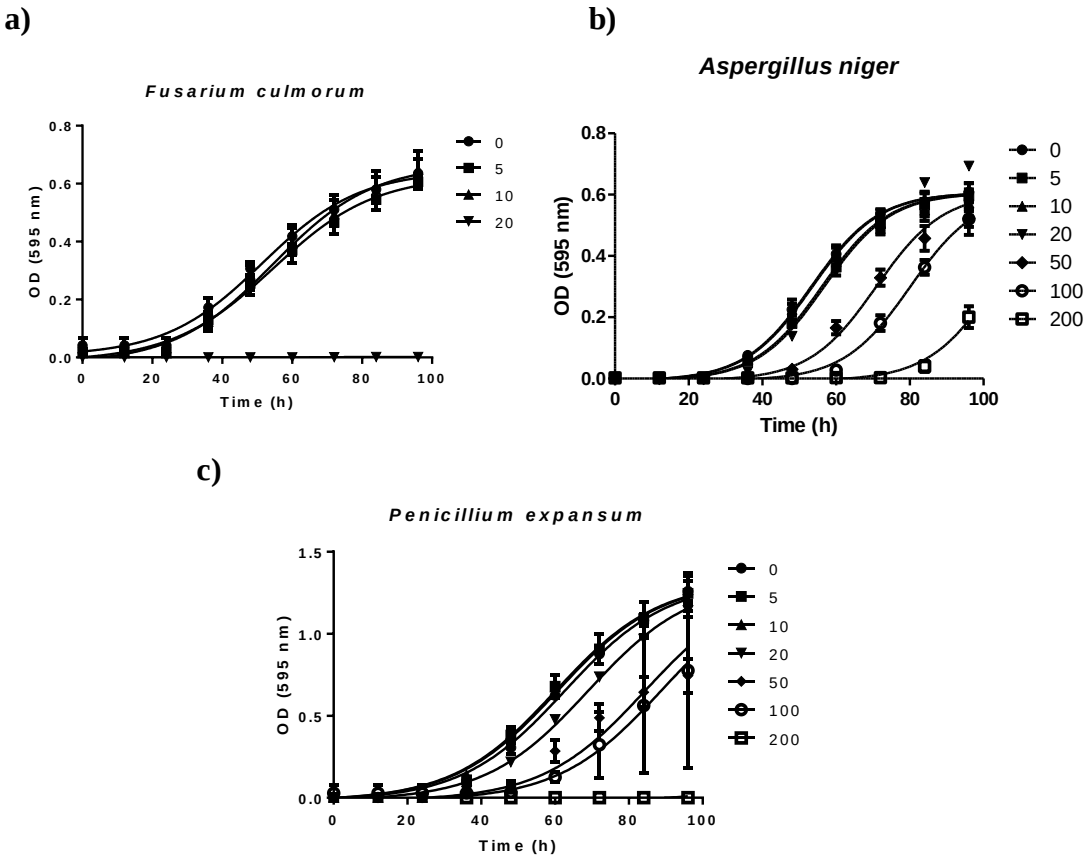
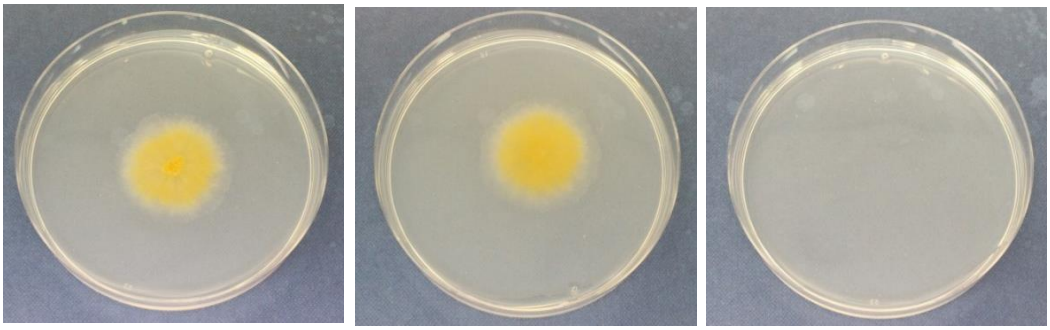


Figure 4.1. Fungal growth inhibition by KT43C**.** Concentrations of KT43C are indicated in µg.ml^{-1}. **(a)** *Fusarium culmorum* FST 4.05, **(b)** *Aspergillus niger* FST 4.21, **(c)** *Penicillium expansum* FST4.22. All absorbance values are the mean of three replicates.

a)

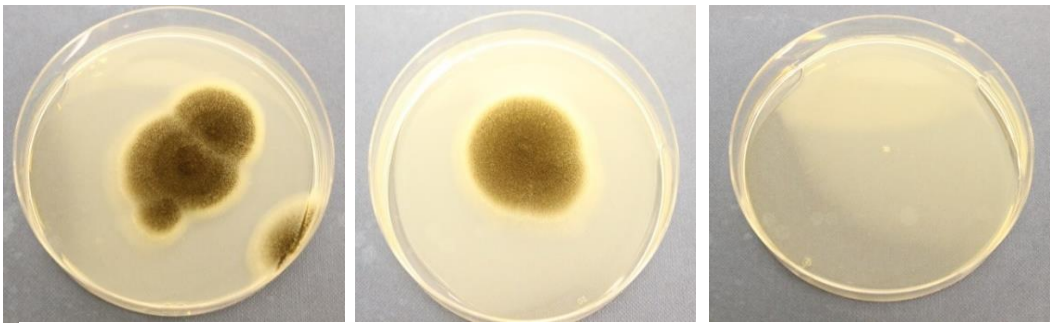
F. culmorum



0 µg.ml^{-1} 10 µg.ml^{-1} 20 µg.ml^{-1}

b)

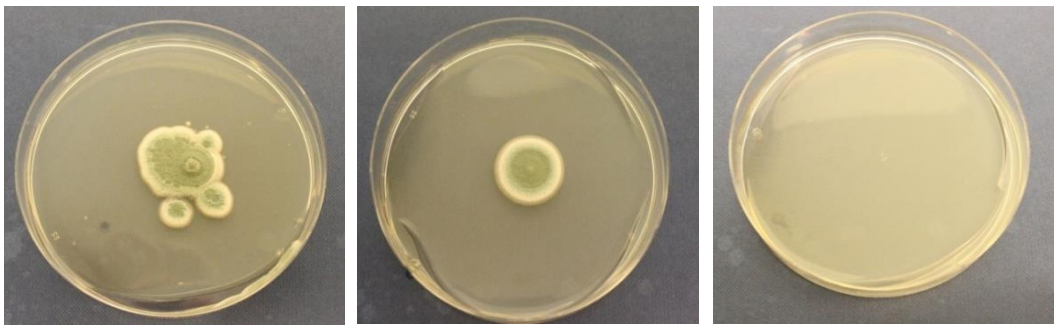
A. niger



0 µg.ml^{-1} 100 µg.ml^{-1} 200 µg.ml^{-1}

c)

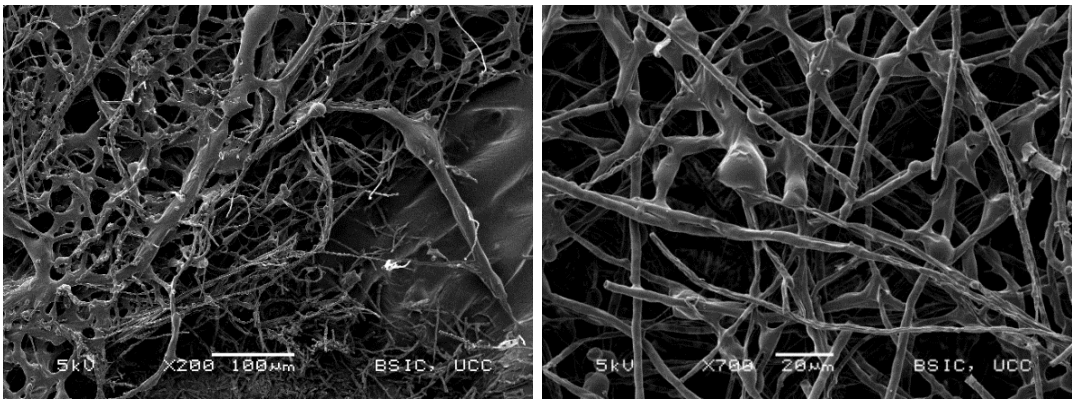
P. expansum



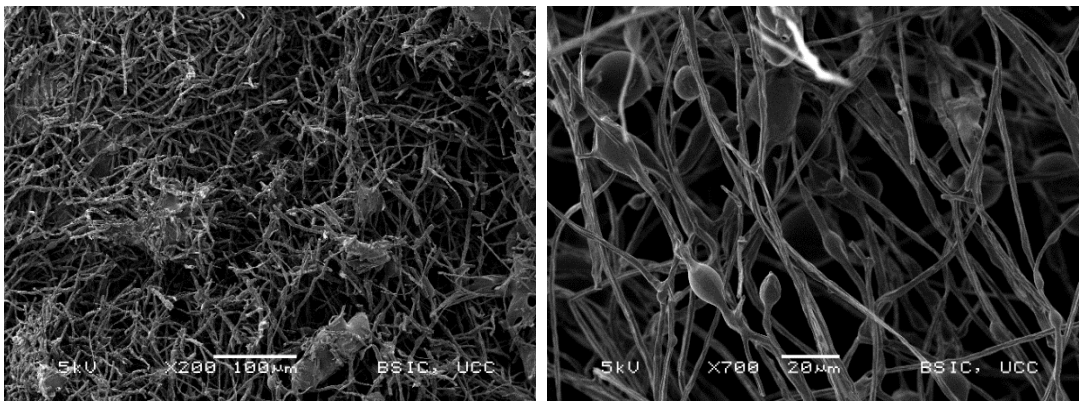
0 µg.ml^{-1} 100 µg.ml^{-1} 200 µg.ml^{-1}

Figure 4.2. Examination of fungal inhibition after 24 h of incubation in ½ PDB with different concentrations of KT43C followed by 72 h of incubation on agar plates. **(a)** *F. culmorum* FST 4.05, **(b)** *A. niger* FST 4.21, **(c)** *P. expansum* FST4.22. The concentrations of KT43C are indicated below the pictures.

a)



b)



c)

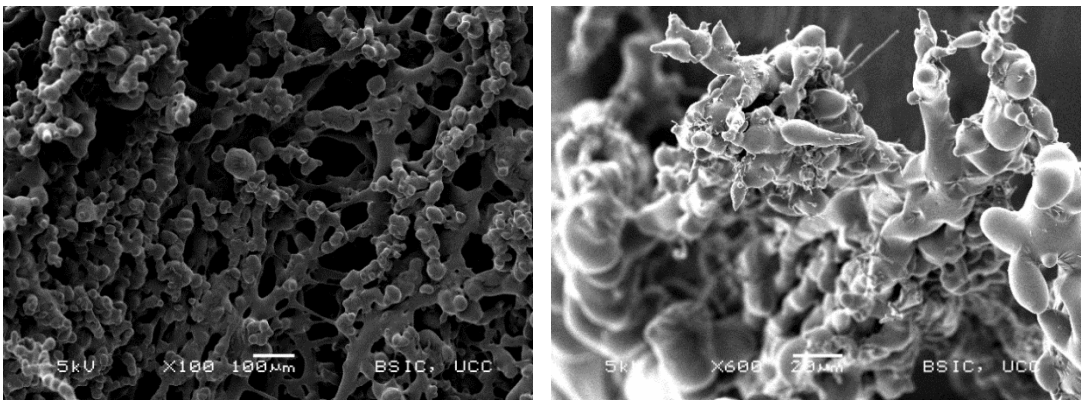


Figure 4.3. Inhibition of *F. culmorum* germination in presence of KT43C observed with a scanning electron microscopy: **a**: Control; **b**: 5 µg.ml^{-1}; **c**: 20 µg.ml^{-1}. The magnifications are indicated on the pictures. The absence of conidial germination and mycelium are clearly visible with a concentration of 20 µg.ml^{-1} of KT43C.

Effect of heat and salt on the antifungal activity of KT43C

A solution of KT43C was treated at 100ºC for 15 min and the antifungal activity was determined against *F. culmorum*. The antifungal activity of KT43C (20 µg.ml^{-1}, with 100% of inhibition) was completely maintained after heat treatment (99.6 ± 0.004% of fungal growth inhibition), demonstrating the thermostability of the synthetic peptide.

The effect of cations on the activity of KT43C against *F. culmorum* is presented in Figure 4.4. The presence of cations, especially divalent cations such as Ca^{2+} and Mg^{2+}, partially or totally inhibited the antifungal activity of KT43C.

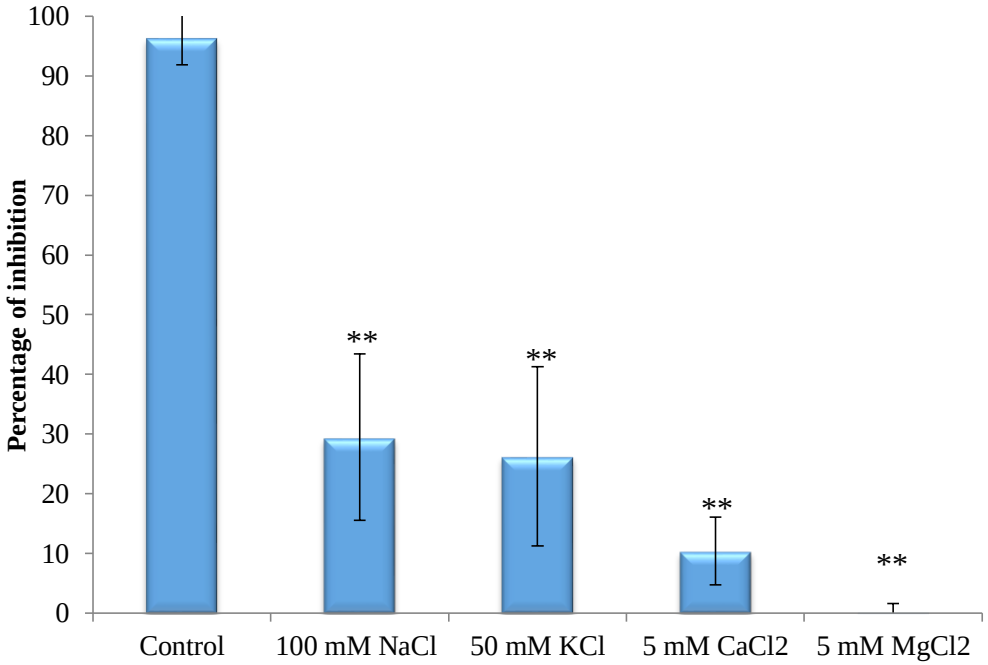


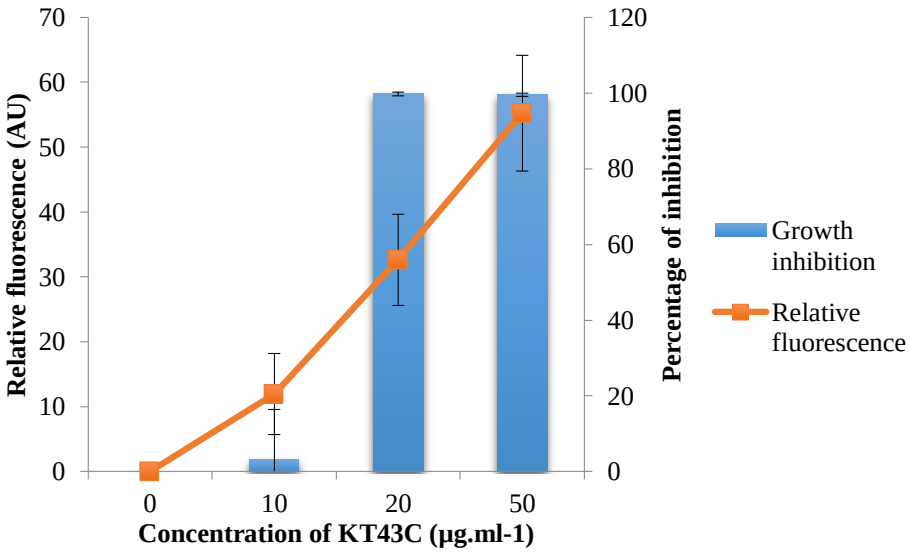
Figure 4.4. Cation sensitivity of the synthetic cowpea defensin, KT43C (20 µg.ml^{-1}). Percentage of inhibition of *F. culmorum* growth after 96 h in growth medium in presence of different salts: **p<0.01, significant difference of the percentage of inhibition between the control and the tested media

Mode of action of KT43C

To identify some characteristics of the mode of action of KT43C, a SYTOX® Green uptake assay was performed on *F. culmorum* with different concentrations of KT43C. This dye emits fluorescence only when bound to nucleic acids, meaning in case of compromised cell membrane. When *F. culmorum* hyphae was incubated with KT43C,

the peptide induced permeabilization of *F. culmorum* hyphae at a concentration of 10 µg.ml^{-1} and higher (Fig. 4.5). Permeabilized hyphae had significant cytoplasmic granulation at higher concentrations. However, KT43C-induced permeabilization appeared to be required for inhibition but was not sufficient to cause cell death.

a)



b)

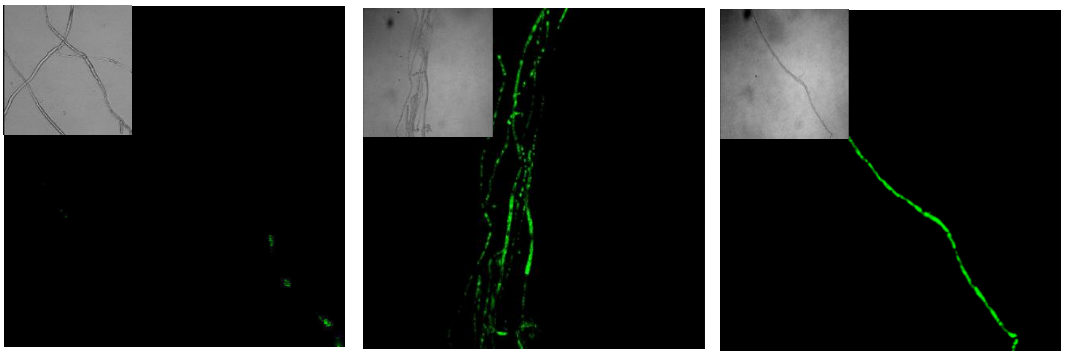
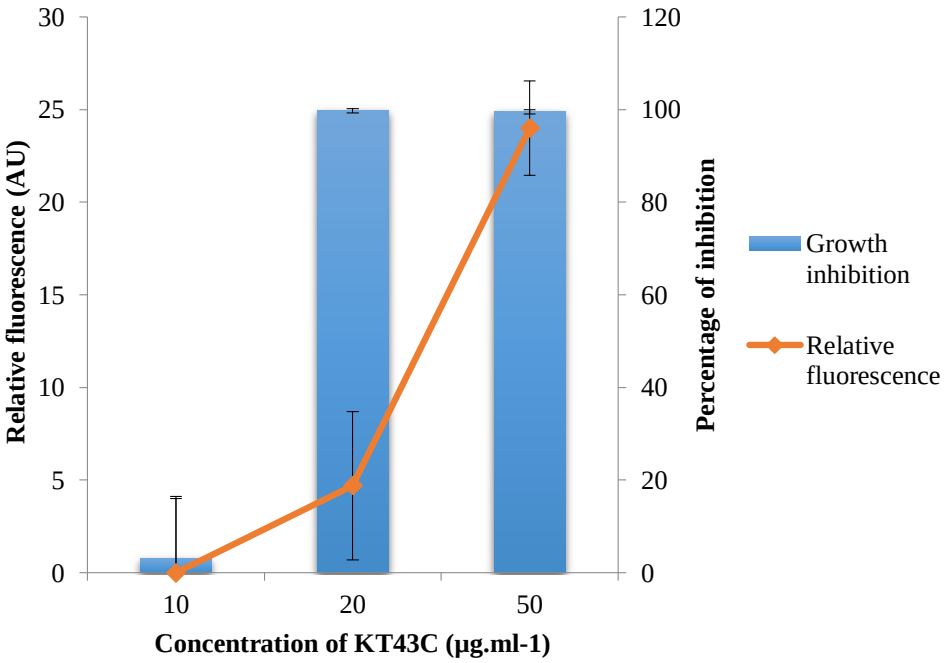


Figure 4.5. SYTOX® Green uptake assay of *F. culmorum* hyphae after treatment with KT43C. **a)** After 18 h of growth, *F. culmorum* hyphae were treated for 2 h with KT43C (0, 10, 20 and 50 µg.ml^{-1}). Permeabilization of the fungal membrane was determined by fluorescence with SYTOX® Green (excitation 488 nm, emission 538 nm) and correlated to the percentage of inhibition. Values are the mean of three different experiments. **b)** *F. culmorum* hyphae were observed with a confocal laser scanning microscope, with an excitation wavelength 460-490 nm. Left: no KT43C; middle: 20 µg.ml^{-1} KT43C; right: 50 µg.ml^{-1}. High concentrations of KT43C induced permeabilization of fungal membrane, highlighted by fluorescence of the dye, and granulation of the fungal cytoplasm

Another inhibitory mechanism is the increased generation of free radicals, mostly from a mitochondrial source, which can lead to an excessive level of reactive oxygen species (ROS), commonly known as oxidative stress. Significant production of ROS appeared only at concentrations of KT43C well above those required to inhibit fungal growth (Fig. 4.6). These results suggest that the overproduction of ROS was not a primary mechanism of antifungal action of KT43C against *F. culmorum*, but may, at high concentrations, enhance its activity.

a)



b)

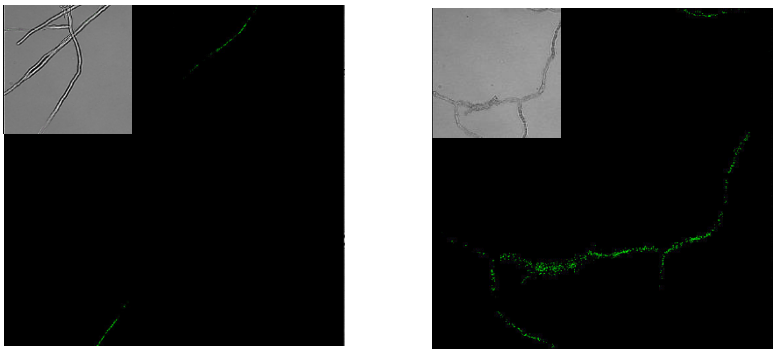


Figure 4.6. Detection of reactive oxygen species (ROS) production. **a)** After 18 h of growth, *F. culmorum* hyphae were treated for 12 h with KT43C (0, 10, 20 and 50 µg.ml^{-1}). Production of ROS was determined by fluorescence of DHR 123 (excitation 488 nm, emission 538 nm), and correlated to the percentage of inhibition. Each value is the mean of triplicates **b)** *F. culmorum* hyphae were observed with a confocal laser scanning microscope, with an excitation wavelength 460-490 nm. Left: no KT43C; right: 50 µg.ml^{-1}

Circular dichroism

Native defensins possess a characteristic CSαβ motif, in which tertiary structures, such as an α-helix and antiparallel β-sheet, are stabilized by disulfide bonds. In aqueous solutions, in the absence of disulfide bonds, the linear analogues of defensins presented random conformations and therefore are thought to be more flexible. In contrast, in trifluoroethanol or SDS micelles (a membrane-mimicking model), the analogues could adopt structured conformations.

The secondary structures of KT43C were studied by CD spectroscopy (Fig. 4.7). In deionized water, the peptide appeared unstructured with a minimum at ~200 nm, which is consistent with the presence of transient β-hairpin or turn-like conformations. A transition was observed in presence of SDS with a crossover at ~195 nm that correlates with the presence of more structured conformers. The slightly positive peak at ~190 nm and the double minima at ~205 nm and ~223 nm indicated a propensity for α–helical conformations with the presence of a β–hairpin.

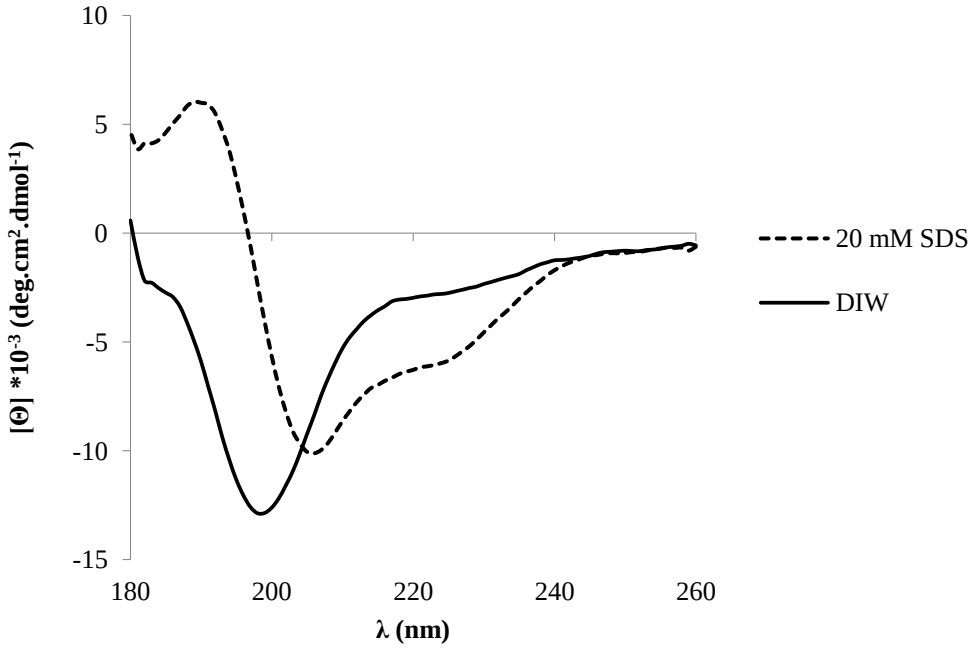


Figure 4.7. CD spectra of KT43C in presence of DIW (solid line) or 20 mM SDS/DIW (dashed line). In DIW, the absence of crossover and the minimum at ~200 nm indicated unstructured conformation with transient β-hairpin. In SDS, KT43C adopted a more structured conformation with the presence of α–helical and β-hairpin conformers

Application of KT43C in dough

Spoilage fungi, such as *Fusarium* spp. in wheat or barley, are ubiquitous in cereal environments. Although the baking step essentially kills spoilage fungi, the presence of heat-stable mycotoxins remains a potential health issue. The protection of raw material or a microbiologically unstable cereal-based product against fungal growth is therefore an absolute necessity.

The synthetic peptide, KT43C, was used as an ingredient in the preparation of bread dough (20 $\mu g.ml^{-1}$), based on total flour weight. The monitoring of the shelf life of chilled dough, previously inoculated with *F. culmorum* spores is presented in Figure 4.8. For 14 days, dough was monitored each day and classified into different categories based on the percentage of fungal contamination present on the surface. The development of fungal colonies on the treated dough was delayed by 2 days compared to the dough without peptide. To quantify the fungal contamination, the concentration of ergosterol in the dough was determined. After 14 days of storage, the level of ergosterol in the control dough was twice as high as in the treated dough (17.5 ± 3.5 and 7.6 ± 0.9 $\mu g.ml^{-1}$, respectively). The synthetic defensin was thus shown to be an efficient tool against fungal spoilage of dough. In addition, the thermostability of KT43C could allow its exploitation as a novel preservative in baked goods.

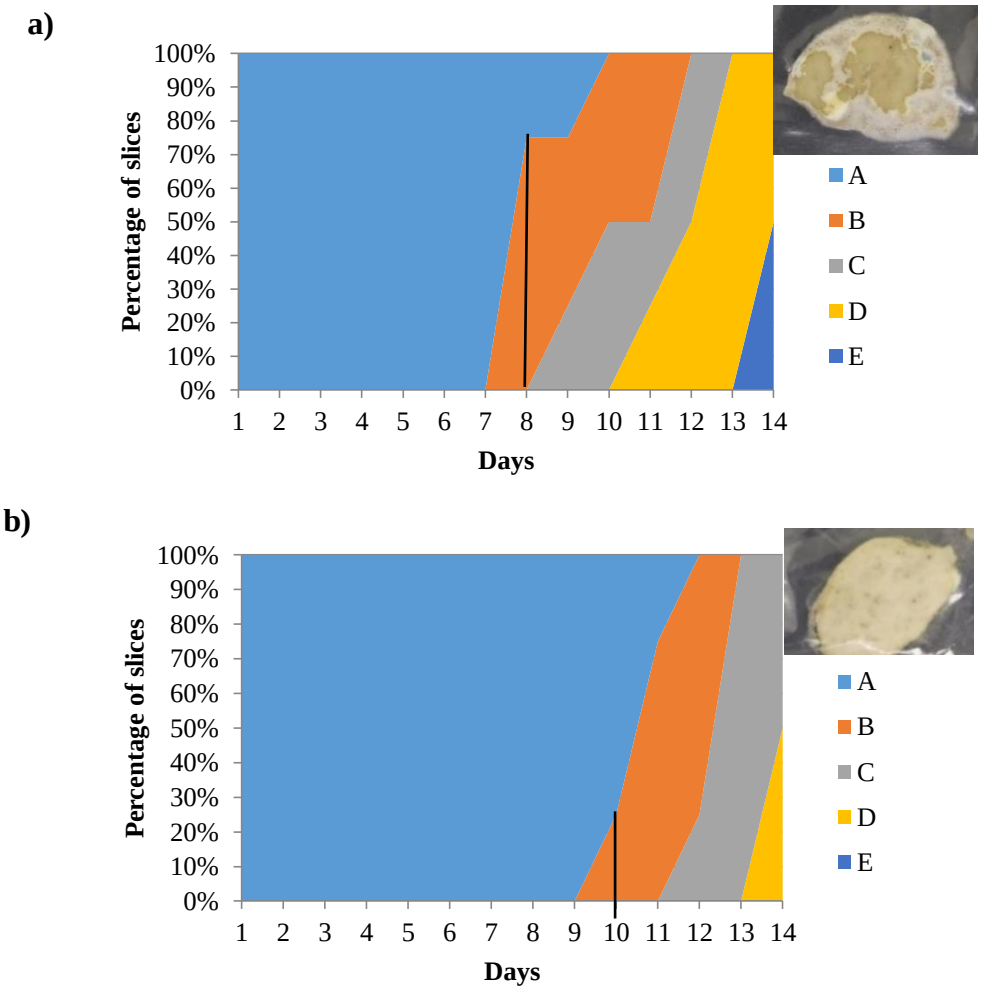


Figure 4.8. Shelf life of chilled dough inoculated with *F. culmorum* conidia ($1x10^2$ spores.ml^{-1}). The development of fungal mycelium was monitored over 14 days and the dough was classified according to the percentage of contamination (from A (0% contamination) to E (50% contamination)). **a)** Control dough; **b)** Treated dough with KT43C (20 µg.ml-1). Three doughs were used in each experiment. **Insert**: pictures of fungal contamination on chilled dough after 14 days of storage are presented with the charts

Haemolysis assay

In order to determine the safety of KT43C against mammalian cells, the activity of the peptide against red blood cells was measured. The release of haemoglobin from fresh sheep erythrocytes after treatment with KT43C was measured in a microtiter plate assay. At concentrations up to 200 µg.ml^{-1}, KT43C did not lyse the red blood cells (data not shown). The safety of KT43C against mammalian blood cells is an essential parameter for potential applications in food products.

4.4 Discussion

Previous studies have highlighted the antifungal (Carvalho et al. 2001) and antibacterial (Franco et al. 2006) activity of antimicrobial peptides isolated from cowpea seeds, such as the defensin, Cp-thionin II as described by Franco et al. (Franco et al. 2006). In this study, a synthetic linear analogue of Cp-thionin II, namely KT43C, exhibited antifungal activity against *F. culmorum*, *P. expansum* and *A. niger* (Fig. 4.1); while KT43C has been previously shown to inhibit Gram-positive bacteria (Kraszewska et al. 2016), to our knowledge, the antifungal activity of cowpea antimicrobial peptides or derivatives has never been described for the fungi studied here. Despite the absence of disulfide bonds in its linear structure, KT43C displayed antifungal and antibacterial activity. Disulfide bonds are determinants of defensins' integrity and have been reported to increase the antimicrobial activity (Jenssen et al. 2006). However, some linear derivatives of AMPs (Wu & Hancock 1999) and defensins (Liu et al. 2008) are able to retain partial or complete antimicrobial activity after removal of the disulfide bonds. Other parameters, such as net charge, hydrophobicity, amphipathicity and flexibity, are essential for the antimicrobial activity of AMPs. Although disulfide bridges are not essential for the antimicrobial and antifungal activity of Cp-thionin II, the presence of free cysteine residues may modify the hydrophobicity of the peptide and then change its activity. However, this effect seems unclear for linear analogues of defensins (Liu et al. 2008, Nagano et al. 1999). Dimerization of plant defensins is also a highly significant criterion for their antifungal activity (Song et al. 2011). Plant defensins that form dimers become highly efficient molecules against pathogenic fungi due to a stronger interaction with the negatively charged proteins of the fungal cell wall and membrane (Lay et al. 2003). However, the oligomerization of defensins does not appear to be crucial, as shown for the antibacterial activity of Cp-thionin II (Franco et al. 2006).

KT43C displayed antifungal activity against *F. culmorum*, *A. niger* and *P. expansum*. These three fungal species belong to the same subdivision, *Pezizomycotina*, but *F. culmorum* belongs the class of *Sordariomycetes* while *P. expansum* and *A. niger* both belong to the class of *Eurotiomycetes*. Although the mechanistic action of KT43C on these fungi may have similarities, differences in cell/wall composition or fungal physiology between these species may be pertinent to the mode of action of KT43C

and its antifungal potency. Differences in the mode of action of the plant defensin MtDef4 against *Neurospora crassa* and *F. graminearum* have been described by El-Mounadi et al. (El-Mounadi et al. 2016). KT43C inhibited growth of *F. culmorum* without inducing morphogenic changes in the hyphae (Fig. 4.3). This finding is in agreement with the ability of KT43C to inhibit Gram-positive bacteria (Kraszewska et al. 2016) and the antibacterial activity of native peptide (Franco et al. 2006). Indeed, only non-morphogenic defensins appear to have an effect on bacteria (Carvalho & Gomes 2009).

After heat treatment, KT43C retained its antifungal activity against *F. culmorum*. The heat stability of KT43C has also previously been shown regarding its antibacterial potency (Kraszewska et al. 2016). Terras et al. (Terras et al. 1992) and Broekaert et al. (Broekaert et al. 1995) have reported the thermal stability of defensins from radish and other plant species. The role of disulfide bonds in defensins stabilization was highlighted by Terras et al. (Terras et al. 1992). KT43C appeared unstructured in aqueous solutions but adopted an α-helical conformation in a membrane environment (Fig. 4.7). The random conformations and flexibility of the peptide could protect from thermal denaturation. The adoption of a structured conformation in presence of SDS may indicate possible interactions between KT43C and a membrane-mimic environment. This new conformation could be related to its antifungal activity (Domingues et al. 2008, Liu et al. 2008). The presence of ions, especially divalent cations, has been proven to decrease the antifungal activity of native plant defensins (Vriens et al. 2014). The antifungal activity of this linear analogue of Cp-thionin II was also demonstrated to be affected by the presence of cations (Fig. 4.4). Kraszewska et al. (Kraszewska et al. 2016) have reported that the peptide keeps its antibacterial activity in the presence of NaCl, up to 50 mM, but loses it at 100 mM. The loss of activity in presence of cations is a common feature for plant defensins and AMPs linear derivatives in general (Adem Bahar & Ren 2013, Vriens et al. 2014). This effect is due to the weakening of electrostatic interactions between the cationic peptides and the negatively charged membrane of microbial cells (Wu et al. 2003). Other potential effects may include structural changes in the peptide (Oard & Karki 2006), or stabilization of the microbial membrane by cations (Thevissen et al. 1999).

Plant defensins (Vriens et al. 2014) and linear AMPs (Domingues et al. 2015, Liu et al. 2008) have been shown to present several modes of action. Among these different

mechanisms, permeabilization of the fungal membrane has been described as a secondary effect of plant defensin action (Vriens et al. 2014), but is primarily involved in the antimicrobial activity of linear peptides (Bechinger & Lohner 2006). Like native plant defensins, such as NaD1 or linear antimicrobial peptides (Van Der Weerden et al. 2010), KT43C caused fungal membrane permeabilization of *F. culmorum* at the MIC (Fig. 4.5), but at a slower rate of action than the native peptide (data not shown). The time difference can be explained by the formation of oligomers of the synthetic peptide or the necessity to reach a sufficient concentration of peptide at the surface of fungal membrane (Thevissen et al. 2004). Another hypothesis would be a limited mobility of aggregates of KT43C that tend to form into the bilayer environment because of changes in hydrophobicity, as described for tachyplesin (Han & Lee 2015). At high concentrations, KT43C induced a high production of ROS in *Fusarium* hyphae (Fig. 4.6). The generation of ROS is involved in mechanisms related to oxidative stress and damage, leading generally to cell-death. The interaction with intracellular targets and the overproduction of ROS in the fungal cytoplasm has been highlighted for several defensins (Vriens et al. 2014) and linear AMPs (Huang et al. 2010). In addition, a model, involving pore-formation and intracellular target strategy, has already been considered for several linear AMPs (Mason et al. 2007). Like KT43C, ROS production with NaD1 was only observed at concentrations greater than the MIC, even when membrane permeabilization was observed, suggesting a partial role for oxidative stress in fungal inhibition. (Hayes et al. 2013).

A major issue with the use of cAMPs in pharmaceuticals or food applications is their potential toxicity towards mammalian cells. The reduction of hydrophobicity and the absence of disulfide bridges in linear derivatives have been pointed out as key elements in reducing their cytotoxicity (Liu et al. 2008). KT43C did not induce red blood cells lysis in the range of concentration used for the antifungal assays. Due to the presence of cholesterol, mammalian cell membranes have been shown to be less sensitive to destabilization by linear cationic AMPs than fungal membranes (containing mostly ergosterol) (Mason et al. 2007). In the next chapter, a study from our group has shown that that the synthetic cationic peptide OOWW-NH_2 is inactive against gut Caco-2 cell line (Thery et al. 2018) (see Chapter 5). The cytotoxicity of plant defensins has already been proven to be low, even negligible (Thevissen et al. 2004). In addition, Liu et al. (Liu et al. 2008) showed that a linear analogue of HBD-3 displayed lower cytotoxicity

compared to the native form of HBD-3. The decreased cytotoxicity towards mammalian cells of linear derivatives of AMPs and defensins has been attributed to the removal of the disulfide bridges, decreasing the overall hydrophobicity (Liu et al. 2008).

KT43C (20 $\mu g.ml^{-1}$) was used as an ingredient in the preparation of chilled dough and delayed the growth of *F. culmorum* by 2 days in a challenge test. The use of natural (Lucera et al. 2012, Rai et al. 2016, Rydlo et al. 2006) and synthetic (Appendini & Hotchkiss 2000, Thery et al. 2018) AMPs to prevent food products from food spoilage has been reported previously. Thus, the synthetic analogue of the human β-defensin 3 protects bread against environmental contaminants, with a shelf life extension of 3 days (Thery et al. 2016). Although the concentration of KT43C used in this test was the MIC against *F. culmorum*, the conidial germination was not completely inhibited. The presence of other dough ingredients and proteases resulting from the preparation process may affect the antifungal action of the peptide. The sensitivity of AMPs to proteolytic digestion is a major concern for a potential use as food additive to avoid further action once in the intestinal system.

In this study, a synthetic linear analogue of the Cp-thionin II, showed antifungal activity against common fungal contaminants of cereal and cereal-based products. The analogue defensin, KT43C, presented similar characteristics to native plant defensins, such as heat stability and cation sensitivity. The synthetic peptide appeared to be an efficient tool against fungal contamination of dough. No lysis of erythrocytes being detected, KT43C may represent a novel alternative to commonly used chemical preservatives in the baking industry.

4.5 Acknowledgements

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Chapter 5
Optimisation of the antifungal potency of the amidated peptide H-Orn-Orn-Trp-Trp-NH_2 against food contaminants

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Abstract

The design of novel efficient antimicrobial peptides (AMPs) faces several issues, such as cost of synthesis, proteolytic stability or cytotoxicity. The identification of key determinants involved inthe activity of AMPs, such as cationicity and amphipathicity, allowed the synthesis of short peptides with optimized properties. An ultrashort peptide, with the sequence H-Orn-Orn-Trp-Trp-NH_2 (O3TR), showed antifungal activity against several contaminants from food products. This peptide inhibited the growth of the filamentous fungi *Fusarium culmorum*, *Penicillium expansum* and *Aspergillus niger* within a range of concentration of 12.5–50 μg/ml. In addition, O3TR inhibited the growth of the yeasts *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii* and *Kluyveromyces lactis* within the range 12.5–50 μg/ml. A derivative peptide, called C12O3TR, made by the addition of lauric acid at the N-terminus of O3TR was 2- to 8-fold more active than O3TR against every species. In addition to the inhibition of conidial germination, O3TR and C12O3TR killed *F. culmorum* hyphae at 100 and 50 μg/ml, respectively. The MIC of the two peptides against *F. culmorum* and *Z. bailii* after heat treatment at 100°C for 60 min and within the pH range 3–10, were not changed. However, the activity of O3TR against *F.culmorum* and *Z. bailii* was strongly reduced in salt solutions, whereas the lauric acid peptide kept its antifungal activity and resistance to proteolytic digestion. The conjugation with lauric acid reduced the random coiled structure and increased the α-helical content of O3TR. After conjugation with the dye tetramethylrhodamine (TMR), both peptides entered *F. culmorum* spores. They also both induced permeabilization of *F. culmorum* hyphae but only C12O3TR permeabilized *Z. bailii* membrane. In contrast to the lipopeptide, O3TR did not show haemolytic or cytotoxic activity when applied at the concentrations that exhibited antifungal potency. The two peptides were challenged against a yeast cocktail of *S. cerevisiae* and *Z. bailii*, and *A. niger* in different commercial beverages. After 7 days, O3TR was able to inhibit the yeast cocktail in a commercial lager and carbonated drink. Due to its antifungal potency, high stability and low cytotoxicity, the tetrapeptide could represent a promising starting point of a novel food preservative.

5.1 Introduction

In order to provide sustainable and safe food, protection from microbiological contamination is essential. Food supply is facing two major issues, due to microbial safety: the risk to human health and economic losses due to food loss. In 2015, foodborne diseases have been estimated by the WHO to affect 1 in 10 people every year and 420 000 cases resulted in death (WHO [World Health Organization], 2015). Beverage industry is not spared by microbial spoilage. Although the low pH of soft drinks prevents most of microbial spoilage, fungal growth still occurs. Yeasts are the primary contaminants in carbonated products, due to their ability to grow in highly carbonated and with low pH beverages (Tribst et al., 2009; Tserennadmid et al., 2011). In case of poor hygiene, mould can grow thanks to high water availability and acidity (Kregiel, 2015). In addition to health risk, fungal spoilage induces undesirable modifications of flavours and tastes. Chemical preservatives are commonly used to inhibit food spoilage but the intensive overuse of these preservatives has led to a rise of resistant microorganisms. The common preservatives used in soft drinks are weak acid preservatives such as sorbic or benzoic acids (Battey et al., 2002). However, yeast species including *S. cerevisiae* and mostly *Z. bailii* have shown resistance to these acids (Tribst et al., 2009). In addition, the increasing interest of the consumer for products of natural origin generated an increasing development of novel natural antimicrobials. Among these natural compounds, antimicrobial peptides are short (12-50 amino acid residues) peptides of the innate immune system found in every kingdom. They contain 2 to 9 basic residues and approximately 50% hydrophobic residues and a diversity of structures. The main advantage of these peptides is their mode of action which, for most of them, does not involve specific targets on bacteria or fungi thereby avoiding the development of resistance (Bechinger and Lohner, 2006; Huang et al., 2010). *In vivo*, apart from their direct action, some cationic antimicrobial peptides (cAMPs) are involved in immunomodulatory function reinforcing their antimicrobial potential (Hancock et al., 2016). Several AMPs and AMP-derived lipopeptides derived from AMPs have already been approved by the Food and Drug Administration. However, only some bacteriocins, natamycin, lactoferrin, or more recently, the plant protein thaumatin, are AMPs used as food preservative (Domalaon et al., 2014). Some limitations, such as a narrow spectrum of activity or stability, in the absence of

additional treatments, are significant hurdles to their widespread uses (Rydlo et al., 2006).

Positive net charge, hydrophobicity and amphipathicity are the key parameters of the antimicrobial activity of these peptides and many potent peptides have been synthesized based on these properties. However, the high cost of these peptides is a hurdle that has led to research on shorter peptides. The shortest peptides are called ultrashort peptides (USPs) whose length is below seven amino acid residues. Among the different USPs, the 4 amino acid H-Orn-Orn-Trp-Trp-NH_2 has been characterized (Bisht et al., 2007; Laverty et al., 2010). This peptide displays antimicrobial activity against several human pathogens (Laverty et al., 2015). The use of conjugated fatty acids has been revealed by different groups to increase the antimicrobial potential and act syngergistically with USPs (Laverty et al., 2010; Yadav et al., 2013).

In this study, the *in vitro* activity of the USP Orn-Orn-Trp-Trp-NH_2 (O3TR) was characterized against some representatives of fungal contaminants found in cereal-based products, such as beer, or soft drinks. In addition to potency and safety, promising antimicrobials in food must present an interesting "cost in use". As such, a synthetic O3TR peptide (12 mg, 80% purity) can be purchased for a price of $38.00.Thus, the efficiency of this low-cost 4-residue peptide and a derived lipopeptide to reduce the contamination of pre-inoculated drinks was also determined.

5.2 Material and methods

Peptides

The ultrashort amidated tetrapeptide H-Orn-Orn-Trp-Trp-NH_2 (O3TR) and its derivatives, (C_2H_3O)-Orn-Orn-Trp-Trp-NH_2 (Ac-O3TR) and a lauric acid ester of O3TR, the lipopeptide ($C_{12}H_{23}O$)-Orn-Orn-Trp-Trp-NH_2 (C12O3TR), were synthesized by and purchased from GLBiochem, Shanghai, China. Synthesis was performed by solid-phase peptide synthesis technique on Fmoc MBHA Rink amide resin, followed by removal of the Fmoc group. The purity of all peptides exceeded 90% according to the manufacturer. The lyophilized peptide was reconstituted in deionized water and stored in vials of different concentrations at -20°C.

Fungal strains and growth conditions

The following fungal strains were used in this study: *Fusarium culmorum* FST 4.05, *Aspergillus niger* FST 4.21 and *Penicillium expansum* FST 4.22. These fungi were chosen because they represent some of the main fungal contaminants of beverages (Kregiel 2015). *F. culmorum* is an important contaminant of barley used in brewing industry and residual mycotoxins can be found. Fruit juices are also regularly tested for the content of mycoxotins, produced by *Aspergillus* or *Penicillium* species (Kregiel 2015). The three strains originated from the culture collection of School of Food and Nutritional Sciences, University College Cork (Cork, Ireland). Spore suspensions were prepared according to Oliveira et al. (Oliveira et al. 2012). Briefly, fungal mycelium was cultured on Potato Dextrose Agar (PDA, Sigma-Aldrich) plates at 28°C for 3 days. Following incubation, hyphae was inoculated in nutrient-poor broth until sporulation. Fungal spores were collected by filtration (30 µm), counted on a haematocytometer and inoculated in fresh nutrient-poor broth.

The yeast strains were purchased from the Leibniz Institute DSMZ (Braunschweig, Germany) and Fermentis (Belgium). *Zygosacharromyces bailii* DSM 70492, *Zygosaccharomyces rouxii* DSM 2531, *Kluyveromyces lactis* DSM 4394 and *Debaryomyces hansenii* DSM 3428, brewing *Saccharomyces cerevisiae* (Fermentis) were chosen since they represent some of the main contaminants of food and beverages

products (Wareing & Davenport 2007). The yeasts were cultured on Yeast Potato Dextrose Agar (YEPD, Sigma-Aldrich) at 25°C for 2-5 days, depending on the strain.

Antifungal activity assays

Antifungal *in vitro* asssays were conducted as described by Muñoz et al. (Muñoz et al. 2014) with some modifications. Briefly, fungal conidia (1 $x10^4$ conidia/ml) were inoculated in synthetic fungal media (SFM) ($MgSO_4/7H_2O$, 5 µM $FeSO_4/7H_2O$, 0.1 µM $CoCl_2$, 0.1 µM $CuSO_4/5H_2O$, 2 µM $Na_2MoO_4/2H_2O$, 0.5 µM H_3BO_3, 0.1 µM KI, 0.5 µM $ZnSO_4/7H_2O$, 0.1 µM $MnSO_4/1H_2O$, glucose, asparagine, 20 mg of methionine, 2 mg of myo-inositol, 0.2 mg of biotin, 1 mg of thiamine-HCl, 0.2 mg of pyridoxine-HCl, 0.5 mM $K_2HPO_4/3H_2O$), as described previously (Thevissen et al. 1999).

Fungal suspension (90 µl) was added to 96-well microplate wells followed by the addition of 10 µl of peptides (0-200 µg/ml). Deionized water was used as negative control. Fungal growth was monitored at 595 nm over 96 h at 28°C on a microplate reader (Multiskan TM, Thermo Scientific). The MIC was defined as the lowest concentration of peptide that showed no growth at the end of the experiment (4 days of incubation) in all the experiments. The IC_{50} was defined as the concentration required inhibiting 50% of fungal growth and calculated via PRISM software based on the microplate data.

A similar procedure was used for yeasts cells with slight differences. The initial yeast concentration was $1x10^5$ cfu/ml in yeast minimal media (YMM), as described by previously (Thevissen et al. 1999) and the yeast growth was monitored at 30°C. YMM was made of 0.8 g of complete supplement mixture – URA [CSM-URA] per liter, 6.5 g of yeast nitrogen base (YNB) without amino acids per liter (Formedium) 10 g of glucose (Sigma) per liter.

Scanning electron microscopy

Yeast cells *S. cerevisiae* ($1x10^6$ cfu/ml) were mixed with O3TR and C12O3TR (12.5 µg/ml) for 2 h in YMM. Following washing twice with 0.1 M phosphate buffer, cells

were fixed for 30 min at room temperature in a solution containing 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3. Cells were rinsed three times, post-fixed for 30 min with 1.0% osmium tetroxide (OsO_4) and dehydrated using a gradient ethanol series. Upon dehydration, the cells were air dried after immersion in hexamethyldisilazane (Sigma). The lyophilized samples were then analysed using a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan), as described by Oliveira et al. (Oliveira et al. 2012), with some modifications. Briefly, dried yeasts were mounted onto plain aluminium stubs using carbon double surface adhesive and coated with a 7 nm gold layer using a Gold Sputter Coater (BIO-RAD Polaron Division, SEM coating system, England) and observed under a constant accelerating voltage of 5 kV.

Fungal cell viability assays

Cell viability assays were performed as described by van der Weerden et al. (Van Der Weerden et al. 2010) with some modifications. F. culmorum hyphae were grown in SFM from a concentration of 5 x 10^4 spores/ml for 18 h at 28°C with some modifications. Hyphae were collected by centrifugation (10,000 *g* for 5 min) and resuspended in fresh medium in an equal volume. Hyphal suspension (90 µl) was transferred in wells of a 96-well microtiter plate with 10 µl of peptides (0-200 µg/ml). Deionized water was used as negative control as described above. After 2 h of incubation at 28°C, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Sigma) was added to the wells. After 16 h of incubation at room temperature, medium was removed and 100 µl of MTT solvent (0.1 N HCl in anhydrous isopropyl alcohol) were added to the wells and left for 15 min until cell lysis. The presence of MTT/formazan was monitored by measuring absorbance at 570 nm and subtracting background at 690 nm.

Modification of fungal cell-wall

In order to identify a potential receptor for the peptides on the fungal cell-wall, the latter was modified as described by Van der Weerden et al. (Van Der Weerden et al. 2010). *F. culmorum* hyphae were grown from a starting solution of 5x10^4 spores/ml

for 18 h at 28°C. The hyphae were collected by centrifugation (10,000 *g* for 10 min) and treated with β-glucanase (1 ml of 2 mg/ml) for 1 h at 30°C. After 3 washings (10 min) in SFM, hyphae were treated with the two peptides as described above. Cell viability assay was monitored using MTT assay after 2 h of incubation.

Heat and pH resistance

To investigate their stability, the peptides were treated at a range of temperatures from 25-100°C for 15, 30 or 60 min, in a heating block (Grant Instruments, Cambridge). After cooling at room temperature for 30 min, the antifungal activity of the peptides was determined against *F. culmorum* and *Z. bailii*, as described above.
The inhibitory effect of the non-heated peptides was determined in the respective media at pH of 3-10.

Effect of cations on the antifungal activity

To determine the effect of cations on the activity of O3TR and C12O3TR, an antifungal assay against *F. culmorum* and *Z. bailii* was performed in different salt solutions, as described in previous work (López-Garcia et al. 2000). Fungal spores ($1x10^4$ spores/ml) and yeast ($1x10^5$ cfu/ml) were inoculated in SFM and YMM respectively, supplemented with 50 mM/150 mM NaCl or 1 mM/5 mM $CaCl_2$. After the addition of the peptide, the inhibitory activity of the peptide/lipopeptide was determined as described above.

Resistance to proteolytic digestion

Resistance to chymotrypsin was conducted as described by Kim et al. (Kim et al. 2014) in a growth inhibition assay. Peptides were incubated with α-chymotrypsin (Sigma, St Louis, MO, USA) at different molar ratios peptide:enzyme (60:1, 250:1, 2500:1), in a digestion buffer (50 mM Tris–HCl, pH 7.4, 5 mM $CaCl_2$) at 37°C for 4 h. To stop enzymatic reaction, the solution was treated at 80°C for 10 min and 10 µl of this solution (0-50 µg/ml) were added to *F. culmorum* spores solution (90 µl). Fungal growth was monitored as described above.

Circular dichroism spectroscopy

The analysis of the peptides secondary structure was conducted as described by Liu et al. (Liu et al. 2008). Circular dichroism spectroscopy was performed by using a Chirascan™ spectropolarimeter (Applied Photophysics) with the peptides dissolved in deionized water (DIW), in 50% trifluoroethanol (TFE) in DIW or 20 mM sodium dodecyl sulphate (SDS). The two peptides were analysed at a concentration of 1 mg/ml. CD measurements were performed at 27°C within a wavelength range of 180-260 nm. Every sample and solvent was scanned three times and solvent CD was subtracted from the sample CD.

Membrane permeabilization

To characterize the mode of action of the synthetic peptides against *F. culmorum*, a SYTOX® Green (SG) (Molecular Probes) uptake assay was performed as described by van der Weerden et al. (Van Der Weerden et al. 2010) with some modifications. *F. culmorum* hyphae were grown in SFM from a starting concentration of 5 × 10^4 spores/ml for 18 h at 28°C. Hyphal suspension (90 µl) was then transferred to 96-well microtiter plates and exposed at O3TR and C12O3TR. Water alone and Triton X-100 (0.1%) were used as negative and positive controls respectively. After 1 or 2 h of incubation, SG (final concentration 0.5 µM) was added to the wells and incubated for 10 min before visualization. SG uptake was visualized by fluorescence microscopy (Olympus FluoView™ FV1000) (excitation wavelength 460-490 nm).

A kinetic procedure was used to measure SG uptake in yeast cells (10^5 cfu/ml in YMM). Peptides and SG (final concentration of 20 µM) were added simultaneously and the SG uptake was measured every 10 min for 2 h, using a fluorospectrophotometer (VarioSkan) with excitation and emission wavelength of 488 and 538 nm, respectively.

Internalization assay

In order to detect internalization of labelled O3TR and C12O3TR, tetramethylrhodamine (TMR) was added at the N-terminal of the original peptides.

The labelled peptides were provided by GLBiochem (Shanghai, China). The antifungal activity of the labelled peptides was determined as described above.
Labelled peptides (10 µl) were added to *F. culmorum* spores solutions ($1x10^5$ conidia/ml) in a 96-well microplate. After 10, 30 and 60 min of incubation, spores solutions were visualized with confocal laser-scanning microscopy (Olympus FluoView™ FV1000). TMR-O3TR and TMR-C12O3TR were excited at 543 nm and fluorescence detected at 570-700 nm.

Haemolysis assay

O3TR and C12O3TR were assayed for their ability to induce haemoglobin release from fresh sheep erythrocytes (Oxoid) according to the method of Papanastasiou et al. (Papanastasiou et al. 2009). Fresh blood cells (1 ml) were prepared by centrifugation at 1,620 *g* and the pellet was then resuspended in an equal volume of PBS. This process was repeated three more times. After a final centrifungation at 900 *g* for 15 min, the pellet was resuspended at 4% (v/v) in PBS. Erythrocytes (100 µl) were then combined in the wells of 96-well microtiter plate with peptides at different concentrations, previously diluted in PBS. The solutions were incubated for 1 h at 37°C followed by centrifugation at 1,000 *g* for 2 min. Aliquots of the supernatant were transferred to a fresh 96-well microtiter plate and the absorbance (haemoglobin release) was measured spectrophotometrically at 405 nm. PBS and 0.1% Triton X-100 were used as negative and positive controls, respectively. All peptide concentrations are reported as the mean of six replicates. The percentage of haemolysis was calculated as followed:

$$\% \text{ Haemolysis} = \frac{(Abs405\ peptide\ treatment)-(Abs405\ PBS)}{(Abs405\ 0.1\%\ Triton\ X-100)-(Abs\ 405\ PBS)} \text{ x } 100$$

Cytotoxicity assays

The cytotoxicity of O3TR and C12O3TR was performed as described by Laverty et al (Laverty et al. 2010) by measuring cell viability using a MTT cell viability kit (Roche, Switzerland). Human colonic cells, Caco-2 (ECACC) were cultured in a 96-well microtiter plate in Dulbecco's modified Eagle's medium (DMEM), supplemented with non-essential amino acids (1%) and FBS (10%), at 37°C in 5% CO_2. Cells were allowed to adhere overnight, media was removed and 200 µl of fresh medium

supplemented with peptides (0-200 µg/ml) was added to the wells and incubated for 24 h at 37°C. After removal of the medium, fresh medium (100 µl) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (10 µl) (Cell viability kit, Roche) was added to each well and incubated for 4 h at 37°C. The solubilisation solution (100 µl) was added to each well and the plate was incubated overnight at 37°C. Cell viability was measured spectrophotometrically at 570 nm with a background reading at 690 nm. The cytotoxicity of each peptide was determined in 4 wells for 5 replicates.

Applications in beverages

Cultures of *S. cerevisiae* and *Z. bailii* were grown in malt extract broth solutions (for 2 days at 25°C at 160 rpm). Cell counting was performed with decimal dilutions on malt extract agar (Oxoid Ltd.), incubated at 25°C for 5 days. The individual solutions were blended together mixed thoroughly to form the yeast cocktail at a final concentration of $5x10^4$ cfu/ml, as described previously (Battey et al. 2002). The cocktail was stored at 5°C until further use.

Four beverages were used: commercial lager [Heineken (g/per 100 g): protein: 0.4; carbohydrates: 3.4; alcohol: 4; pH= 4.7]; carbonated orange soft drink [Fanta: carbohydrates (=sugars) 12.2 g/100 mL; pH = 2.73]; orange juice (Tesco) [10 g sugars/100 ml; pH =3.7]; apple juice (Tesco) [9.6 g sugars/100 ml, pH =3.4]. The filter-sterilized beverages (were inoculated with the yeast cocktail (10^2 cfu/ml) or *A. niger* spores (10^2 conidia/ml). Beverage solutions (90 µl) were transferred to the wells of a 96-well microtiter plate and 10 µl of O3TR or C12O3TR were added to the wells. Fungal growth was monitored spectrophotometrically at 595 nm for 7 days at RT.

5.3 Results & Discussion

Antifungal activity

The growth of some representatives of fungal food and beverage contaminants was conducted in the presence of ultrashort peptide O3TR and two derivatives: a lipopeptide C12O3TR and an acylated Ac-O3TR. O3TR and C12O3TR displayed antifungal activity against all the species at different levels (Table 5.1).

Table 5.1. MIC (μg/ml) of O3TR and derivatives against fungal and yeast contaminants

	O3TR	**C12O3TR**	**Ac-O3TR**
F. culmorum	12.5	6.25	>200
P. expansum	50	12.5	>200
A. niger	50	25	>200
Z. rouxii	50	6.25	N.D.
K. lactis	25	6.25	N.D.
Z. bailii	12.5	3.12	N.D.
D. hansenii	25	6.25	N.D.
S. cerevisiae	12.5	3.12	N.D.

All peptides possess a NH_2 group at the C-terminus; Fmoc-protection was removed at the N-terminus.
C12 = $C_{12}H_{23}O$, is lauric acid tail; Ac is an acetyl group (CH_3CO)
>200 : no antifungal activity observed in the range of peptide concentrations (0-200 μg/ml)
N.D.: not determined

The presence of the NH_2 group removes the negative charge at the C-terminus, increasing the net charge of the peptide. Amidation can be used to increase antimicrobial activity of AMPs, such as lactoferrin (Pan et al. 2007), or restore the activity of an inactive peptide (Wu & Hancock 1999). The acylated peptide (C_2H_3O)-OOWW-NH_2 did not show any antifungal activity in the range of concentrations (0-

200 µg/ml) used in this study. The addition of the acetyl group removes the positive charge at the N-terminus of O3TR. This removal has been shown to abrogate the antimicrobial activity of several peptides, such as dermaseptin (Radzishevsky et al. 2005) or lactoferrin (Lu et al. 2016), mainly due to a reduced electrostatic interaction. On the contrary, Bisht et al (Bisht et al. 2007) showed that the *N*-acylation with acetic anhydride improves the antmicrobial activity of the amidated peptide H-OOWW-NH_2.

The viability of *F. culmorum* hyphae after peptide treatment was determined with a MTT cell viability assay. Both peptides induce hyphal death but at higher concentrations than the ones required for inhibition of conidial germination (Fig. 5.1).

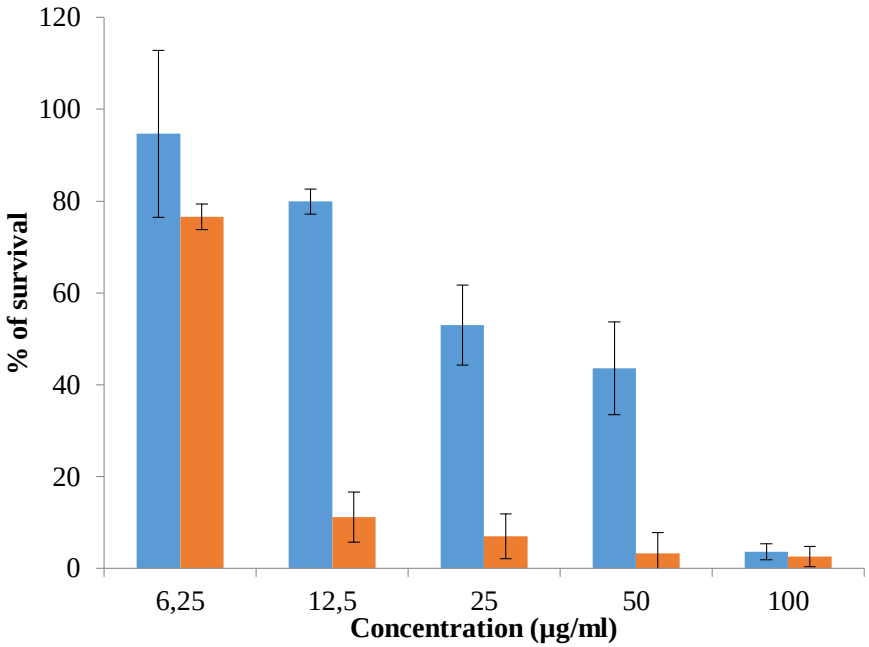


Figure 5.1. Survival of *F. culmorum* hyphae challenged with O3TR (blue) or C12O3TR (orange). Errors bars represent the standard deviation between three replicates.

In order to identify a peptide receptor on fungal cell-wall, a pre-treatment of *F. culmorum* hyphae with β-glucanase was performed to degrade the B-glucans of fungal cell wall. This treatment did not modify the percentage of hyphal survival, after challenging with both peptides (data not shown), which indicated that the absence of glucans did not affect the interaction of the peptides with the cell wall.

The net charge and the hydrophobicity of O3TR are carried by the ornithine and the tryptophan residues, respectively. The use of the unnatural amino acid residue ornithine reduces its degradation by proteases (Bisht et al. 2007). N-terminal

modification of the peptide increased synergistically the antifungal potential of the peptide and of lauric acid (Table 5.1). The effect of the peptides on yeast cells morphology was also examined by scanning electron microscopy (SEM). In the control (Fig. 5.2A) and after treatment with O3TR (Fig. 5.2B), the observed cell shapes appeared intact with clear budding yeasts. In contrast, the treatment with C12O3TR (12.5 µg/ml) modified drastically the size and the shape of *S. cerevisiae* (Fig. 5.2C). The fatty acid chain tail of lauric acid was shown to be the optimal chain tail for O3TR regarding its antimicrobial activity (Laverty et al. 2010). With longer fatty acid chains, high hydrophobicity may induce an important oligomerization of the peptide which prevents it entering the microbial membrane (Matsuzaki et al. 1997). The improvement of the activity has been attributed to an increase of the overall hydrophobicity of the lipopeptide.

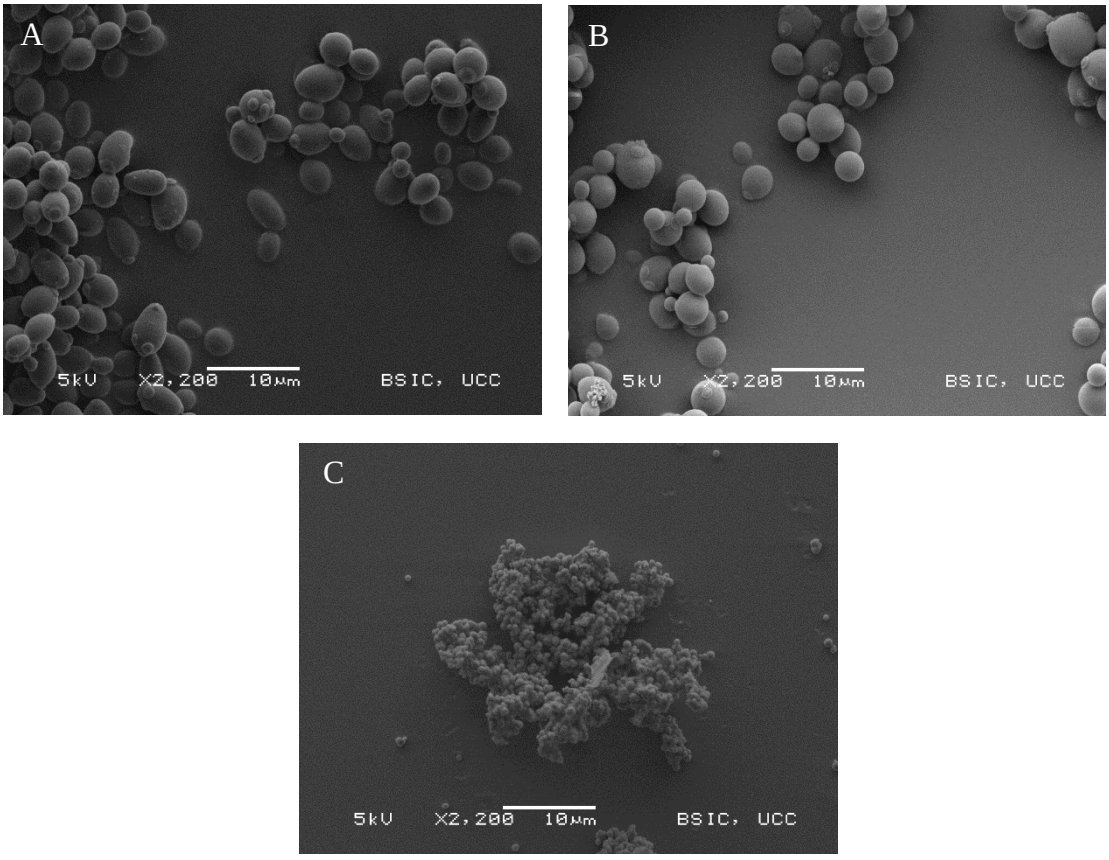


Figure 5.2. Scanning electron microscopy observation of *S. cerevisiae* after 2 h-incubation in YMM without peptides (**A**), with O3TR (12.5 µg/ml) (**B**) or C12O3TR (12.5 µg/ml) (**C**)

Resistance of the antifungal peptides

The resistance of the two antifungal peptides was tested after different heat treatments, at different pH and in presence of salt solutions. Both peptides showed heat resistance up to 100°C for 1 h and pH resistance in the range 3-10. They presented the same MIC against *F. culmorum* and *Z. bailii* as showed in Table 5.1. The ultrashort peptide O3TR has been reported to keep its antibacterial activity after heat treatment (Kraszewska et al. 2016). As shown on the CD spectra, the two peptides presented different conformations in aqueous solutions. While O3TR is mostly unstructured in aqueous solutions, C12O3TR adopted a more structured conformation. The absence of structure in aqueous solutions has been highlighted both for natural and synthetic AMPs (Liu et al. 2008). In addition to the absence of structural elements (*e.g.*, disulfide bonds), the limited size of the peptides may contribute to the difficulty of creating defined structure. However, small derivative proteins (Nedwidek & Hecht 1997) or linear hexapeptide (Guo et al. 2013) have been observed to form ordered structures. In addition, self-assembly has been reported for several AMPs (Bechinger & Lohner 2006), including short peptides (Han & Lee 2015).

In salt solutions, O3TR lost most of its antifungal potency (Table 5.2). O3TR was only able to inhibit *F. culmorum* growth in presence of 50 mM NaCl and 1 mM $CaCl_2$ at concentrations of 50 and 100 µg/ml, respectively, well above the concentration required in non-supplemented SFM. C12O3TR kept its antifungal potency against *F. culmorum* and *Z. bailii*, regardless of the salt concentrations. The reduction of the antibacterial and antifungal activity with an increase of ionic strength is a common feature for antimicrobial peptides, linear or non-linear (Jenssen et al. 2006). Several hypothesis have been suggested: the increase in the ionic strength increase the net charge of the membrane, reducing electrostatic interactions; the changing in the global zeta-potential of the membrane; binding of some ions to the membrane, reducing the ability of the peptides to find receptors; changes in the configuration of the peptide. Thus, AMPs with a high positive net charge, such as HBD-3, are thought to be less sensitive to the presence of cations. Despite the reduction of electrostatic interactions between peptides and fungal membrane, in presence of cations, the antifungal activity of C12O3TR is suggested to be maintained by the high hydrophobicity of the lipopeptide.

Table 5.2. MIC (µg/ml) of O3TR and C12O3TR in presence of different salt concentrations

	F. culmorum				***Z. bailii***			
	50 mM NaCl	150 mM NaCl	1 mM $CaCl_2$	5 mM $CaCl_2$	50 mM NaCl	150 mM NaCl	1 mM $CaCl_2$	5 mM $CaCl_2$
O3TR	50	>100	100	>100	>100	>100	>100	>100
C12O3TR	6.25	6.25	6.25	6.25	3.12	3.12	3.12	3.12

The resistance of the peptides to proteolytic digestion was performed by measuring antifungal activity after treatment with α-chymotrypsin in a digestion buffer (Table 5.3). Due to the presence of Ca^{2+} in the digestion buffer and the sensitivity of O3TR to divalent cations (Table 5.2), the use of such buffer is a limiting factor for the study of the peptide resistance to proteolytic digestion. C12O3TR showed total resistance to chymotrypsin digestion at a ratio peptide:enzyme of 2500:1, and partial resistance with a ratio at 250:1. The antifungal activity of C12O3TR was largely inhibited using a peptide:enzyme ration of 60:1, since the MIC against *F. culmorum* was over 50 µg/ml. When used as food preservatives, the degradation of peptides by proteolytic enzymes, including the ones found in the intestinal tract, is desirable to avoid undesirable effect on gut microbiota and to limit allergenic reactions, as found for peanut defensins (Petersen et al. 2015).

Table 5.3. MIC (µg/ml) against *F.culmorum* after treatment with α-chymotrypsin

Ratio peptide/chymotrypsin	**O3TR**	**C12O3TR**
0 = digestion buffer	>50	6.25
60/1	>50	>50
250/1	>50	12.5
2500/1	>50	6.25

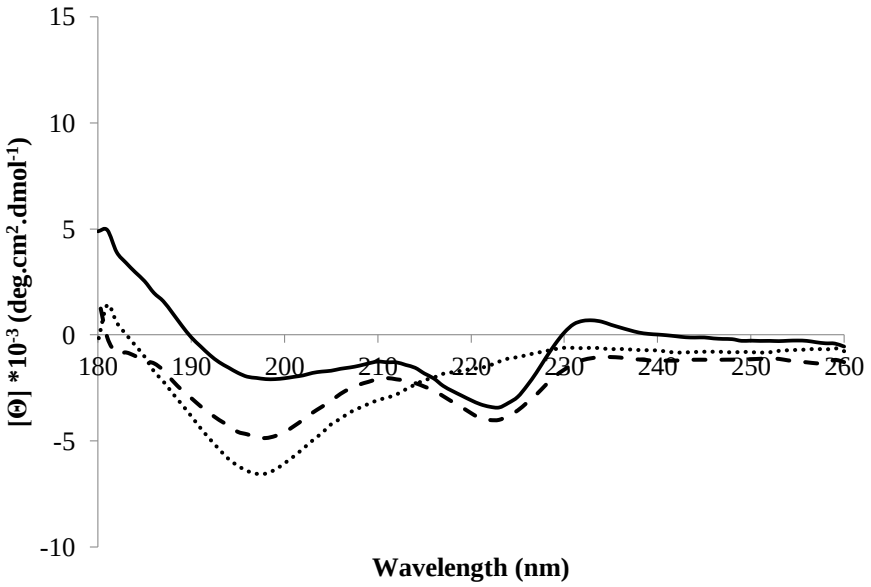
Secondary configurations of O3TR-NH2 and C12-O3TR-NH2

The secondary structures of O3TR-NH2 and C12-O3TR-NH2 were analysed by CD spectroscopy in DIW, 50%TFE and 20 mM SDS micelles (Fig. 5.3). In aqueous solution, O3TR profile showed a negative peak at ~200 nm, which indicates a mostly unstructured configuration or transient B-turn or polyproline II (PPII) motif (Rozek et al. 2003). In presence of TFE, known to stabilize confiugrations, the reduction of the peak intensity suggested a reduction of unordered conformation. In addition, a second negative peak appeared at ~222 nm, possibly due to the tryptophan residues (Rozek et al. 2003). In presence of SDS, used to mimick a membrane-environment, the conformation was more ordered with a potential increase of α-helix content, as suggested by the second negative peak and the presence of positive peak. A small peak at ~230 nm could correlate with PPII structural features.

The presence of the positive peak below 200 nm and a reduced negative peak at ~220 nm in CD spectra of C12O3TR in DIW and TFE suggested a more ordered conformation than O3TR. In SDS, the negative peak at ~200 nm moved to ~205 nm, and the second negative peak moved to ~225 nm separated by a maximum at ~218 nm. This change suggested a more structured conformation. In addition, a strong positive peak appeared at ~190 nm with a crossover at 200 nm, indicating a mix of α-helical structures and random coil. The presence of Trp residues could explain the positive peak at ~235 nm in both peptides (Crabtree effect).

C12O3TR conformation comprised α-helix and β-turn structural elements. The fatty acid conjugation has been reported to increase the α-helicity of peptides which then display higher antimicrobial activity (Chu-Kung et al. 2004). Indeed, this conformation enhances the amphipathicity of the peptide improving its antimicrobial action, enabling a better interaction and penetration into the membrane (Chu-Kung et al. 2004). However, Yadav et al. showed that a linoleic acid derivative of OOWW-NH_2, did not show α-helical or β-sheet configuration (Yadav et al. 2013).

A)



B)

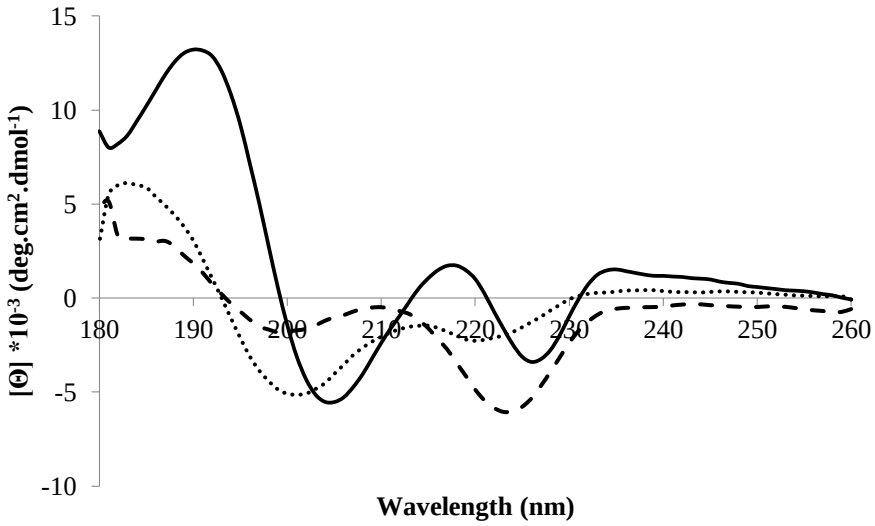


Figure 5.3. CD spectra of O3TR (**A**) and C12O3TR (**B**) in DIW (⋯), 50% TFE (━ -) or 20 mM SDS (▬)

Permeabilization of fungal membrane

In order to characterize the mode of action of the peptides O3TR and C12O3TR, a SG assay was performed on *F. culmorum* hyphae (Fig. 5.4 and 5.5) and *Z. bailii* (Fig. 5.6).

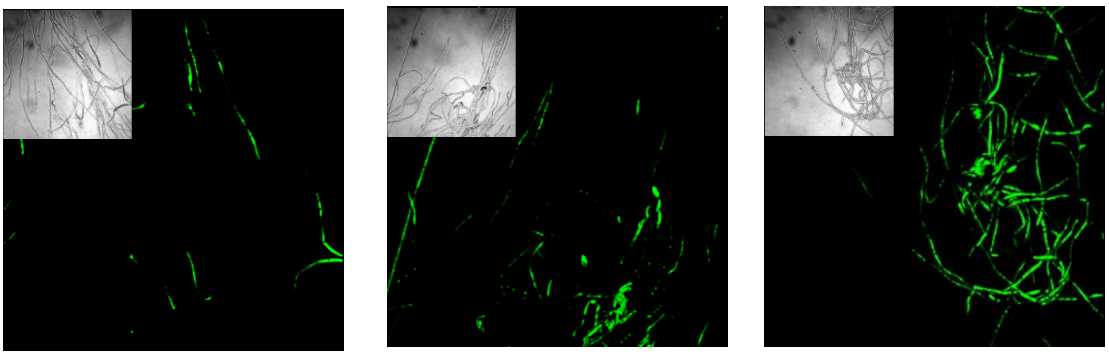
After 1 h of incubation, C12O3TR started to induce membrane permabilization at 6.25 µg/ml. At higher concentration, the permeabilization was more important (Fig. 5.4A). O3TR only induced permeabilization of fungal membrane at high concentrations (100 µg/ml) after 1 h of incubation (Fig. 5.5A). After 2 h of incubation, both peptides induced membrane permeabilization at concentrations that induce hyphal death (Fig. 5.4B and 5.5B).

The kinetics of membrane permeabilization of *Z. bailii* was determined by fluospectrophotometry for 4 h (Fig. 5.6). The yeast solutions treated with O3TR did not show fluorescence for 4 h even at peptide concentration of 100 µg/ml, much higher than its MIC against *Z. bailii*. On the contrary, fluorescence was rapidly detected in solutions treated with C12O3TR indicating a rapid permeabilization of yeast membrane. The permeabilization appeared to be correlated to the peptide concentration. In addition, a plateau was reached after almost 3 h of incubation, which suggests a potential saturation in membrane permeabilization.

In addition to the inhibition of the conidial germination, O3TR and C12O3TR killed fungal hyphae of *F. culmorum*, involving permeabilization of the fungal membrane. Membrane permeabilization has been identified for many short synthetic peptides (Matsuzaki et al. 1997). Interestingly, despite its small size (compared to the 7-10 nm of cell membrane), O3TR permeabilized the membrane so is thought to create at least transient pores. Self-assembly has been observed for antimicrobial peptides, increasing the size of the peptide and facilitating the pore creation (Bechinger & Lohner 2006).

However, no fluorescence was detected in SG assay performed on yeast cells with O3TR. Yeast resistance to the antifungal protein AFP has been attributed to the activation of several signalling pathways (Ouedraogo et al. 2011). On the contrary, C12O3TR induced a rapid permeabilization of the yeast membrane. The two peptides appeared to use different mechanisms to inhibit yeast and this may explain the higher activity of C12O3TR. The addition of fatty acid chain tail increases the ability of the peptide to induce membrane permeability, generally correlated to antimicrobial activity of the peptide. However, an excessive hydrophobicity can drastically reduce the activity if the peptide cannot penetrate the outer membrane of the microbial target (Makovitzki et al. 2006). The formation of aggregates at the surface of the membrane may result in a too large compound for membrane penetration or change the mode of action of the peptide (Han & Lee 2015).

A.



B.

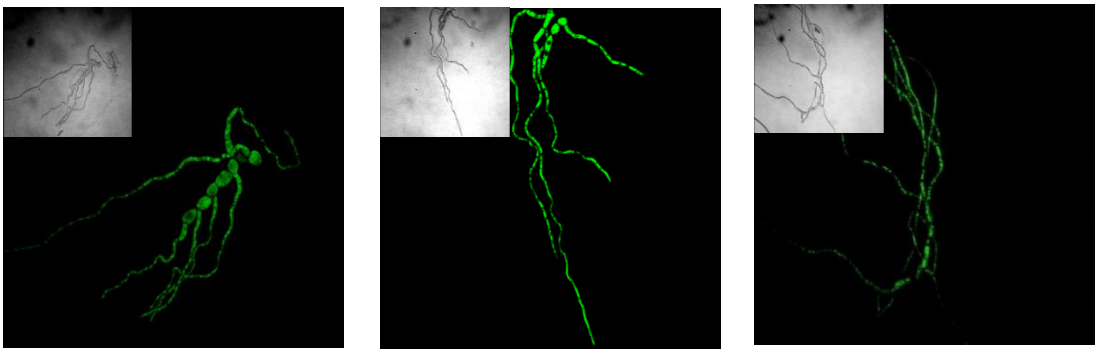
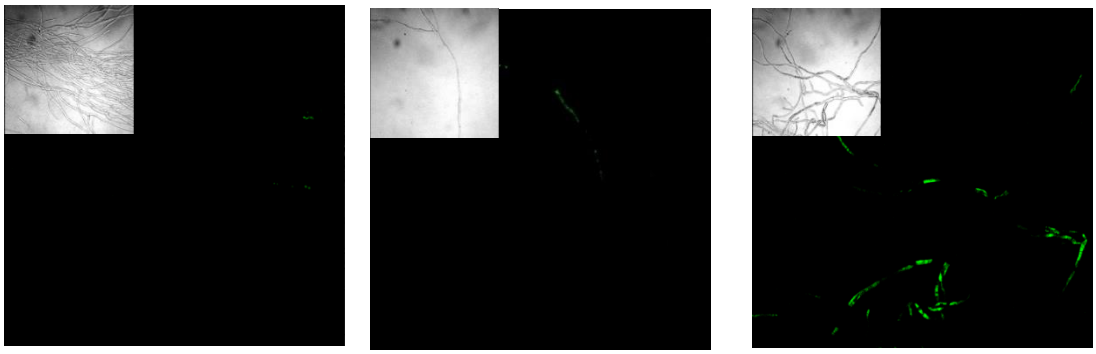


Figure 5.4. Sytox Green uptake in *F. culmorum* hyphae after incubation with C12O3TR for 1 h (**Fig. 5.4A**) or 2 h (**Fig. 5.4B**), at concentrations of 6.25 (left), 12.5 (middle) or 25 µg/ml (right).

A.



B.

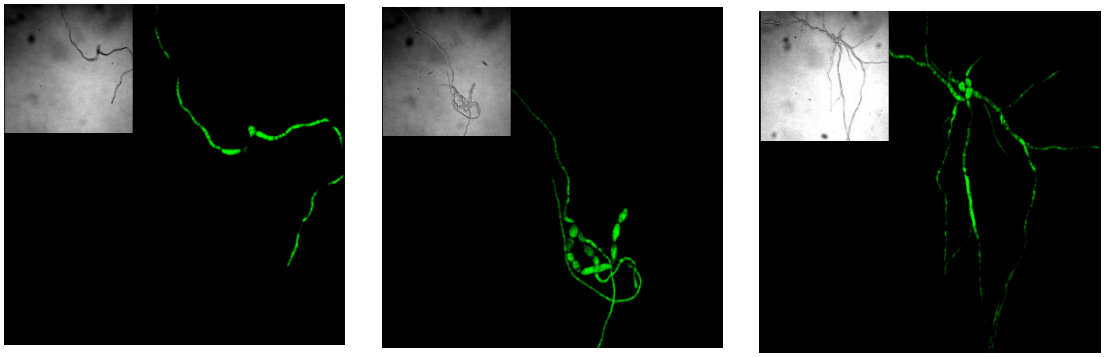


Figure 5.5. Sytox Green uptake in *F. culmorum* hyphae after incubation with O3TR for 1 h (**Fig. 5.5A**) or 2 h (**Fig. 5.5B**), at concentrations of 25 (left), 50 (middle) or 100 µg/ml (right).

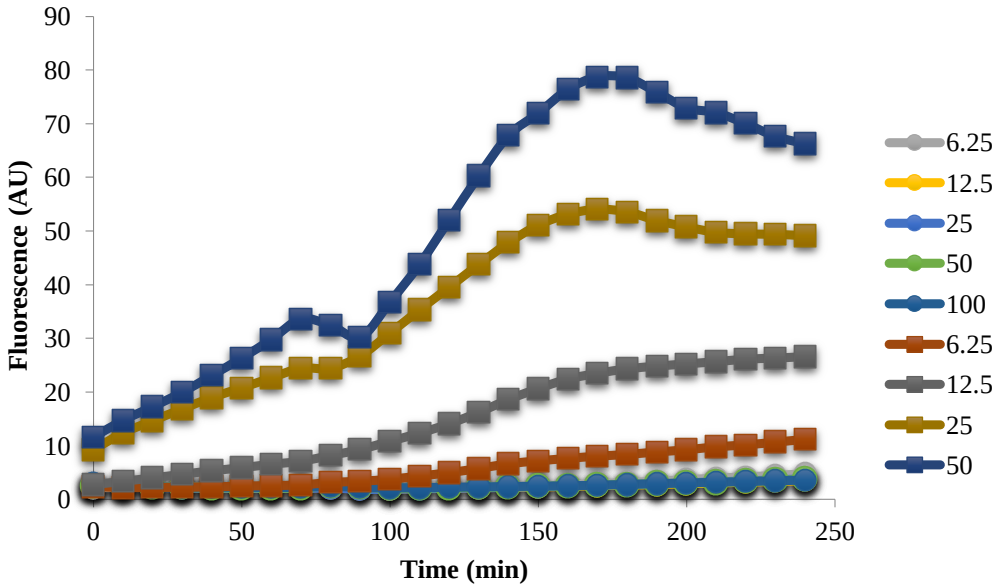


Figure 5.6. Kinetics of membrane permeabilization of *Z. bailii*, in presence of C12O3TR (squares) or O3TR (circles). Peptide concentrations (µg/ml) are indicated in the legend.

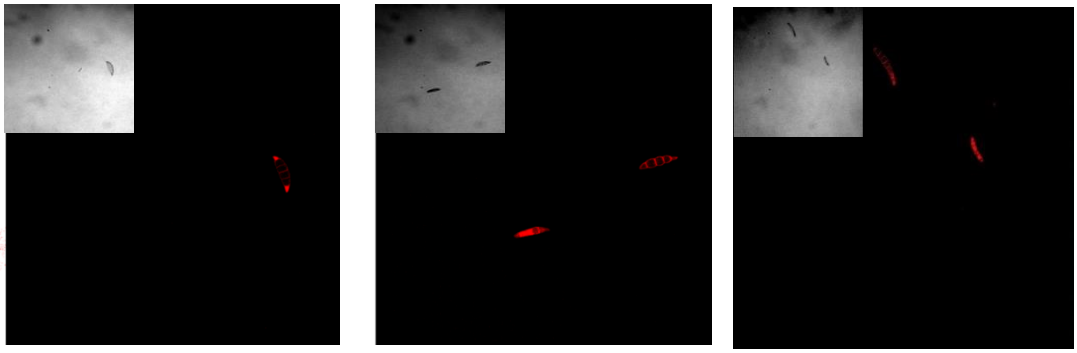
Internalization assay

Apart from the modification of fungal membrane permeability, the presence of intracellular targets has been highlighted for several AMPs, as well as the mechanisms of entry (El-Mounadi et al. 2016). To highlight their internalization into *F. culmorum* conidia, the peptides were labeled with TMR at the N-terminus. The labeled peptides inhibed *F. culmorum* germination with a MIC of 12.5 µg/ml for both peptides.

Within 10 min of treatment, the labelled peptides, highlighted by the red dye TMR, had already bound to the conidial surface and slowly entered the fungal cells (**Fig. 5.7** and **5.8**). After 30 min of incubation, TMR-C12O3TR completely entered some of the fungus cell whereas TMR-O3TR remained mostly at the conidial surface After 1 h of treatment, with a concentration of 6.25 µg/ml, the internalization of TMR-O3TR was reduced, compared to TMR-C12O3TR. At a concentration of 12.5 µg/ml, TMR-O3TR and TMR-C12-O3TR were internalized in all the *F. culmorum* conidia.

It is noteworthy that the entry of the labelled peptide appeared at concentrations below the ones required to inhibit germination. This suggests that a minimum concentration is required to inhibit specific pathways causing germination. TMR-C12O3TR entered into the fungus cell faster than TMR-O3TR, which can also explain its higher activity. The internalization of the peptides was not affected by a cold pre-treatment, which suggests an energy-independent mechanism. Energy-dependent mechanisms such as endocytosis should be inhibited following these treatments (El-Mounadi et al. 2016). Instead of creating membrane permeabilization, AMPs can interact with intracellular targets (Adem Bahar & Ren 2013). Furthermore, maintenance of the antifungal activity after β-glucanase treatment indicates that the peptides do not require a cell-wall receptor (Van Der Weerden et al. 2010).

A.



B.

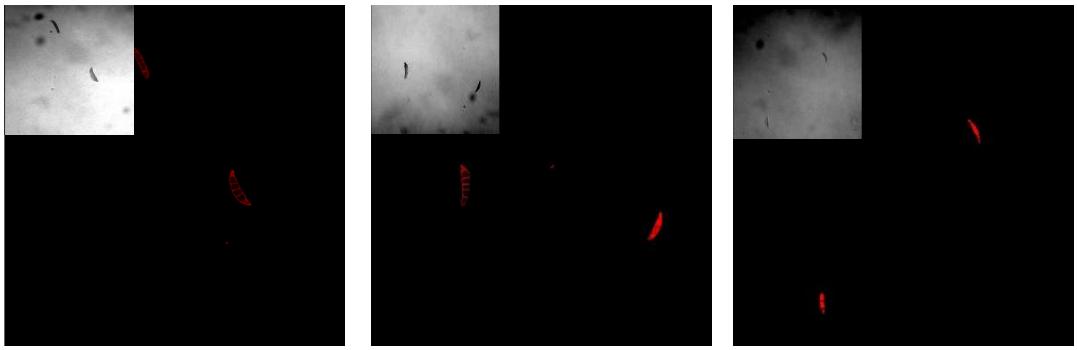


Figure 5.7. CLSM pictures of labelled peptides TMR-C12O3TR at concentrations of 6.25 (**Fig. 5.7A**) and 12.5 µg/ml (**Fig. 5.7B**). Pictures were taken at different incubation times: 10 min (left), 30 min (middle) and 60 min (right).

A.



B.

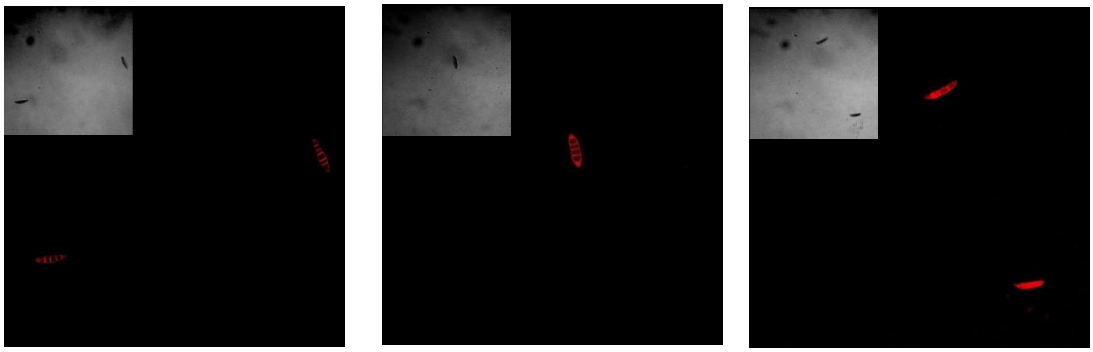


Figure 5.8. CLSM pictures of labelled peptides TMR-O3TR at concentrations of 6.25 (**Fig. 5.8A**) and 12.5 µg/ml (**Fig. 5.8B**). Pictures were taken at different incubation times: 10 min (left), 30 min (middle) and 60 min (right).

Haemolytic and cytotoxic activities

In addition to important costs of synthesis, the limited use of AMPs as novel pharmaceuticals is due to their resistance and toxicity to human cells. O3TR and C12O3TR were tested against fresh sheep RBCs and Caco-2 human cell line. In presence of O3TR, RBCs and Caco-2 cells remained alive at peptide concentrations up to 200 µg/ml (**Fig. 5.9** and **5.10**).

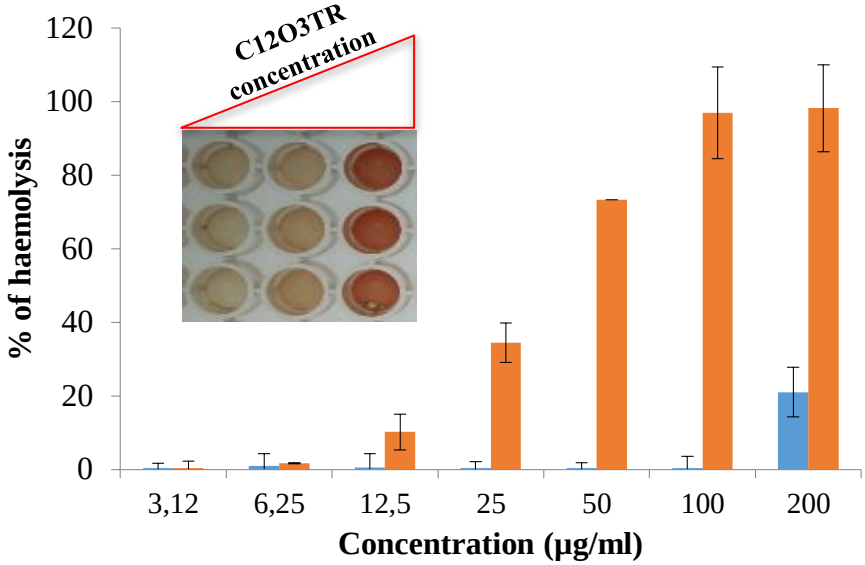


Figure 5.9. Haemolytic activity of O3TR (blue) and C12O3TR (orange) towards sheep RBCs. Error bars represent the standard deviation between six replicates. Insert: wells showing the haemoglobin release from sheep RBCs upon treatment with increased concentrations of C12O3TR

On the contrary, a C12O3TR concentration of 50 µg/ml induced total RBCs lysis and significant cytotoxic activity towards Caco-2 cells (**Figs. 5.9** and **5.10**).

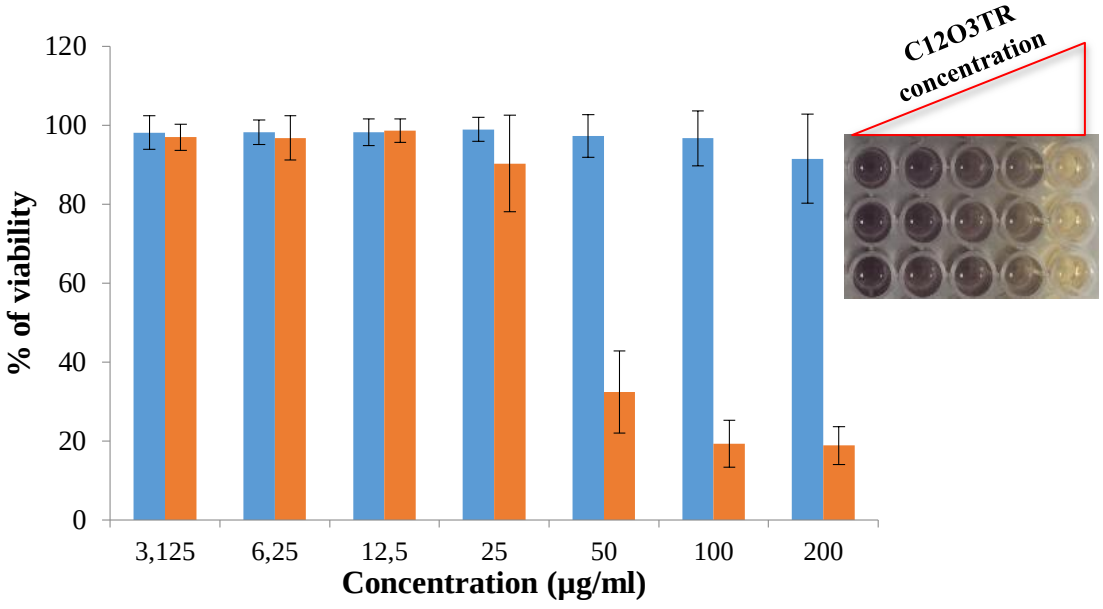


Figure 5.10. Percentage of viability of Caco-2 cells in presence of O3TR (blue) or C12O3TR (orange). The experiment was performed in 4 wells in 5 independent experiments. Insert: wells showing the viability of Caco-2 cells (conversion of MTT to purple formazan) upon treatment with increased concentrations of C12O3TR

C12O3TR lysed sheep RBC and induced Caco-2 cell death at low concentrations in contrast to O3TR. In agreement with these results, Laverty et al. (Laverty et al. 2010) showed that the acyl chain addition increases the cytotoxicity of the peptides towards keratinocytes and horse RBC. The presence of cholesterol or ergosterol in mammalian or fungal membranes, respectively, has been shown to reduce the potency of these peptides (Mason et al. 2007) in comparison to antimicrobial activity. Indeed, sterols stabilize the interaction between the phospholipids of the bilayer and prevents degradation. Structural differences (*e.g.*, fused rings) in yeast ergosterol, fungal ergosterol or cholesterol, are involved in the lipid acyl chain order and consequently in their sensitivity to AMPs (Shrivastava & Chattopadhyay 2007). Despite their cholesterol-rich zwitterionic membranes, mammalian cells were sensitive to C12O3TR. Lipopeptides are non-selective so an increase of their hydrophobicity and helical structural features increase their cytotoxicity towards human cells (Makovitzki et al. 2006). It is thought that peptides with suitable level of hydrophobicity can penetrate deeper in the bilayer and are able to induce membrane lysis (El-Mounadi et al. 2016).

Although desired in certain treatments, such as cancer, this toxicity is usually a limiting factor in the use of cAMPs (Haug et al. 2016). To reduce the toxicity of C12-OOWW-NH_2, modifications can increase the specificity of the peptide. Thus, the addition of specific receptor (Yang et al. 2015) or a pH-dependent conjugate (Burns et al. 2016) have been proven to be efficient.

Application as beverage preservatives

Despite hostile environment for most microorganisms, beverages can be suitable for some yeast and mould growth, leading to undesirable flavour and taste (Wareing & Davenport 2007). In addition, yeast species such as *Z. bailii* shows resistance to common weak-acid preservatives used in soft drinks. To avoid this resistance, the multiple mode of action of AMPs is a promising way. To date, only a few antimicrobial peptides are allowed for food applications, but their use is limited, due to a narrow spectrum (Rydlo et al., 2006).

To foresee potential applications, the two peptides were added to different commercial beverages in a challenge test against yeast and fungi contaminants. After 7 days of

incubation, O3TR at concentrations of 100 and 25 µg/ml inhibited yeast growth in the commercial lager and the carbonated soft drink, respectively. The peptide was not efficient in preventing yeast growth in apple or orange juice and slightly reduced *A. niger* growth (Tables 5.4 and 5.5).

After 7 days, C12O3TR inhibited the growth of yeast and *A. niger* in all the drinks, except in orange juice, where *A. niger* was not affected (Table 5.5). The inhibition of yeast growth in orange juice required higher concentration of C12-O3TR (100 µg/ml) (Table 5.4).

Table 5.4. Yeast growth in commercial beverages in presence of O3TR and C12O3TR

	Day 2				Day 7			
	Commercial lager	Carbonated soft drink	Apple juice	Orange juice	Commercial lager	Carbonated soft drink	Apple juice	Orange juice
Control	+	-	++	++	++	+	++	++
O3TR (µg/ml)								
25	+	-	++	++	++	-	++	++
50	-	-	++	++	++	-	++	++
100	-	-	++	++	-	-	++	++
200	-	-	+	++	-	-	++	++
C12O3TR (µg/ml)								
25	-	-	-	+	-	-	-	++
50	-	-	-	+	-	-	-	++
100	-	-	-	-	-	-	-	-
200	-	-	-	-	-	-	-	-

-: no growth (OD_{595}<0.2; +: moderate growth (OD_{595}: 0.2-0.6); ++: strong growth (OD_{595} > 0.6)

O3TR was able to inhibit yeast growth in beer and carbonated drink. However, high concentrations were required. Interactions between antimicrobials and food components, including sugars, have been highlighted, resulting in a limited activity. To avoid these interactions, novel formulations such as encapsulation are currently used for nisin (Carmona-Ribeiro & Carrasco 2014). C12O3TR was efficient in preventing or delaying fungal growth in all the beverages, even at concentrations that did not induce cytotoxicity. Other rationally designed peptides have been proven to delay fungal growth on food products (López-Garcia et al. 2000).

Table 5.5. *A. niger* growth in commercial beverages in presence of O3TR and C12O3TR

	Day 2			Day 7		
	Commercial lager	Apple juice	Orange juice	Commercial lager	Apple juice	Orange juice
Control	+	++	++	++	++	++
O3TR (µg/ml)						
25	+	+	++	++	++	++
50	+	+	++	++	++	++
100	+	+	+	++	++	++
200	+	+	+	++	++	++
C12O3TR (µg/ml)						
25	-	-	-	-	++	++
50	-	-	-	-	-	++
100	-	-	-	-	-	++
200	-	-	-	-	-	++

-: no growth (OD_{595}<0.2; +: moderate growth (OD_{595}: 0.2-0.6); ++: strong growth (OD_{595} > 0.6)

5.5 Conclusion

Antimicrobial peptides represent promising alternatives to common antibiotic and food preservatives. The ease and the reduction of costs for the synthesis of ultrashort peptides make them ideal candidates. The addition of a C12 fatty-acid chain tail at the N-terminus of the tetrapeptide amine H-Orn-Orn-Trp-Trp-NH_2 improved its antifungal activity. Structural differences between the two peptides and in their mode of action were correlated to their antifungal potency. The high hydrophobicity of the lipopeptide was suggested to play a role in these differences as well as in its cytotoxicity and haemolytic activity. By optimizing their properties, these peptides could represent a novel preservative against beverage contaminations. Although the use of novel additives requires food regulatory approval, this study shows the feasibility of using an ultrashort peptide to protect beverages and more generally food products from fungal spoilage, especially against preservative-resistant species.

5.6 Acknowledgements

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Chapter 6
Antifungal activity of a *de novo* synthetic peptide and derivatives against fungal food contaminants

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Abstract

The development of novel solutions to fight microbial food contaminants rests upon two pillars, which are the development of resistant strains and consumers' desire for a reduced consumption of synthetic drugs. Natural antimicrobial peptides possess the qualities to overcome these issues. *De novo* synthesis of novel antifungal compounds is a major progress that has been facilitated by the identification of parameters involved in the antimicrobial activity. A 14-residue peptide named KK14, with the sequence KKFFRAWWAPRFLK-NH_2, was designed and inhibited conidial germination and fungal growth of food contaminants within the range 6.25-50 µg/ml and 6.25-100 µg/ml, respectively. The study of three analogues of the peptide highlighted the role of some residues in the structural conformation of the peptide and its antifungal activity. The substitution of a Pro residue with Arg increased the helical content of the peptide and its antifungal activity but also its cytotoxicity. The insertion of an unnatural bulky residue β-diphenylalanine or a full D-enantiomerization overall increased the antifungal potency. The four peptides showed similar behaviour towards salt increase, heat treatment and pH decrease. Interestingly, the D-enantiomer remained the most active at high pH and after proteolytic digestion. The four peptides did not present haemolytic activity up to 200 µg/ml but had different behaviours of cytotoxicity against colonic cells and macrophages. These differences could be crucial for potential application as pharmaceutical or food preservatives.

6.1 Introduction

Although less commonly associated with grave symptoms in humans than bacteria or viruses, fungi are frequently involved in severe infections (Perlroth et al. 2007, Spampinato & Leonardi 2013, Vandeputte et al. 2012). In humans, fungal infections which generally involve *Candida*, *Aspergillus* and *Cryptococcus* species are rarer than infections from bacteria (Matejuk et al. 2011, Spampinato & Leonardi 2013). However, in case of disseminated infections, they can reveal life threatening. Food-related fungi are not lethal but detrimental effects have been observed in immuno-suppressed people. Fungal infections of the eye are also classified as mucosal fungal infections, but are caused more frequently by *Fusarium* or *Aspergillus* species rather than *Candida* species (Vandeputte et al. 2012). In addition, a prolonged exposure to mycotoxin-producing fungi, such as *Aspergillus niger*, may lead to the development of several diseases, such as gastrointestinal disorders, to neurological issues or cancers (Miedaner et al. 2017). When submitted to inefficient or inappropriate treatments, the fungal threat remains and represents a health hazard and may cause major food spoilage. Either in a therapeutic setting or as a food preservative, there is a need for novel antifungal compounds.

Cationic antimicrobial peptides (cAMPs) are members of the innate immune system and act as primary agents against infective pathogens. These peptides play an important role in novel treatments and many are currently under clinical trials (Da Silva & MacHado 2012, Desbois et al. 2011, Haney & Hancock 2014) or used in food products (Losso 2008). However, few of the latter target filamentous fungi. Plants and insects are particularly powerful producers of antifungal peptides effective against filamentous fungi (Lacerda et al. 2014, Van Der Weerden et al. 2008). Natural cAMPs possess three main properties to fulfil the need of novel antifungal drugs: a generally broad spectrum of antimicrobial targets; a mechanism of activity that, for many, does not rely on a specific compound or at least an essential compound for the survival of the microorganism; a diversity of activity that includes immunomodulation and wound healing properties. However, the use of natural AFPs faces several issues, such as poor stability, potential toxicity and a time-consuming extraction. To overcome these problems, synthetic peptides with a broad antimicrobial activity have been developed. However, the high cost of production of long peptides reduces the potential

applications. Thus, d*e novo* peptides have been generated based on previous work on the key effectors of peptide efficacy, such as cationicity and hydrophobicity, carried by Hodges and coworkers (Chen et al. 2007; Kondejewski et al. 1996, 1999). To a lesser extent, other parameters such as flexibility, with a non-defined but inducible structure in hydrophobic environment, or amphipathicity play a role in the antimicrobial and cytotoxic activities (Jiang et al. 2009).

Peptide production is achieved via recombinant and chemical synthesis, both methods presenting advantages and limitations. Recombinant production peptides are usually cheaper with an useful material for large peptides and are expected to play a major role in novel drug development. However, post-translational modification (PTMs), generally essential for the activity of the peptide, require a more complex eukaryotic system. In addition, recombinant methods are more demanding in terms of process and personnel (Lax 2010, Vale et al. 2014) than chemical synthesis. A major hurdle of the current most popular method of solid-phase synthesis, based on fluorenylmethoxycarbonyl (Fmoc) chemistry, is the cost of synthesis. However, this cost has decreased significantly and Fmoc synthesis appears easier to apply at large scales. It also facilitates the insertion of unnatural peptides, commonly recognised as necessary for the design of drugs.

Based on these parameters and on the comparison of several peptides sequences and, we designed novel peptides sequences (see Appendix). Among the numerous sequences used as frameworks, indolicidin is a potent 13-mer Trp-rich peptide with an hydrophobic core, ILPWKWPWWPWRR (Falla & Hancock 1997, Hilpert et al. 2006); LTX-315 (KKWWKKDipK) is an anticancer 9-mer cationic peptide comprising an unnatural β-β diphenylalanine residue (Haug et al. 2016); V13KL (Chen et al. 2007) is a 26-residue amphipathic α-helical peptide, studied to highlight the role of hydrophobic residues in the activity of helical peptides. The insertion of Lys and Arg residues was meant to increase the cationicity while the choice between Lys and Arg was due to a will to improve reduce haemolytic activity. A similar rationale was used to select the hydrophobic residues Trp and Phe. Due to its helix propensity, Ala residue was used whereas the presence of Pro and its associated helix-breaking properties aimed at reducing the toxicity. Ultimately, after comparison of predicted antimicrobial activity (accessible online http://www.camp.bicnirrh.res.in/prediction.php), we decided to work with a sequence

of a 14-residue novel peptide, KKFFRAWWAPRFLK-NH_2, named KK14. In the present study, the influence of specific residues and conformation on the antifungal activity of the peptide was studied with the generation of 3 analogues. The peptide presented the following characteristics: presence of cationic residues with an hydrophobic centre; a 10-20 residue length to avoid dimerization but to give a defined structure and limit the cost of synthesis. The peptide was amidated at the C-terminus to increase the net charge of the peptide. The choice between Lys/Arg and Trp/Phe was made to limit haemolytic activity while maintaining antimicrobial potency (Subbalakshmi et al. 1998, Yang et al. 2003). The positions of the polar and apolar residues were also chosen to limit the haemolysis of the peptides. Indeed, Jiang et al. showed that disruption of a perfect α-helix by the substitution of Val to Lys on the non-polar face reduced toxicity while maintaining the antimicrobial potency (Jiang et al. 2009). The modifications of the initial peptide KK14 included: a substitution with the unnatural residue β, β-diphenylalanine (Dip) (KK14(Dip)), in order to improve salt stability and activity; D-enantiomerization of KK14 (kk14), known to give protease resistance; the substitution of Pro_{10} with Arg_{10} (KK14(R)), to increase the net charge of the peptide. The effect of sequence modifications on the antifungal potency, mechanisms of antimicrobial action, the structure and the toxicity of the peptides were studied. The rational design and the correlation with predictors is an effective way to create powerful antifungal agents and to improve the understanding of the mechanisms involved in antifungal activity. The generation of this novel antifungal peptide and derivatives could aid in the cost-effective development of novel, efficient and safe antifungal agents.

6.2 Materials and Methods

Peptides

Four newly designed peptides (purity > 90%, as specified by manufacturer) were synthesized by Fmoc solid-phase synthesis on resin (GLBiochem, Shanghai, China). The characteristics of the peptides, determined via the online tool (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py), are indicated in Table 6.1. The antimicrobial nature of the peptides was predicted with online tools available on the different databases. The analogues of the initial peptide KK14 (KKFFRAWWAPRFLK-NH_2) included a peptide where Trp in position 8 was replaced by an unnatural bulky residue β,β- diphenylalanine (Dip) (KK14(Dip)), a D-enantiomer of KK14 (kk14) and KK14(R) where Pro_{10} substituted with Arg_{10}. The peptides were resuspended in sterile deionized water at an initial concentration of 2 mg/ml.

Table 6.1. Predicted characteristics of the synthetic peptides

Peptide	Sequence	Net charge[a]	Hydrophobicity[a]	Hydrophobic moment[a]	AMP activity[b]	pI[c]	MW (Da)[d]	Purity (%)
KK14	KKFFRAWWAPRFLK-NH_2	+6	0.566	0.175	0,999	14	1880.3	92.2
KK14(Dip)	KKFFRAW**Dip**APRFLK-NH_2	+6	ND	ND	ND	ND	1880.3	95.2
kk14	kkffrawwaprflk-NH_2	+6	ND	ND	ND	ND	1917.4	95.5
KK14(R)	KKFFRAWWA**R**RFLK-NH_2	+7	0.442	0.174	0,999	14	1868.3	94.5

[a]: The different parameters were determined with the online tool http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py (using the non amidated peptide)

[b]: The AMP potential of the peptides was predicted with the SVM algorithm (available at http://www.camp.bicnirrh.res.in/predict_c/)

[c]: The isoelectric point was calculated with the online tool PepCal.com

[d]: Molecular weight (MW) was provided by the supplier GLBiochem

ND: not able to be determined with the online tools

Antifungal assays

Fusarium culmorum FST 4.05, *Aspergillus niger* FST 4.21 and *Penicillium expansum* FST 4.22 were chosen as representatives of filamentous fungal contaminants of ingredients and food. Fungal strains were originated from University College Cork (UCC, Cork, Ireland) collection. The inhibition of conidial germination was determined as described previously (Thery et al. 2018). Fungal spores (final concentration, $1x10^4$ spores/ml) were inoculated in 90 µl of synthetic fungal media (SFM: 50 µM $MgSO_4$/7H2O, 5 µM $FeSO_4$/7 H_2O, 0.1 µM $CoCl_2$, 0.1 µM $CuSO_4/5H_2O$, 2 µM $Na_2MoO_4/2H_2O$, 0.5 µM H_3BO_3, 0.1 µM KI, 0.5 µM $ZnSO_4/7H_2O$, 0.1 µM $MnSO_4/1H_2O$, glucose, asparagine, 20 mg of methionine, 2 mg of myo-inositol, 0.2 mg of biotin, 1 mg of thiamine-HCl, 0.2 mg of pyridoxine-HCl, 0.5 mM $K_2HPO_4/3$ H_2O), as described previously (Thevissen et al. 1999). Peptide solution (10 µl, 0-100 µg/ml) was added to the wells and OD_{595} of the solution was monitored for 120 h at 28°C using a Varioskan microplate reader (ThermoFischer Scientific).

The inhibition of fungal growth was performed on germinated conidia as described by Thevissen et al. (Thevissen et al. 1999). Fungal spores were incubated for 24 h in half-strength potato dextrose broth (½ PDB, Sigma-Aldrich) at 28°C. Following incubation, hyphae were washed twice with SFM. Aliquots of hyphal suspension (90 µl) were mixed with 10 µl peptides (0-100 µg/ml) and growth was monitored as described above. For each experiment, the minimum inhibitory concentration (MIC) was determined as the minimum concentration that entirely inhibited conidial germination or mycelium growth in 3 replicates.

Peptides stability

The stability of KK14 and analogues was determined upon heat treatment, at low and high pH solutions and in different salt solutions. The four peptides (0-50 µg/ml) were heated at 100°C for 15 min using a heating block and left for cooling at room temperature for 30 min. Following cooling, the antifungal activity of the peptides was determined against *F. culmorum* conidia as described above. The pH stability of the antifungal activity of the peptides was tested in SFM at pH 3.5 and pH 9.5. The conidial germination in presence or absence of peptides (0-25 µg/ml) was monitored by

absorbance as described above. Finally, the antifungal activity of the peptides was also determined in salt solutions with monovalent (50 mM and 150 mM of KCl) and divalent (1 mM and 5 mM $CaCl_2$) cations as described by Terras et al. (Terras et al. 1992).

Effect of trypsin on antifungal activity

The protease effect on the antifungal activity was performed as described by Rozek et al. (Rozek et al. 2003). Due to its presence in the gastrointestinal tract, trypsin was chosen to mimic the fate of the peptides when used as food preservatives. KK14 and analogues were incubated at 37°C for 2 h in the presence of trypsin (Sigma-Aldrich) at a peptide-to-trypsin ratio of 256:1 in 100 mM Tris buffer, pH 7.3. After treatment, trypsin was heat-inactivated at 80°C for 10 min. The remaining antifungal activity was performed against *F. culmorum* conidia (10^4 conidia/ml) as described above.

Membrane permeabilization

F. culmorum conidia ($5x10^4$ spores/ml) were cultured for 24 h in ½ PDB at 28°C. Fresh *F. culmorum* mycelia was collected by centrifugation (8,000 *g* / 10 min), washed twice with SFM and resuspended in 100 µl of SFM. Mycelium was then treated with different concentrations of peptides (0, 6.25, 12.5 and 25 µg/ml) for 2 h. Following incubation, mycelium was resuspended in 100 µl of SFM and incubated in 2 µM of propidium iodide (PI, Sigma-Aldrich) for 10 min, and rinsed with distilled water 3 times. The mycelium was visualized by fluorescence microscopy (Olympus FluoView™ FV1000) (excitation 535 nm/emission 615 nm).

Circular dichroism (CD) spectroscopy

The secondary structures of KK14 and analogues were analyzed by circular dichroism (CD) spectroscopy. The analysis of the peptides secondary structures was conducted as described by Liu et al. (Liu et al. 2008). CD spectroscopy was performed by using a Chirascan™ spectropolarimeter (Applied Photophysics) with the peptides dissolved in deionized water or 20 mM sodium dodecyl sulphate (SDS) micelles using a quartz

cell of 1 mm path length. CD measurements were performed at 27°C within a wavelength range of 190-260 nm. Samples and solvents were scanned 3 times and baseline was corrected by subtracting corresponding solvent spectrum. Ellipticities were converted to mean residue molar ellipticities [θ] in units of deg.cm^2/dmol and percentage of secondary structures obtained with deconvolution program CD spectroscopy software. A 3D model of KK14 and KK14(R) was generated via PEP-FOLD3 online tool (Lamiable et al. 2016). In date of use, PEP-FOLD 3 did not process peptides with D-amino-acids, or unnatural L-amino acids, such as Dip. The structure of the four peptides was also predicted via *in silico* prediction using the online tools PEPstrMOD accessible online (Harpreet Kaur et al. 2007, Singh et al. 2015)

Haemolytic activity assay

Fresh sheep erythrocytes (Oxoid, ThermoScientific) were centrifuged at 1,620 *g* and pellet was resuspended in an equal volume of 1 x PBS. Washing steps were repeated 3 times and the final pellet was resuspended at 4% (v/v) in PBS. Erythrocytes solution (180 µl) were added with 20 µl of peptides (0-200 µg/ml), diluted in PBS. The solutions were then incubated for 1 h at ººC. Supernatant (100 µl) was collected after centrifugation (1,000 *g* / 2 min) and absorbance was measured at 405 nm (Varioskan, ThermoFischer Scientific). PBS and 0.1% Triton X-100 were used to induce 0 and 100% haemolysis, respectively. The percentage of haemolysis was calculated as follows:

$$\% \text{ Haemolysis} = \frac{(Abs405\ peptide\ treatment)-(Abs405\ PBS)}{(Abs405\ 0.1\%\ Triton\ X-100)-(Abs\ 405\ PBS)} \text{ x } 100$$

Cytotoxicity assays towards Caco-2 and mouse RAW 264.7 cell line

The cytotoxicity of KK14 and analoguewas performed against Caco-2 human colonic cell line (1x10^5 cells/ml) (ECACC), using the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (Thery et al. 2018). Briefly, Caco-2 cells were cultured in a 96-well microtiter plate in Dulbecco's modified Eagle's medium (DMEM), supplemented with non-essential amino acids (NEAA, 1%) and FBS (10%), at 37°C in 5% CO_2. A similar procedure was used to test the cytotoxicity of peptides towards mouse macrophages RAW 264.7 cell line

($1x10^5$ cells/ml). Cells were allowed to adhere and 200 µl of fresh medium (with 2.5% FBS) supplemented with peptides (0-200 µg/ml) was added to the wells and incubated for 24 h at 37°C. The MTT reagent (Cell proliferation Kit I MTT; Sigma, Ireland) was added for 4 h, followed by an overnight incubation with lysis solution. Cell viability was measured spectrophotometrically at 570 nm with a background reading at 690 nm.

Applications in beverage systems

The potential application of the peptides was tested in several beverages upon challenges with fungal spores, as described previously (Thery et al. 2018). Acid foods such as apple juice have been associated with foodborne illness outbreaks (Rydlo et al. 2006). *A. niger* has been reported as a fungal contaminant of soft drinks, including apple juice, in poor hygiene conditions (Wareing & Davenport 2007). Filter-sterilized apple juice (for 100 mL, < 0.5 g fat, 11 g carbohydrates, <0.5 g proteins, <0.01 g salt, pH=3.7) was inoculated with spores of *A. niger* (10^5 spores/ml). Beverage solutions (90 µl) were transferred in the wells of a 96-well microtiter plate and peptides were added at a concentration range of 0-200 µg/ml (final concentration). After 24 h of incubation at 28°C, beverage solution (50 µl) was drop-inoculated on a PDA plate and fungal growth monitored over 3 days at 28°C.

Statistical analysis

Antifungal and stability experiments were conducted in three technical replicates. Haemolysis and cytotoxicity assays were conducted in three technical replicates and repeated at three occasions. Values are reported as means ± standard deviations of three technical replicates for antifungal and stability assays. A two-tailed t-test was performed was conducted for each peptide to determine significant difference in cytotoxic assays. Significant difference was considered for *p*-values of <0.05.

6.3 Results

Antifungal activity

The reduction of food spoilage due to fungal contaminants is a health and economic necessity that requires novel approaches, such as the use of antimicrobial peptides. Based on the key determinants of the antifungal activity of antimicrobial peptides, a short AFP, named KK14, was constructed and tested against several food fungal contaminants. KK14 inhibited the germination of *F. culmorum*, *A. niger* and *P. expansum* conidia with a MIC of 6.25-50 µg/ml (Table 6.2). The substitution of Trp in position 8 with the bulky unnatural residue Dip did not significantly modify the activity of KK14 (MIC: 12.5-25 µg/ml). A similar effect was obtained with the enantiomerization of KK14. The replacement of Pro (in position 10) by a polar residue Arg increased the MIC by 2- to 4-fold against *P. expansum* and *A. niger* (Table 6.2). The potency of the peptides to inhibit hyphal growth was only important against *F. culmorum* with a MIC of 6.25-25 µg/ml. *A. niger* and *P. expansum* hyphae appeared only sensitive to high concentrations of kk14 and KK14(R).

Table 6.2. Antifungal activity of KK14 and analogues against fungal food contaminants. The IC_{50} is indicated into brackets if reached.

Peptide	**Minimal Inhibitory Concentration (µg/ml)**					
	Non germinated conidia			Germinated conidia		
	F. culmorum	*P. expansum*	*A. niger*	*F. culmorum*	*P. expansum*	*A. niger*
KK14	6.25	25	50 (25)	25 (12.5)	>100	>100
KK14(Dip)	12.5	25 (12.5)	25	25 (12.5)	>100	>100
kk14	6.25	25 (12.5)	25	6.25	>100 (100)	100 (50)
KK14(R)	6.25	12.5	12.5	25 (12.5)	>100 (50)	100 (50)

Peptides resistance

The stability of the peptides was studied in salt solutions, low and high pH solutions, after heat treatment and following trypsin digestion. The antifungal potency of the peptides was determined as the ability to inhibit the germination and growth of *F. culmorum*. The antifungal activity of the peptides was altered in the presence of 150 mM KCl, 1 mM and 5 mM $CaCl_{2,}$ with a MIC increase by 2- to 16-fold. However, in less concentrated solutions (50 mM KCl) the peptides showed the same activity against *Fusarium* conidia (Table 6.3) as in synthetic fungal media.

Table 6.3. Antifungal activity of KK14 and analogues for the inhibition of *F. culmorum* conidial germination after 120 h of incubation in different salt solutions.

Peptide	**Minimal Inhibitory Concentration (**µg/ml)			
	50 mM KCl	150 mM KCl	1 mM $CaCl_2$	5 mM $CaCl_2$
KK14	6.25	50 (25)	25 (12.5)	> 100 (25)
KK14(Dip)	12.5	25 (6.25)	25 (12.5)	100 (25)
kk14	6.25	25 (12.5)	12.5	100 (25)
KK14(R)	6.25	25 (6.25)	12.5	100 (25)

At pH 3.5, *F. culmorum* showed a similar sensitivity to the 4 peptides as at pH 6.5, despite a slight reduction of KK14 potency. On the contrary, the activity of the peptides was reduced at pH 9.5, with an increase of MIC by 2- to -4 fold. This trend was even clearer for KK14(Dip) which did not inhibit *Fusarium* germination up to 25 µg/ml (Table 6.4).

Table 6.4. Percentage of fungal growth inhibition at pH 3.5 and pH 9.5 in presence of the peptides. Results are expressed as means of three technical replicates ± standard deviation.

	Percentage of fungal growth inhibition					
	pH 3.5			*pH 9.5*		
Concentration (µg/ml)	6.25	12.5	25	6.25	12.5	25
KK14	**71.1** ± 14.3	**63.1** ± 4.6	**69.5** ± 10	**20.6** ± 23.7	**47.1** ± 20.8	**86.3** ± 1.2
KK14(Dip)	**84.2** ± 6.1	**98.1** ± 0.3	**97.3** ± 0.2	**-18.9** ± 11.7	**-6.81** ± 3.6	**-23.6** ± 5.3
kk14	**93.8** ± 4.6	**95.7** ± 5.2	**97.3** ± 0.4	**62.7** ± 10.7	**95.1** ± 1.7	**97.6** ± 0.7
KK14(R)	**81.6** ± 15.2	**97.8** ± 0.6	**98.7** ± 0.3	**25.9** ± 30.0	**63.4** ± 18.8	**80.7** ± 6.4

The peptides did not appear to be affected by heat treatment of aqueous peptide solutions, although the MIC of KK14 was slightly increased (Table 6.5). Finally, following trypsin digestion, only the D-enantiomer kk14 kept its antifungal activity after 1 h treatment. The other peptides did not inhibit conidial germination at a concentration up to 50 µg/ml. (Table 6.5).

Table 6.5. Antifungal activity of the peptides after heat treatment and trypsin treatment. The remaining antifungal activity is expressed as the percentage of inhibition of *F. culmorum* conidial germination, based on OD monitoring. Results are expressed as means of three technical replicates ± standard deviation.

	Percentage of fungal growth inhibition							
	Heat treatment				*Trypsin treatment*			
Compound	6.25	12.5	25	50	6.25	12.5	25	50
KK14	**15.7** ± 29.3	**77.4** ± 41.0	**96.8** ± 4.3	**94.9** ± 2.8	**8.3** ± 2.8	**3.2** ± 9.5	**-2.2** ± 5.6	**12.7** ± 7.5
KK14(Dip)	**51.0** ± 12.5	**97.8** ± 3.5	**93.8** ± 3.5	**100.0** ± 5.5	**3.0** ± 8.0	**0.1** ± 3.4	**-1.7** ± 7.5	**8.0** ± 3.5
kk14	**93.7** ± 1.7	**88.7** ± 3.3	**86.2** ± 7.7	**86.1** ± 8.3	**79.4** ± 5.6	**84.5** ± 3.7	**84.9** ± 5.6	**93.6** ± 8.9
KK14(R)	**93.5** ± 16.5	**98.1** ± 4.4	**93.9** ± 0.8	**95.4** ± 8.3	**1.8** ± 5.1	**-2.3** ± 4.4	**-10.4** ± 3.7	**-10.8** ± 5.9

Secondary structure of the peptides

The secondary structure of KK14 and analogues was determined in aqueous and hydrophobic environment to evaluate the structural changes induced by sequence modifications (Fig. 6.1). KK14 appeared unstructured in aqueous environment with a negative peak around 200 nm and positive peak at 220 nm. In presence of SDS

micelles, a clear transition between coil-helix and helix was observed with two negative peaks at 205 and 220 nm, and a slightly positive peak at 195 nm and a crossover at 200 nm. However, the low value and the undefined positive peak showed the remaining of a high proportion of random coil. The transition was clearer for KK14(R) with high values of ellipticities and clear peaks, which suggest a high α-helical content. The D-enantiomer of KK14 showed a mirror spectrum in aqueous environment. However, the D-enantiomer appeared even more structured than KK14 in hydrophobic environment with a more defined peak at 195 nm.

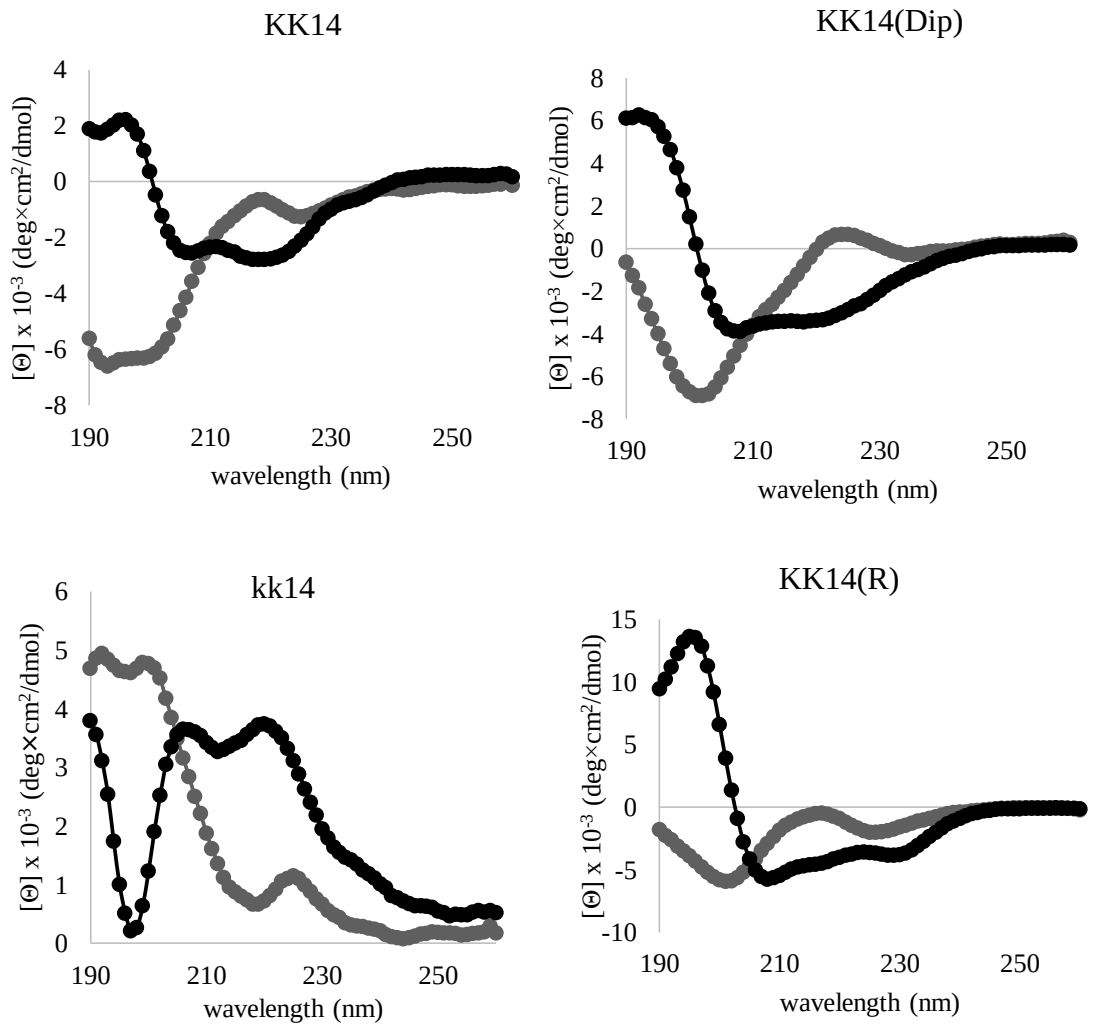


Figure 6.1. CD spectra of the peptides dissolved in deionized water (grey) or 20 mM SDS micelles solution (black) in the 190-260 nm region. Each curve is the average curve of three runs.

Deconvolution analysis revealed a high proportion of random coil and antiparallel structure in aqueous and SDS solutions (Table 6.6). The values of structural features for KK14(R) showed a clear transition with an increase in helical content (17 to 29.8% of helical content in deionized water (DIW) and SDS, respectively). However, the high percentage of predicted secondary structures (>110% of total structure) make conclusions for the other peptides weak.

Table 6.6. Percentage of secondary structures of KK14 and analogues in DIW and SDS solution. The total sum is indicated, highlighting the delicate interpretation of these results. Results are calculated based on three runs of each sample.

	Percentage of secondary structures							
Type of structures	*KK14*		*KK14(Dip)*		*kk14*		*KK14(R)*	
	DIW	*SDS*	*DIW*	*SDS*	*DIW*	*SDS*	*DIW*	*SDS*
Helix	15.9	20	17.3	23.4	17.6	14.9	17	29.8
Antiparallel	57.4	32.5	54.3	22.7	32.2	47.2	50.1	10.6
Parallel	11.9	11.7	10.8	10.7	14.1	14.6	11.7	9.7
Beta-Turn	22.3	20.1	22.2	19.1	19.8	21	21.6	17.5
Random. Coil	36.2	39.6	32.8	37.8	48.9	47.9	36.8	36.9
Total Sum	143.6	124	137.4	113.8	132.7	145.6	137.3	104.4

An *in silico* prediction was also performed using the online tools PEP-FOLD3 and PEPstrMOD (Fig. 6.2).

The 3D model prediction of KK14 with PEP-FOLD3 online tool indicated the presence of random coil after the Pro residue in KK14. On the contrary, KK14(R) was represented as a perfect helix (Fig. 6.2A-B). The secondary structure prediction of analogues kk14 and KK14R could not be performed with PEP-FOLD3, due to limitations of the tool. Using PEPstrMOD, only KK14(R) presented helical features in its secondary structure, whereas the other peptides presented a mostly random coiled configuration (Fig. 6.2C-F).

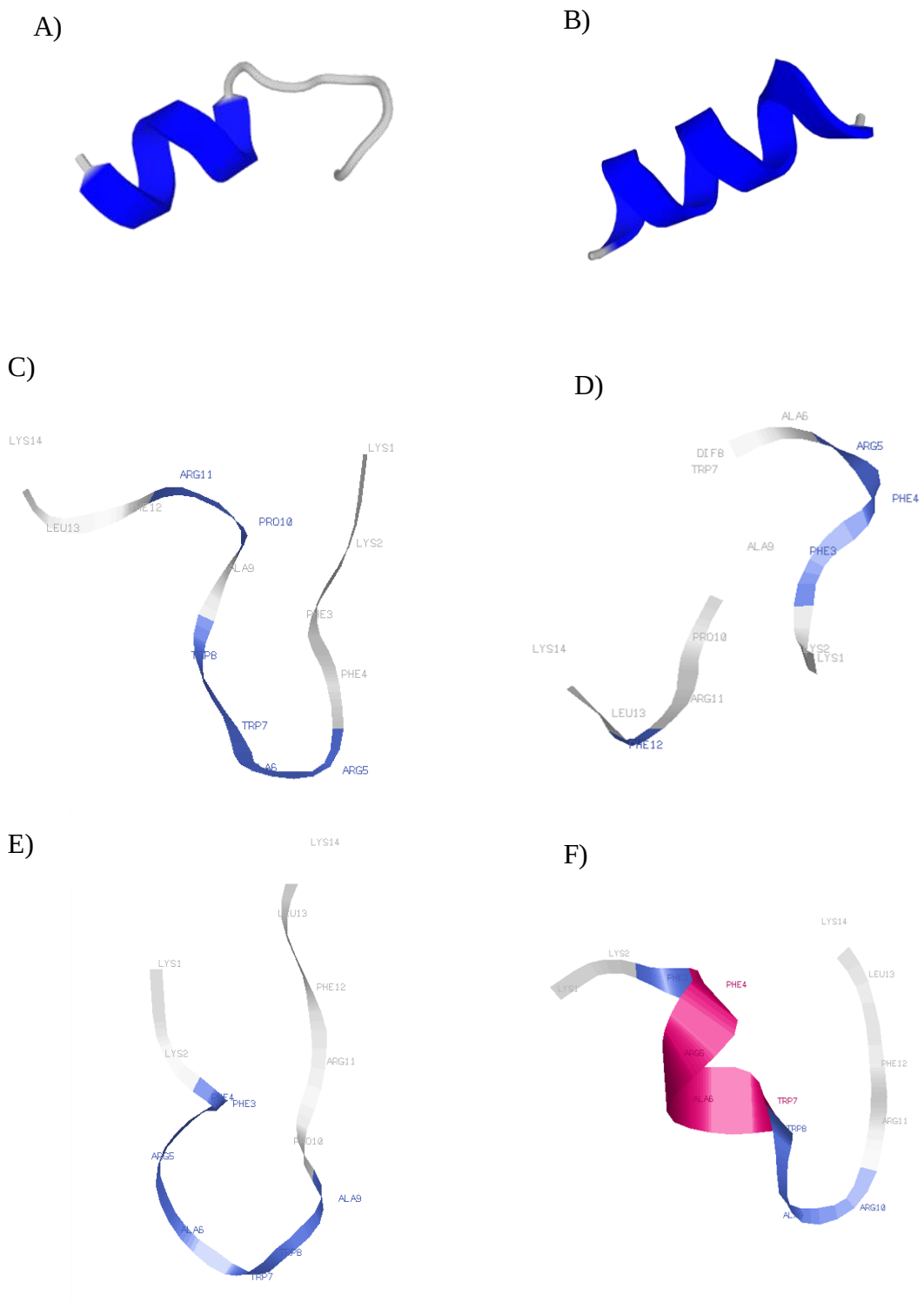


Figure 6.2. *In silico* prediction of secondary structure of the peptides using PEP-FOLD 3 tool (A and B) or PEPstrMOD (C-F). A) KK14; B) KK14(R); C) KK14; D) KK14(Dip); E) kk14; F) KK14(R)

Membrane permeabilization

A PI uptake assay was performed on *F. culmorum* hyphae in presence of the four peptides. After 2 h of incubation, microscopic observation revealed the presence of PI into the mycelium treated with the peptides from a concentration of 6.25 µg/ml. The peptides kk14 and KK14(R) appeared as the most active peptides to permeate fungal membrane with a higher content of permeabilized hyphae at 12.5 µg/ml (Fig. 6.3, Table 6.7).

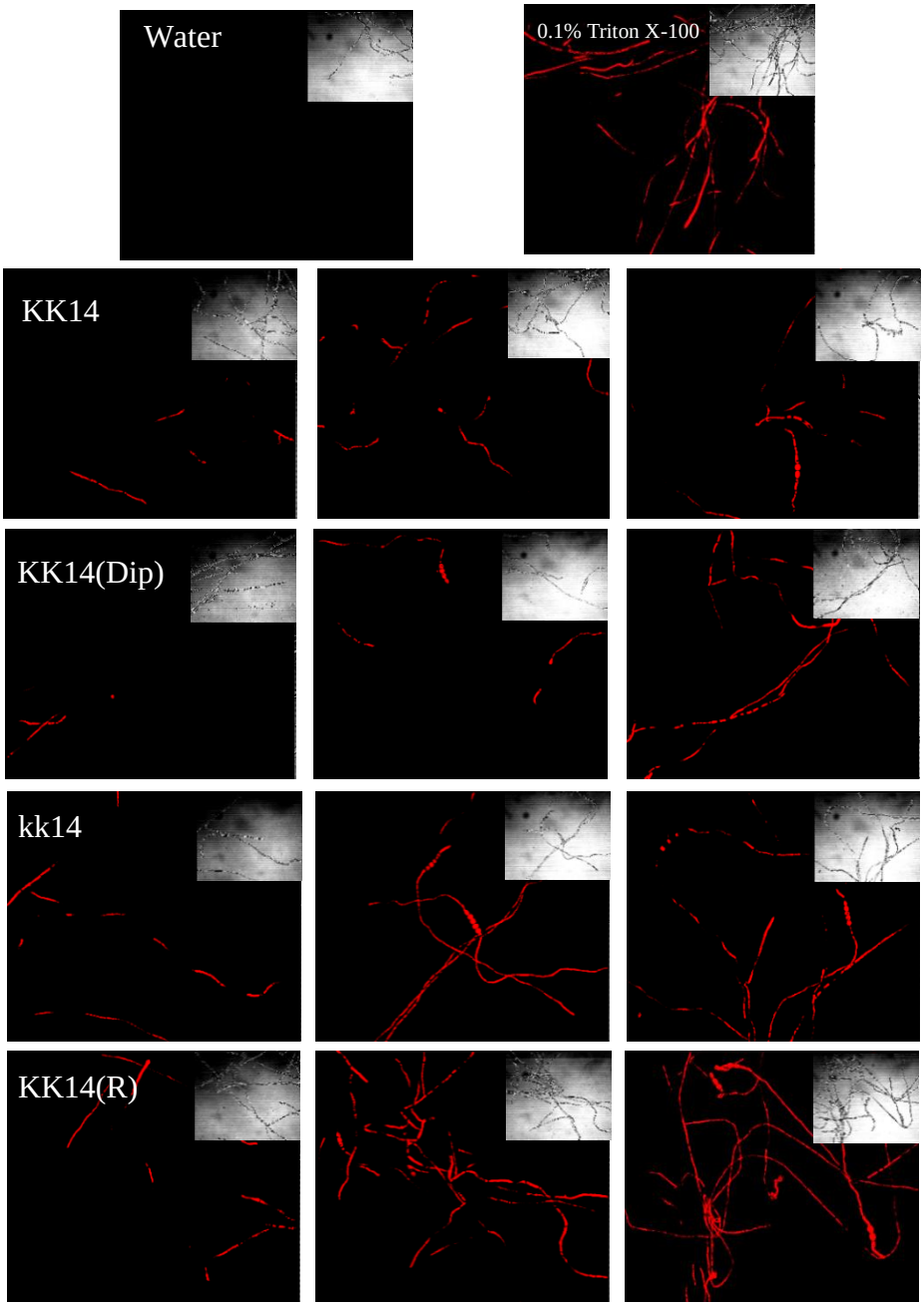


Figure 6.3. Propidium iodide uptake in *F. culmorum* after treatment with KK14 and analogues: 6.25 µg/ml (left), 12.5 µg/ml (middle), 25 µg/ml (right). Insets: *F. culmorum* hyphae with transmitted light

Table 6.7. Mean Intensity of propidium iodide fluorescence

Compound	Concentration (ug/ml)	Mean Intensity
Water	-	3.1E-04
Triton X-100	-	10.4
KK14	6.25	1.1
	12.5	3.1
	25	2.7
KK14(Dip)	6.25	1.1
	12.5	1.1
	25	5.7
kk14	6.25	1.8
	12.5	5.6
	25	4.4
KK14(R)	6.25	2.4
	12.5	7.1
	25	10.9

*Results are based on Mean Gray Intensity, calculated via ImageJ software

Haemolytic and cytotoxic activity

The haemolytic and cytotoxic activity of the four peptides was tested against fresh sheep erythrocytes, colonic Caco-2 cells and mouse macrophages RAW 264.7. The peptides showed no or slight haemolytic activity (~10% at 200 µg/ml) with KK14 being the least haemolytic.

Among the four peptides tested, only kk14 appeared toxic to Caco-2 cells at a concentration of 100 µg/ml, with a percentage of survival of cells of 55.4 ± 16.8% at 100 µg/ml and a 20.4 ± 10.4% viability at 200 µg/ml. KK14(R) showed only toxicity towards Caco-2 cells at a concentration of 200 µg/ml. Macrophages RAW 264.7 were also sensitive to KK14(Dip), kk14, KK14(R) at a concentration 200 µg/ml, with a viability percentage of 29.8, 13.5 and 5.8%, respectively (Fig. 6.4). At lower concentrations, percentages higher than 100% of viability (based on the negative control DMEM) of cells are observed. An increase of cells concentration can be observed in reaction to the presence of a foreign/dangerous compound.

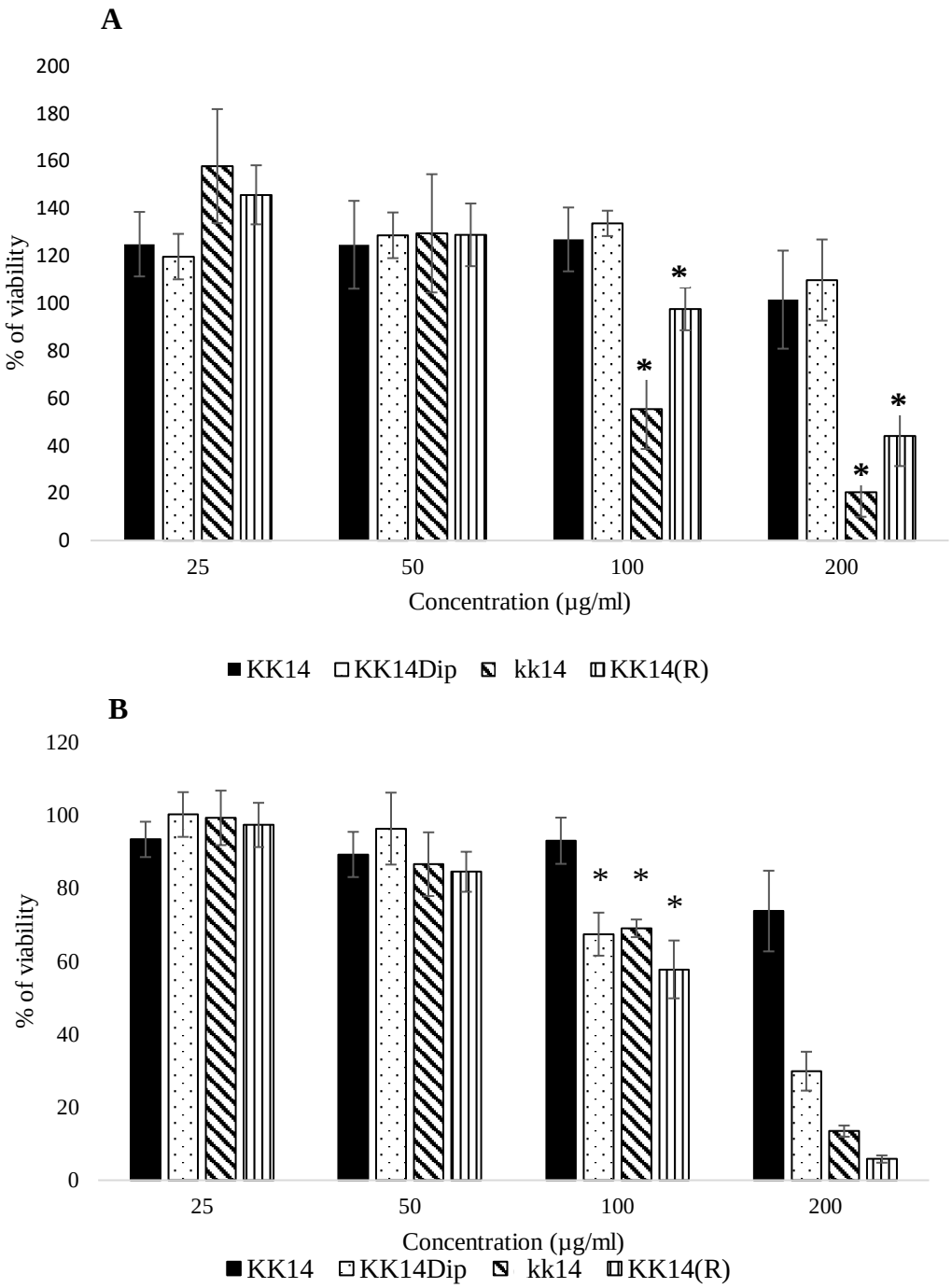


Figure 6.4. Viability of macrophages Caco-2 **(A)** and RAW 264.7 **(B)**. Statistical analysis was calculated with a two-tailed t test. $P < 0.05$ was considered significant. An asterisk (*) indicates significant difference from negative control (100% viability). Results are expressed as means of three biological replicates ± standard deviation.

Applications in beverages

Aspergillus species, the most frequent being *Aspergillus versicolor*, have been reported a fungal contaminant of soft drinks, including in apple juice (Kregiel 2015, Wareing & Davenport 2007). Many fruits including apples are also subject to contamination by the saprophytic fungus *A. niger* (Zhang et al. 2018). The efficacy of the peptides to limit fungal spoilage of beverages was tested in an apple juice solution, contaminated with *A.niger* spores. After 3 days of incubation on PDA, the parental peptide KK14 and the D-enantiomer kk14 failed to reduce the growth of *A. niger*. A reduction of fungal growth was only observed after treatment with KK14(Dip) and KK14(R) at the highest concentration (200 µg/ml) (Fig. 6.5). At lower concentrations (up to 100 ug/ml) of peptides, no inhibition of fungal growth was observed, which indicated an impairment of antifungal activity by, at least, 4- to 8- fold.

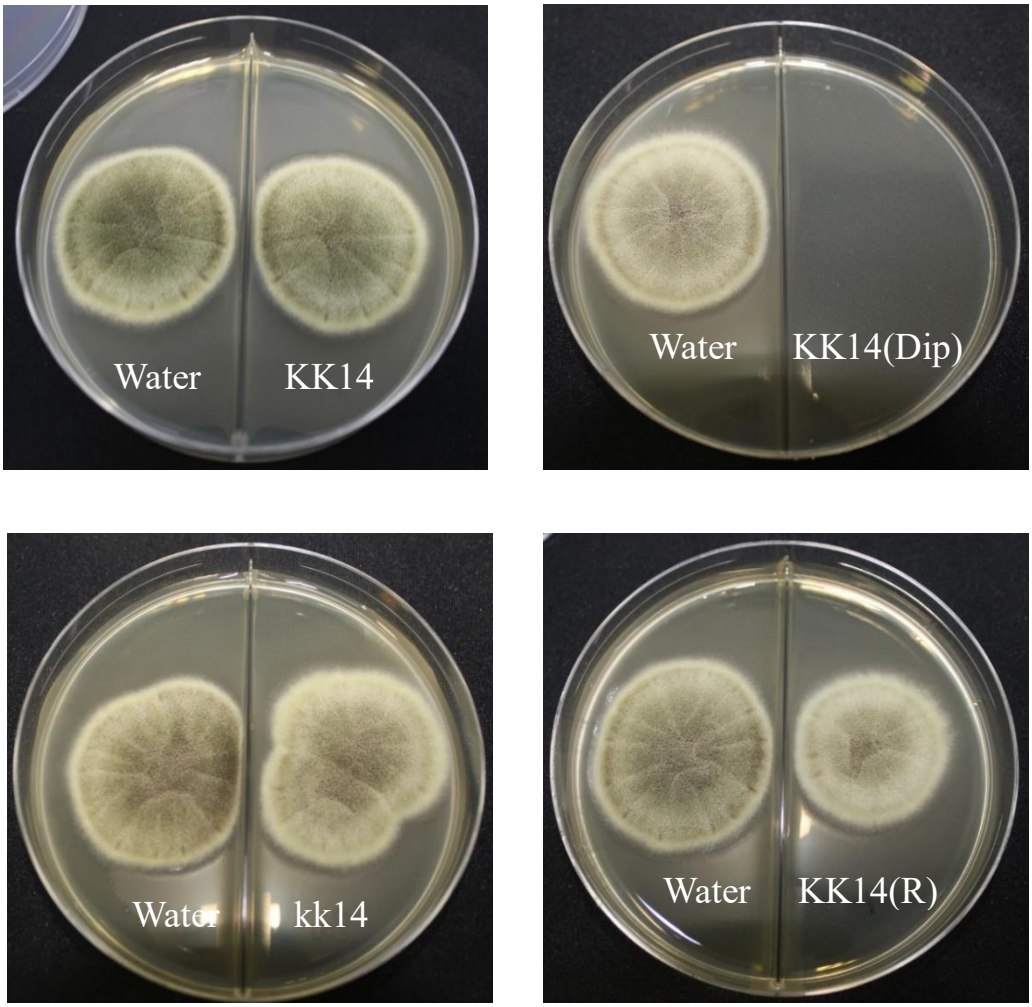


Figure 6.5. Growth of *A. niger* after incubation in apple juice in the absence (left compartment) or presence of peptides (200 µg/ml) (right compartment).

6.4 Discussion

A novel synthetic peptide KK14 (KKFFRAWWAPRFLK-NH_2) and analogues inhibited the germination and hyphal growth of *F. culmorum*, *A. niger* and *P. expansum*. KK-14 possesses a C-terminal amidation which is a common PTM used to increase the net charge and the stability of peptides. However, its role in serum stability and improvement of antifungal activity is relative (Nguyen et al. 2010, Strömstedt et al. 2009). N-terminal acylation is another common PTM and, while it is used to improve the stability (Nguyen et al. 2010, Pan et al. 2007), it decreases the net charge and can reduce antifungal activity.

The substitution of Trp_{10} by the unnatural residue Dip (peptide 2) induced a slightly stronger antifungal activity against *A. niger* and *P. expansum* and a change in the structure of the peptide. Dip increased the hydrophobicity of the peptide, probably resulting in higher antifungal activity. In addition, Dip possesses additional side chains involved in interactions such as hydrogen bonds (HB) and salt bridges. Indeed, Dip interacts more efficiently than Trp with Arg to facilitate the insertion into the fungal membrane as revealed by the better membrane permeabilization of *F. culmorum* hyphae. However, the presence of only one Dip residue did not change drastically the antifungal activity of the peptide, as observed for histatin P-113 derivatives (Chu et al. 2013). Unnatural amino acids, such as 1-benzyl (1-Bzl), 2,4 diaminobutyric acid (Dab), β-naphthylalanine (Nal) are also used to increase salt stability (Chu et al. 2013, Yu et al. 2011), rigidity (André et al. 2015) and antimicrobial (Maurya et al. 2013) potency. However, these substitutions can lead to a higher membranolytic activity, resulting in cytotoxicity to normal and cancer cells and haemolysis (Haug et al. 2016, Yu et al. 2011). Generally, the position and size of a bulky aromatic residue is critical. Its hydrophobic or charged side chains and their fit in position on the peptide affects the overall structure of the peptide, which regulates its activity. In addition, the additional side chains and the resulting HB and salt bridges influence the binding of peptides with membranes and other compounds (Bzymek et al. 2016). These bridges also direct the lifetime of peptides/membrane interactions (Bzymek et al. 2016). A common way to increase lifetime of peptides and serum stability is the blockage of terminal functional groups via cyclization. Thus, cyclization of the short peptide RRWWRF maintained its antibacterial activity in serum (Nguyen et al. 2010).

However, cyclization of short peptides might restrict peptide flexibility inducing limited activity. The lack of secondary structure in aqueous environment allied to the presence of defined structures in hydrophobic environment was added to the list of determinants of antifungal activity of small peptides. The presence of a cationic residue on the non-polar face and the role of the self-association (to a certain extent) in membrane also regulate the activities of the peptides (Chen et al. 2007, Jiang et al. 2009).

The replacement of the Pro_{10} by Arg_{10} (KK14(R)) increased the antifungal activity by 2- to 4- fold. In addition to an increase in cationicity, KK14(R) acquired a more defined structure with an increase in α-helical content, as highlighted by CD spectroscopy. Pro is recognised as an helix breaker due to its inability to establish HB, with the absence of side chains. The breaking ability of Pro is shown by the undefined spectrum at 190-200 nm. The higher cationicity and helical content are associated with the increase of antimicrobial activity and explains the higher toxicity of KK14(R) (Gopal et al. 2013, Jin et al. 2016). With mirror structures, the L and D-enantiomers showed an overall similar antifungal activity as demonstrated for other synthetic peptides (Choi & Moon 2009, López-Garcia et al. 2000, Muñoz et al. 2007). This indicates that KK14 and its D-enantiomer did not use a specific receptor to inhibit fungal growth but rather use, at least partially, a membrane-permeabilizing mechanism. Although D-enantiomerization of a single residue can reduce haemolytic activity, full enantiomerization is usually more advised to avoid the loss of activity and structural deficiencies of the peptide as shown with polybia –MPI (Wessolowski et al. 2004, Zhao et al. 2016). Interestingly, L- and D-enantiomers of thanatin display the same antifungal activity, although the D-enantiomer is much less active against Gram-negative bacteria (Fehlbaum et al. 1996).

A major drawback of the use of cAMPs is their sensitivity to high concentrations of salt. Mono- and divalent cations are thought to act either on the structure of the peptide and/or decrease electrostatic interactions with microbial membrane. KK14 and its analogues were sensitive to salt increase, with a high reduction of their antifungal potency in KCl and $CaCl_2$ solutions. However, the peptides remained fully active in 50 mM KCl solution and partially active in 150 mM KCl and 1 mM $CaCl_2$, which may indicate that their high cationicity (+6) and their hydrophobicity are sufficient to ensure antifungal potency (Vylkova et al. 2007). Interestingly, KK14 appeared as the most sensitive peptide to the salt increase. The substitution of Trp residues with unnatural

residues, such as Nal, has been successfully applied to increase salt stability of the histatin derivative P-113 (Yu et al. 2011). However, the number of Nal and their position appears critical to confer salt stability (Chu et al. 2013). pH changes appeared to affect the activity of the peptides, with a stronger activity at low pH. Despite a high pI, a basic solution (pH 9.5) partially altered the activity of the peptides, with a full abrogation of KK14(Dip) antifungal activity (up to 25 µg/ml). The better activity at a low pH enhances the single net charge of the residues, favouring electrostatic interactions. However, a low pH has been shown to be responsible for supplementary side chains interactions and thus changes in secondary structures, responsible for a decrease in activity. The peptides showed moderate stability after heat treatment in an aqueous environment, due to the probable flexibility of the peptide and the absence of secondary structure, as highlighted by the CD spectra. Bigger AMPs are generally stable after heat treatment due to the presence of stabilizing structural elements, such as disulfide bonds.

The four peptides modified the permeability to the plasma membrane of *F. culmorum* mycelium. Despite their short size, other peptides have been shown to retain their ability to disturb phospholipidic membrane (André et al. 2015) usually correlated with a high proportion of helical content and high amphipathicity. Indeed, a length of 22 residues is generally recognized as a minimal size for an a-helix to penetrate plasma membrane, which could indicate that the peptides are unable to penetrate the membrane as monomers. After a correct orientation and a minimal concentration, peptide is thought to induce membrane permeabilization (André et al. 2015). As shown for V13-K, self-association appears essential to induce activity (Jiang et al. 2009). However, additional mechanisms, such as membrane depolarization, may be involved.

KK14 and analogues were either little or not haemolytic, suggesting that the activity of the peptides was quenched by the salt in PBS. Their low amphipathicity (0,175-0,184) of the peptides has also been correlated to a low haemolytic activity of the peptides. The presence of the polar residue on the hydrophobic face of the peptides, particularly the predicted helical-rich KK14(R), limits a high amphipathicity and is generally used to lower the haemolytic properties of AMPs (Hollmann et al. 2016). However, KK14 Dip, kk14 and KK14(R) showed toxicity towards colonic cell lines Caco-2 and macrophages RAW 264.7. The percentage viability of macrophages after incubation with the peptides KK14(Dip), kk14 and KK14(R) at 200 µg/ml was 29.8%,

13.5% and 5.8%, respectively. The phagocytic macrophage cells initiate the immune response, since they are some of the first cells to be exposed to invading microorganisms. When exposed to inflammatory stimuli, they secrete low molecular weight cytokines which possess a chemotaxis role among immune and non-immune cells and which mediate the unleashing of an effective immune response. The toxicity of these peptides may deregulate the secretion of the numerous cytokines, which will have a detrimental effect on the inflammatory response. Interestingly, a 13-mer peptide (KKFFRAWWRRFLK-NH_2), with a higher amphipathicity (0.729), showed higher cytotoxicity (data not published). Aggregation of KK14 and KK14(Dip) with serum proteins might explain the absence of activity, although interaction of molecules with the fungal membrane might limit the activity of these 2 peptides (André et al. 2015). The increase in net charge and/or the helicity content of the peptide are thought to explain the cytotoxicity of KK14(R). Indeed, perfect helices are usually more cytotoxic than less structured peptide. However, the toxicity of KK14(R) must be limited by its sensitivity to proteases in contrast to kk14, which retained its antifungal activity after trypsin treatment. The use of D-enantiomers are common ways to improve the resistance of peptides to proteolytic digestion (André et al. 2015, Strömstedt et al. 2009). Trypsin usually cleaves preferentially at the C- terminal of Arg or Lys, which are protected in D-form of residues. The proteolytic resistance of kk14 is a drawback for its application as food preservative, due to its potential stability in the gut environment and cytotoxicity.

In conclusion, the novel peptide KK14 inhibited the growth of food spoilage fungi and presented essential stability and safety parameters to be considered as a food preservative. However, as highlighted by a challenge test in an apple juice inoculated with *A. niger*, its application might be limited by the presence of other food ingredients, such as sugars. Interactions between peptide and food components, such as lipids, proteins and sugars are a common hurdle to the food applications of antimicrobial peptides. As such, the preincubation of the AMP dermaseptin S4 derivatives in apple juice hampered by 2- to 8-fold its activity against *Escherichia coli* O157:H7 compared with standard incubation conditions (Rydlo et al. 2006).

Peptide-based strategies have been developed to respond to the increase of invasive fungal diseases over the past 50 years while preventing the undesirable side effects of current prolonged treatments and resistance to the common antifungal agent

amphotericin B. These modifications such as cyclization (Nguyen et al. 2010), backbone modification (e.g., β-peptides) or delivery modes (e.g., liposomes, phosphate groups) aim to improve the stability and the half-life of small peptides. Peptide-based vaccine strategies against *major fungal pathogens* (Nosanchuk & Taborda 2013) and the development of peptibodies (Chongsiriwatana et al. 2011, Ciociola et al. 2016, Kim et al. 2014, Shi et al. 2016) are also considered. In this study, similar peptide-based strategies were used to generate KK14 derivatives with enhanced abilities to inhibit the food contaminants, which are reportedly involved in invasive fungal diseases. Thus, the D-enantiomer kk14, due to its trypsin resistance, could represent an interesting candidate for therapeutic applications, where proteolytic sensitivity of small peptides is a major issue. Although an extended characterization of its antifungal spectrum and *in vivo* studies are necessary, this study highlights the potential use of KK14 and its derivatives in therapeutic application or food preservation.

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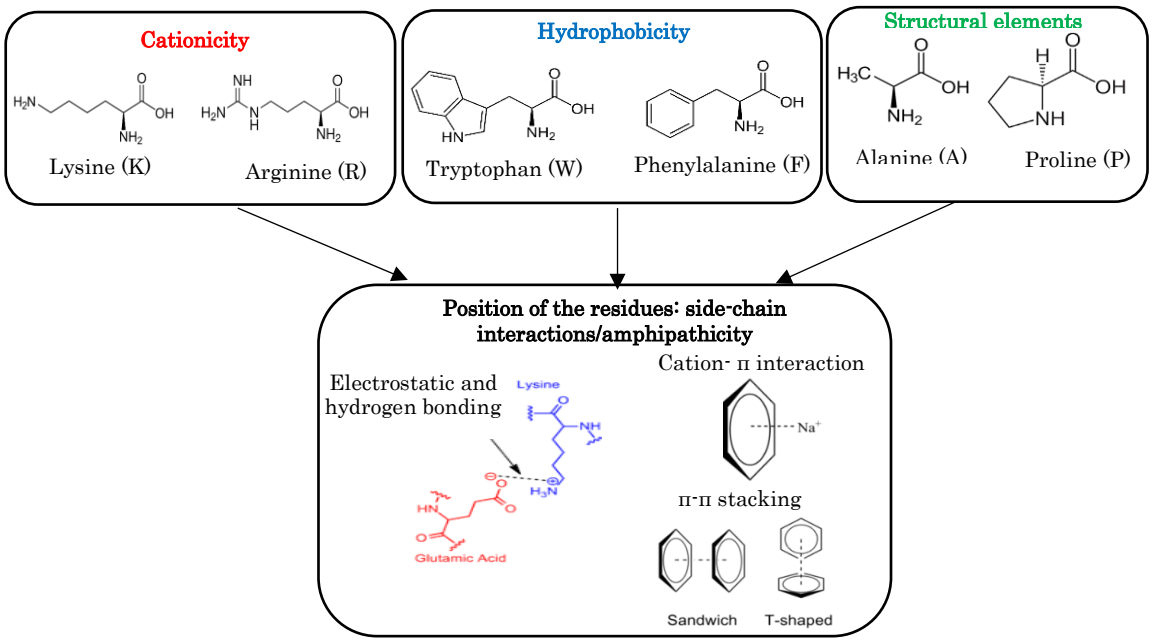
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Appendix: Design of the novel peptide KK14 via key determinants

The first determinant was the net charge of the peptide usually carried by Lys and Arg residue in rational design. These residues possess at neutral pH a positive net charge on their side chain. The second aspect is the hydrophobicity found in a majority of AMPs. Similarly to net charge determinant, and although many residues have with hydrophobic properties, two commonly used residues are Phe and Trp due to their aromatic side chains. The side chains of all residues are also involved in interactions of different nature. The position of the different residues will affect these interactions and the amphipathicity, leading to differential membrane penetration properties. Residues can also be employed for structural properties, Cys for disulphide bonds formation, and Ala and Pro for their helix propensities. A length of 14 residues was selected to provide structural features to the peptide, to limit the cost of synthesis and potential oligomerization. Based on the sequence of previously reported antifungal peptides, a parental sequence was designed and improved via online predictor algorithms (http://www.camp.bicnirrh.res.in/prediction.php), indicating the predicted antimicrobial nature of the new compound. The 14-residue peptide KK14 with the sequence was used as a parental peptide. The sequence was then amidated at its C-terminal to improve both stability and net charge.



KKFFRRAWWAPRFLK

Chapter 7
Isolation, characterisation and application of a new antifungal protein from broccoli seeds – new food preservative with great potential

Manuscript to be submitted to npj-Science of Food, 2018

Abstract

The worldwide problem of food loss and food-related diseases caused by microbial contamination has highlighted the need of better preservation methods. The limits of chemical preservatives and the mistrust by consumers has led to the screening of novel natural compounds as food preservatives. In this regard, the richness of *Brassicaceae* plant family in antifungal compounds and their multiple health properties make them promising candidates. In this study, a water-soluble extract of broccoli (*Brassica oleracea* var. *italica*) seeds showed strong antifungal activity. An antifungal 15-kDa polypeptide with high similarity to the storage protein, napin, found in rapeseed, was isolated and purified from the broccoli seed extract. Assembly of broccoli seed genome revealed the sequence of this newly identified napin-like protein, called BoNap. The extraction and partial purification of the broccoli napin was facilitated in a low pH buffer. *In vitro* assays indicated that BoNap inhibited conidial germination of *Fusarium culmorum* and *Penicillium expansum* with a MIC value of 2.31 µM, but lacked antibacterial activity. The napin-like protein had the ability to permeabilize the fungal membrane of *F. culmorum* and to inhibit trypsin, with an IC_{50} of 9.25 µM. In spite of salt sensitivity, the heat stable protein limited fungal spoilage of several ingredients and foods. The discovery of a non-cytotoxic and readily extractable plant polypeptide could contribute to its application as a natural stable preservative. This study is the first report of a successful application of a broccoli napin, extracted from a water-soluble extract of seeds, to reduce fungal contamination of food products and to limit food waste in a safe way.

7.1 Introduction

Data compiled annually by the Food and Agriculture Organization (FAO) estimate that around 30% of cereals and 40–50% fruits and vegetables are lost along the food production and consumption chain (FAO [Food and Agriculture Organization of the United Nations] 2017). This waste is mainly related to consumer's behaviour in medium/high–income countries, whereas in low-income countries, food loss is mostly attributed to microbial contamination. Saving a third of the estimated 1 billion tons of lost edible goods would theoretically solve the problem of undernutrition. Malnutrition particularly afflicts the poorest regions of the world with around 900 million people suffering from hunger. The paradox of food production is that such a high number of people are malnourished, while food production is almost doubly sufficient to feed everyone on the planet. Improvements in food preservation are necessary to reduce food waste and limit foodborne diseases, which each year cause over hundreds of thousands of deaths (WHO [World Health Organization] 2015). Although foodborne illnesses are mostly due to bacteria, mycotoxin-producing fungi represent an immense challenge. Long-term or chronic exposure to mycotoxins, such as the crop-born aflatoxins or thrichothecenes, promotes several health-damaging effects, with large doses potentially life threatening (Wild & Gong 2009). Due to insufficient food storage or preservation methods, such invasive cereal mycotoxins affect approximately 500 million people of the poorest regions in Sub-Sahara Africa, Latin America and Asia (Stenmarck et al. 2016). The preservation of food is essential, and the use heat treatment and chemicals such as acid-based preservatives are the most common techniques to prevent fungal growth in the food industry; however, acid-based preservatives have several limitations, such as a narrow spectrum of activity or are pH-dependent. An increase in food production is likely to cause an overuse of chemical preservatives, with the potential risk of the development of resistant strains. In addition, the growing mistrust of consumer's to the food industry coupled with increasing awareness around health-related issues of chemical preservatives has led to an increased desire from the food industry for natural preservatives.

There has been an increasing interest in the use of plant extracts to limit food spoilage. In addition to the natural aspect, plants have developed a potent innate immune system to fight biological and chemical stresses. Indeed, many plant extracts or essential oils

are active against a range of bacteria and fungi. In addition to the recognition of foreign molecules/antigens, plants are able to respond to pathogen virulence factors. In the absence of an adaptive immune system, the innate immune response requires the use of potent agents, including pathogenesis-related proteins (PR proteins), whose "accumulation at protein level upon challenge" has been demonstrated upon pathological conditions and related situations in at least two or more plant–pathogen combinations''(van Loon & van Strien 1999). The PR family encompasses a large diversity of proteins, including the small antimicrobial peptides (AMPs) or larger proteins such as chitinases or thionins. These antimicrobial properties have been successfully exploited in the use of water-soluble plant extracts for food applications (Burris et al. 2012, Giuseppe Rizzello et al. 2009, Negi 2012). An increasing number of reports suggest their potential application in food products to reduce foodborne pathogens and fungal spoilage (Rydlo et al. 2006, Wang et al. 2016). However, the efficacy and the stability of the different phytochemicals need investigation. The modification of organoleptic properties and the toxicity of certain plant compounds are also major drawbacks. There is a critical lack of data about the toxicological values of plant compounds for a safe use as food additives.

Plant PR proteins are particularly effective against fungal phytopathogens, a property attributed to an evolutionary response to their main threat. Peptides and proteins with antifungal properties isolated from radish (*Raphanus sativus*) seeds are among the most studied, since the work of Terras and colleagues (Terras et al. 1992a). The antifungal arsenal of radish seeds includes several defensins,the storage proteins, 2S albumins and non-specific lipid transfer proteins (nsLTPs). Thionins have also been identified in several Brassica species, including Brassica oleracea var. viridis (flowering kale). Interestingly, the antifungal activity of barley or wheat thionins was increased by 2- to 73- fold , when combined to radish 2S albumins (Terras et al. 1993a) . Radish belongs to the *Brassicaceae* family, which includes wild cabbage, rapeseed and broccoli. Broccoli (B*rassica oleracea* var *italica*) has attracted great interest due to its multiple health benefits. Indeed, *Brassica* vegetables are a rich source of healthy bioactive compounds, rich in antioxidant vitamins E, C, K, polyphenols, minerals (iron, zinc, selenium) and organosulfur glycosides, called glucosinolates. The breakdown of glucosinolates produces the biologically active compounds isothiocyanates. The most famous isothiocyanate is sulforaphane that has been

involved in the prevention of carcinogenesis *in vivo*, or t cell-type specific apoptosis in cancer cells *(Tarozzi et al. 2013, Ullah 2015).* The combination of all these compounds is presumably responsible for the health benefits of broccoli, from chemoprevention to cardiovascular protection (Vasanthi et al. 2009, Zaghdoud et al. 2016). Broccoli consumption is especially recommended in patients with vitamin A and C deficiency. The different glucosinolates identified in broccoli sprouts and their different activities have been reviewed by Owis (Owis 2015). Several studies have also shown the benefits of broccoli in the treatment of conditions such as stress, genito-urinary issues, or obesity.

A study of broccoli florets extracts obtained with different solvents highlighted the presence of several compounds, such as flavonoids, alkaloids, steroids, terpenoids or the glycosylated versions saponins, with a broad spectrum of antimicrobial activity (Sibi et al. 2013). The amphiphilic nature of the saponins enables them to act as soaps and detergents as they can dissolve membranes, however, since saponins can hemolyze erythrocytes, they are highly toxic if they reach mammalian blood. The antibacterial activity of broccoli has also been reported against human pathogens and foodborne bacteria (Sibi et al. 2013). The overall goal of this study was to investigate the antifungal properties of a broccoli seed extract and to identify the potential antifungal compounds that could be extracted without the use of chemical purification. In addition, a full characterization of the stability and toxicity of these compounds helped to evaluate the limits of these compounds in food application. The use of such novel, natural and stable plant preservatives could aid in reducing food waste due to fungal contamination from farm to table.

7.2 Material and methods

Antifungal activity of water-soluble broccoli extract

Fungal strains of *Fusarium culmorum*, *Aspergillus niger* and *Penicillium expansum* were obtained from the culture collection of University College Cork collection (UCC, Cork, Ireland). Fungal spores were collected as described previously (Oliveira et al. 2012). Broccoli seeds (100 g) were purchased from GreenValu Ltd (Dublin, Ireland) and milled for 30 s with a coffee blender (Delonghi). Broccoli flour was soaked in 500 mL of sterile distilled water for 2 h at 4°C. Following centrifugation (4,000 *g*/20 min), the supernatant was collected, filter-sterilized and tested for its antifungal activity via two methods. Although diffusion of effective compounds can be limited due to interactions with the agar, these agar plate methods give a qualitative overview of the antifungal potency of the extracts.

The assay for antifungal activity was conducted in 100 mm × 15mm Petri dishes containing 10 ml of potato dextrose agar (PDA, Sigma-Aldrich). The first method consisted of mixing fungal spores (10^4 spores/ml) in slightly warm agar (40°C). Once this agar was transferred to plates and had solidified, holes of 0.5 cm diameter were made in the solid agar and filled with 50 µl extract. Water and buffer (used for selective extraction in the following section) were used as controls. Antifungal activity was evaluated visually with the presence or absence of a halo of inhibition.

As a second method for determining antifungal activity, the inhibition of fungal growth was monitored on PDA (5 ml) containing water-soluble extract of broccoli seeds (10% (v/v)). A 50 µl drop of spore suspension (10^5 spores/ml) was spot-inoculated onto the agar. The plates were incubated at 28°C and fungal growth was observed over 3 days.

Selective extraction of seed antifungal proteins

Seed flour was extracted for 2 h at 4°C with 250 mL of buffer (10 mM Na_2HPO_4, 15 mM NaH_2PO_4, 100 mM KCl, 2 mM EDTA) as described by Terras et al. (Terras et al. 1992b). Polyvinylpolypyrrolidone (1.5% w/v, Sigma-Aldrich) was added to ice cold buffer and gently stirred for 2 h at 4°C. Prior to flour extraction, thiourea and PMSF were added to the buffer at 2 and 1 mM, respectively. Following extraction, solid material was removed by centrifugation at 10,000 *g* for 20 min and solid ammonium

sulphate was added to the supernatant until 30% of relative saturation was reached. All chemicals were purchased from Sigma-Aldrich. The precipitate was allowed to form overnight at 4°C under gentle stirring. After centrifugation at 10,000 *g* for 30 min, the supernatant was adjusted to 70% ammonium sulphate saturation. The final precipitate formed overnight at 4°C under gentle stirring was collected by centrifugation at 10,000 *g* for 30 min and re-dissolved in 40 ml distilled water. The suspension was clarified by centrifugation at 10,000 *g* for 30 min and the resulting supernatant heated at 80°C for 15 min. A final centrifugation (10,000 *g* for 15 min) was performed to remove the coagulated material and the supernatant was extensively dialyzed against distilled water for 3 days using dialysis tubing with a molecular mass cut-off of 2,000 Da (Sigma-Aldrich).

The dialyzed solution was adjusted to 10 mM Tris-HCl (pH 7.4) by addition of the ten-fold concentrated buffer and isocratically passed over an anion-exchange DEAE-Sepharose Fast Flow (GE Healthcare) column (12x5cm) in equilibrium with 10 mM Tris-HCl (pH 7.4) at a flow rate of 1 ml/min in an ÄKTA system (GE Healthcare). The choice of pH was based on the isoelectric point of several plant antifungal peptides and proteins previously isolated such as plant defensins (Cammue et al. 1992). Elution was initially achieved by the equilibrium buffer, when F1 fraction (unbound proteins) was obtained. Based on previous reports showing that antifungal peptides and proteins were found in the unbound fraction of anion exchange chromatography. Proteins (F2) were eluted with 1 M NaCl in the same buffer and discarded. The F1 fraction was pooled and submitted to one cycle of chromatography on a Sp-Sepharose High Performance cation-exchange column (HiPrep SP HP 16/10, GE Healthcare), with Tris-HCl buffer (pH 7.4) at a flow rate of 1 ml/min in an ÄKTA system (GE Healthcare). Elution was performed with 1M NaCl gradient in the same buffer (ml/min). The different peaks (1 ml fraction) found in the eluted fraction were filter-sterilized and tested for their antifungal activity against *F. culmorum*, in the radial diffusion assay described above. The peak containing the highest antifungal activity (as observed on agar plates) was injected onto a Superdex 75 10/300 GL (GE Healthcare) on an ÄKTA explorer system. The chromatography was performed at a flow rate of 0.5 mL/min with 150 mM NH_4HCO_3 (pH 9.4) as an elution buffer, as described by manufacturer. The choice of ammonium bicarbonate for gel filtration was based on its decomposition to volatile compounds upon freeze drying, increasing the

recovery percentage after purification. Proteins were monitored by on-line measurement of the absorbance at 280 nm.

Gel electrophoresis

The purification process was monitored throughout by SDS–glycine-gel electrophoresis performed according to the method of Schägger and Von Jagow (Schägger & von Jagow 1987). SDS-glycine-gel electrophoresis was performed on 12-20% Criterion™ Tris-Glycine Precast Gels (Bio-Rad, Hercules, California, United States), according to manufacturers instructions. The presence of peptides was detected in native and dithiothreitol-reduced fractions.

Protein identification and bioinformatics analysis

N-terminal sequencing was performed by Alphalyse Europe A/S, Denmark. The bands corresponding to the antifungal protein were transferred onto a PVDF membrane and identification of the first 20 residues of the lowest molecular weight protein was performed by N-terminal Edman degradation. The resulted sequence was submitted to BLAST analysis in order to identify the protein.

Putative protein identification and bioinformatic analysis was performed using sequences available through the Sequence Read Archive of the DNA Data Bank of Japan (DDBJ), accessible under the following accession numbers, as references: SRX032205 (whole genome of Big Broccoli (*Brassica oleracea* var. *italica*), SRR1049400 (transcriptome of broccoli seeds). BLAST analysis (www.ncbi.nlm.nih.gov/BLAST/) of the 20 residues identified by N-terminal sequencing was performed with Unipro UGENE (Okonechnikov et al. 2012) software on the whole broccoli genome and transcriptome data to identify the putative complete sequence of the isolated protein.

Improved yield of extraction

A second method of isolation and purification of the antifungal protein was performed as described by Perera et al (Perera et al. 2016). Briefly, broccoli seeds were milled

until finely ground and defatted via a hexane extraction (1:3 ratio). Hexane was removed by air-drying overnight under a laminar flow hood. The defatted ground seeds were mixed with MilliQ water at a ratio of 1:10 for 60 min at 30°C and pH 3.0. The remaining seed flour was collected and submitted to another extraction in a 0.1 M NaCl solution (pH 3.0) (1:5) for 60 min. The choice of a low pH is based on the preferential solubility of several antifungal proteins at low pH (Perera et al. 2016). Protein extract was centrifuged at 4,000 *g* for 10 min and the supernatant filtered through Whatman filters #4 (Sigma-Aldrich). The extracts were dialysed with 2,000 Da cut-off tubing (Sigma-Aldrich). A first step of purification consisted in a separation using hydrophobic interaction chromatography (HIC) with a HiTrap Phenyl Sepharose™ (GE Healthcare Life Science) 6 Fast Flow column (mobile phase A: 50 mM Tris-HCl, 5 mM EDTA, 0.3% NaHSO3 pH 8.5; mobile phase B: 50 mM Tris-HCl 5 mM EDTA, 0.3% NaHSO3 at pH 8.5 containing 0.85 M Na_2SO_4) using an ÄKTA Explorer system. The high salt level of the main fraction was removed by passing through a Sephadex G-25 Hiprep™26/10 desalting column (mobile phase: Milli-Q water). Following purification, the resulting fractions were dialyzed against distilled water as described above, freeze-dried and stored at −20°C until further use.

Protein identification

The isolated fraction was run a gel electrophoresis as described previously to verify the purity of the protein. The identification of the protein was performed by LakePharma (Belmont, CA, USA), with Edman degradation and comparison with the previously identified protein. The purification process was monitored throughout by SDS–Tricine gel electrophoresis performed according to the method of Schägger and Von Jagow (Schägger & von Jagow 1987). SDS-tricine-gel electrophoresis was performed on 16,5% Criterion™ Tris-Tricine Precast Gels (Bio-Rad, Hercules, California, United States), according to manufacturer's instructions. Presence of peptides was detected in native and dithiothreitol-reduced fractions.

Inhibition of conidial germination assays

Fungal spores were collected and antifungal assays were performed as described previously (Thery et al. 2018). Briefly, fungal spores were inoculated in half-strength potato dextrose broth (½ PDB) at a concentration of 10^4 spores/ml. Spore solution (180 µl) was supplemented with 20 µl of broccoli seed protein (0-37 µM, final concentration). Fungal growth was monitored at 28°C for 120 h and absorbance followed by spectrophotometry at 600 nm. Control of conidial germination was performed in ½ PDB supplemented with water. After incubation period, conidial germination was observed under microscope.

Antibacterial assays

The antimicrobial activity was performed against Gram-negative and Gram-positive species, representing common foodborne pathogens, using a broth dilution method as described by Sibi et al., with some modifications (Sibi et al. 2013).Bacterial species, *Escherichia coli* DSM 300083, *Staphylococcus aureus* DSM 13661, *Staphylococcus aureus* DSM 20231 and *Salmonella enterica* DSM 17058 were supplied by DSMZ (Germany). Briefly, bacteria were cultured on Mueller-Hinton agar (Sigma-Aldrich) for 24 h at 37°C. One colony was transferred to Tryptic Soy Broth (Sigma-Aldrich) for 2 h and diluted with PBS until the OD of the media reached McFarland 1. Mueller-Hinton Broth (Sigma-Aldrich) was used as growth media and 2.5 µl of bacterial solution was added to each well. The OD was monitored continuously in a 96-well microtitre plate for 24 h at 37°C.

Protein stability

The stability of the isolated protein was determined as described previously by Perera et al (Perera et al. 2016). The isolated fraction was heat treated at 100°C for 15 min in a dry block heating (GrantInstruments, Shepreth, Cambridgeshire, UK). After leaving at room temperature for 30 min, the activity of the protein against *F. culmorum* conidia was determined as described above.

The antifungal activity of the protein was also determined in presence of different salt solutions as described previously. Half-strength PDB (½ PDB) was supplemented with

NaCl (50 mM and 150 mM) or $CaCl_2$ (1 and 5 mM) and germination of *F. culmorum* conidia was followed by monitoring the absorbance, in presence of the broccoli antifungal protein (AFP).

Structural characterization of broccoli protein

The isolated AFP (1 mg/ml) was submitted to circular dichroism (CD) spectroscopy in deionized water (DIW) or 20 mM SDS micelles. The secondary structure of the protein was determined via Chirascan™ CD Spectrometer (Applied Photophysics, UK). The spectrum was recorded from 190 to 260 nm at a speed of 50 nm/min. Three runs were performed for each sample. The solvent signal was subtracted and the CD spectrum transformed to mean residue molar ellipticity.

Membrane permeabilization

The effect of the protein on the *F. culmorum* cell membrane was followed by propidium iodide uptake (PI, Sigma-Aldrich) assay as described previously (Van Der Weerden et al. 2008). Briefly, *F. culmorum* spores were inoculated in ½ PDB for 20 h at 28°C. Following incubation, fresh mycelium was collected by centrifugation (8,000 *g*/5 min) and washed. Hyphal suspension (90 µl) was then transferred to 96-well microtiter plates and treated with the purified protein (0-18.5 µM). Water and 0.1% Triton X-100 were used as negative and positive controls, respectively. The mycelium were harvested and incubated in 2 µM PI for 20 min, and the unbound dye was removed by excessive washing with distilled water. The mycelium was visualized by fluorescence microscopy (Olympus FluoView™ FV1000) with excitation and emission wavelengths of 535 and 615 nm, respectively. The PI uptake was measured over 2 h with a fluorospectrophotometer (VarioSkan) (excitation 535 nm/emission 615 nm).

Trypsin inhibitory assays

Trypsin inhibitory activity of the protein was measured spectrophotometrically at 405 nm by monitoring paranitroaniline release, using N-benzoyl-DL-arginine-p-

nitroanilide (Sigma) as substrate. Trypsin (Sigma) was dissolved in 100 mM Tris-HCl, pH 8.0 to a final concentration of 0.5 µM. A 90 µl trypsin solution was incubated for 30 min at 37°C with 10 µl of the protein solution (0-18.5 µM). After incubation, the remaining tryptic activity was determined by the addition of 5 mM N-benzoyl-DL-arginine-p-nitroanilide. The release of paranitroaniline was measured at 405 nm for 30 min. Tris-HCl buffer was used as a negative control, meaning100% of residual proteolytic activity.

Haemolytic activity of broccoli AFP

Haemolytic activity of the isolated protein was determined on fresh sheep erythrocytes (Thermo Scientific, Fisher Scientific Ireland, Dublin) as described by Thery et al. (Thery et al. 2018). Fresh defibrinated red blood cells (1 ml) were centrifuged at 1,000 *g* for 10 min and the supernatant discarded. After 3 washings with the same volume of PBS, the final pellet was resuspended in 4% PBS (v/v). After 1 h of incubation at 37°C with serial dilutions of broccoli AFP (0-18.5 µM), the solutions were centrifuged at 1,000 *g* for 15 min and the supernatant collected. Haemolytic activity of the protein was determined by measuring the absorbance of the supernatant at 405 nm. PBS alone and Triton-X (0.1%) were used as negative and positive controls, respectively.

Cytotoxicity assays

The cytotoxicity of the broccoli AFP was determined by measuring cell viability using an MTT cell viability kit (Roche, Switzerland) as described previously (Thery et al. 2018). Human colonic cells, Caco-2, (ECACC) were cultured in a 96-well microtiter plate in Dulbecco's modified Eagle's medium (DMEM), supplemented with non-essential amino acids (1%) and FBS (10%), at 37°C in 5% CO_2. Cells ($1x10^5$ cells/ml) were allowed to adhere overnight; media was removed and 200 µl of fresh medium supplemented with peptides (0-18.5 µM) was added to the wells and incubated for 24 h at 37°C. After removal of the medium, fresh medium (100 µl) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (10 µl) (Cell viability kit, Roche) was added to each well and incubated for 4 h at 37°C. The solubilisation solution (100 µl) was added to each well and the plate was incubated overnight at

37°C. Cell viability was measured spectrophotometrically at 570 nm with a background reading at 690 nm. The cytotoxicity of each peptide was determined in 4 wells for 3 biological replicates.

Barley grain spoilage protection

A low pH extraction of broccoli antifungal compounds was performed as described above with omitting the defatting procedure. Following extraction, the solution was dialyzed with 12,000 Da cut-off tubing (Sigma-Aldrich) against distilled water for 2 days at room temperature. The dialyzed extract was then lyophilized, resuspended in distilled water and filter-sterilized. Total protein concentration of the extract was determined with BCA assay. Barley grains were disinfected according to the method described by Oliveira et al. (2012). Briefly, 50 g of grains were disinfected in 500 mL of 10% (w/v) H_2O_2 solution for 10 min with continuous stirring. Subsequently, the grains were washed for 5 min in 1 L distilled water. This procedure was repeated once. Grains were moved to sterile plastic boxes and dried overnight under vertical sterile laminar flow. Lastly, the grains were exposed to UV light (10 min) and collected aseptically for further use. Disinfected grains (50 g) were plunged in a *F. culmorum* spore solution (500 mL, 10^5 spores/mL) and left for incubation for 1 h at room temperature. Infected grains were collected by filtration (Whatman filters #4.) Infected grains (15 g) were disposed on a sterile paper filter in a 90 mm Petri dish and 2 mL of broccoli seed extract (3, 6 and 60 mg/ml) were spread over the grains. Control was performed with pH 3.0 sterile aqueous solution. Petri dishes were sealed with parafilm and incubated for 1 week at 28°C. At days 2 and 4, sterile distilled water (2 mL) were spread over the grains to maintain high a_w to favour fungal growth. Three Petri dishes were used for each condition. After 1 week of storage, grains (10 g) were collected, milled to a whole grain flour (particle size < 2mm), homogenised and stored at -20°C until further analysis.

Determination of ergosterol

The amount of fungal contamination on the grains was determined by a total ergosterol content measurement after storage, based on the method of Jedlickova et al. (2008)

with some modifications. In brief, milled grains were extracted with 25 mL of methanol under constant shaking at room temperature for 30 min. After centrifugation, 12.5 mL of the supernatant were transferred into a tube containing 1.5 g KOH and shaken until complete dissolution. Subsequently, 5 mL of *n*-hexane were added and the mixture was incubated in a water bath for 30 min at 65°C. After cooling to room temperature, 2.5 mL distilled water were added and the upper layer collected. The extraction with n-hexane was repeated twice, the extracts pooled and evaporated till dry. The residue was then re-dissolved in 2.5 mL of methanol before analysis by HPLC. The RP-HPLC column used was a Nova-Pak C18 (300 x 3.9mm, 4µm) (Agilent 283 Technologies). Peak-identity was verified using the UV-spectra recorded by the DAD. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the signal/noise (s/n) ratio. The LOD was set for s/n of 3:1 and the LOQ was set for s/n of 10:1. For calibration, ergosterol standards between 1.0 and 200 µg/mL in methanol were prepared and analysed.

Statistical analysis

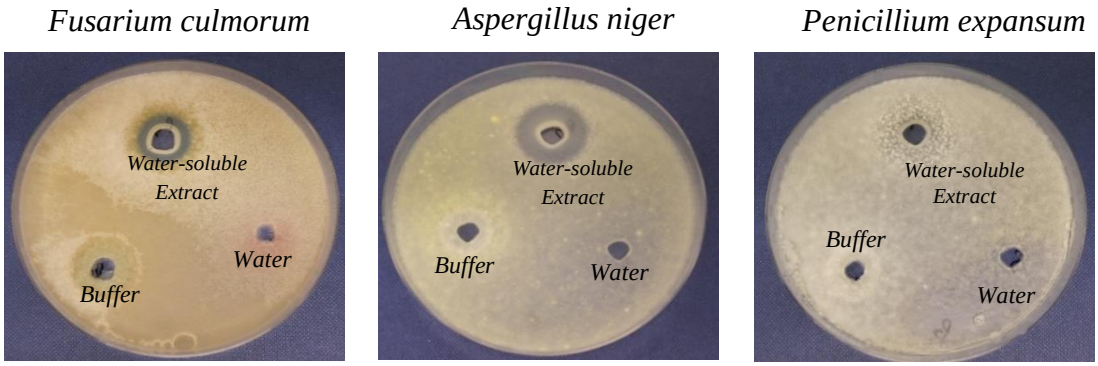
The antifungal assays on Petri dishes or microtitre plates were performed in three technical replicates. The trypsin-inhibitory assay was performed in triplicate for each concentration. The haemolytic and cytotoxic activity of the isolated protein were performed in four technical replicates for 3 biological replicates.

7.3 Results

Antifungal activity of a water-soluble broccoli extract

A water-soluble extract of broccoli seeds was tested for its antifungal activity against *F. culmorum*, *A. niger* and *P. expansum* by different methods. A radial diffusion assay highlighted a clear growth inhibition of *F. culmorum* and *A. niger* with the water-soluble extract (Fig. 7.1 A), whereas *P. expansum* growth was not affected by the extract. In the second assay, the growth medium was supplemented with the full extract (10% (v/v)) and fungal growth was observed after 72 h of incubation. The inhibition of fungal growth was clear for *F. culmorum* and *A. niger*, but was limited for *P. expansum* (Fig. 7.1B).

A)



B)

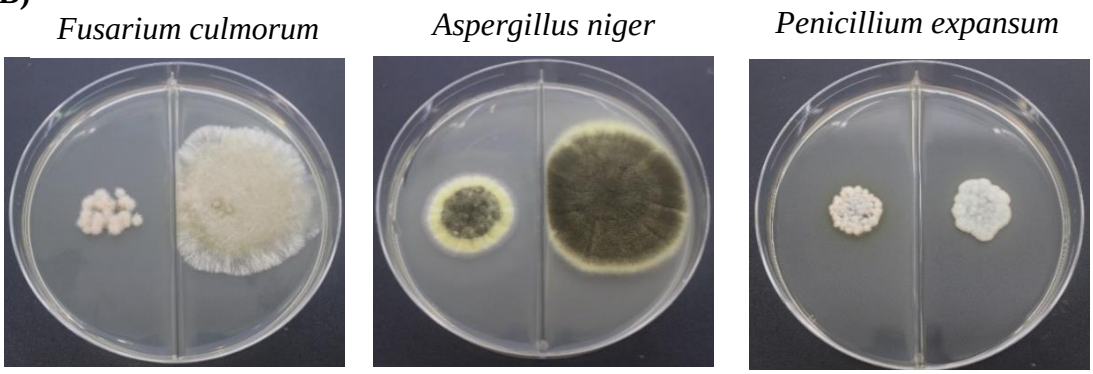


Figure 7.1. Antifungal activity of water-soluble extract of broccoli using radial diffusion assay (**Fig 7.1A.**). The inoculation of 10% extract (left side) in growth medium clearly delayed conidial germination (**Fig. 7.1B.**)

Isolation of an antifungal broccoli protein

Following buffer extraction of broccoli seeds, the extract was submitted to chromatographic purification on an AKTA system, using cycles of ion-exchange and gel filtration chromatography. The unbound fraction (F1) on anion-exchange chromatography (data not shown) was submitted to cation-exchange on a High Performance Sp- Sepharose column (Fig. 7.2A). After elution with 1 M NaCl, only eluted fractions were tested for their antifungal activity, based on previous reports. The fraction B2 showed the highest antifungal activity (Fig. 7.2A and Figure 7.S1), determined visually with the diameter of the halo of inhibition, and was submitted to gel filtration using a Superdex Peptide 10/300 GL Column (Fig. 7.2B). The activity of other fractions against F. culmorum are presented in Figure S1. Due to a possible rich concentration in compounds, the fraction corresponding to the largest peak N1 was selected for collection, dialyzed in 2 kDa cut-off tubing against distilled water overnight and freeze-dried. The concentration of peptide was determined via Pierce BCA assay (ThermoFisher), using reference protein provided (bovine serum albumin) and the sample was resuspended in distilled water at a concentration of 2 mg/ml and kept at -20^{o}C until further use. The graphs presented were directly exported from the software Unicorn (GE Healthcare).

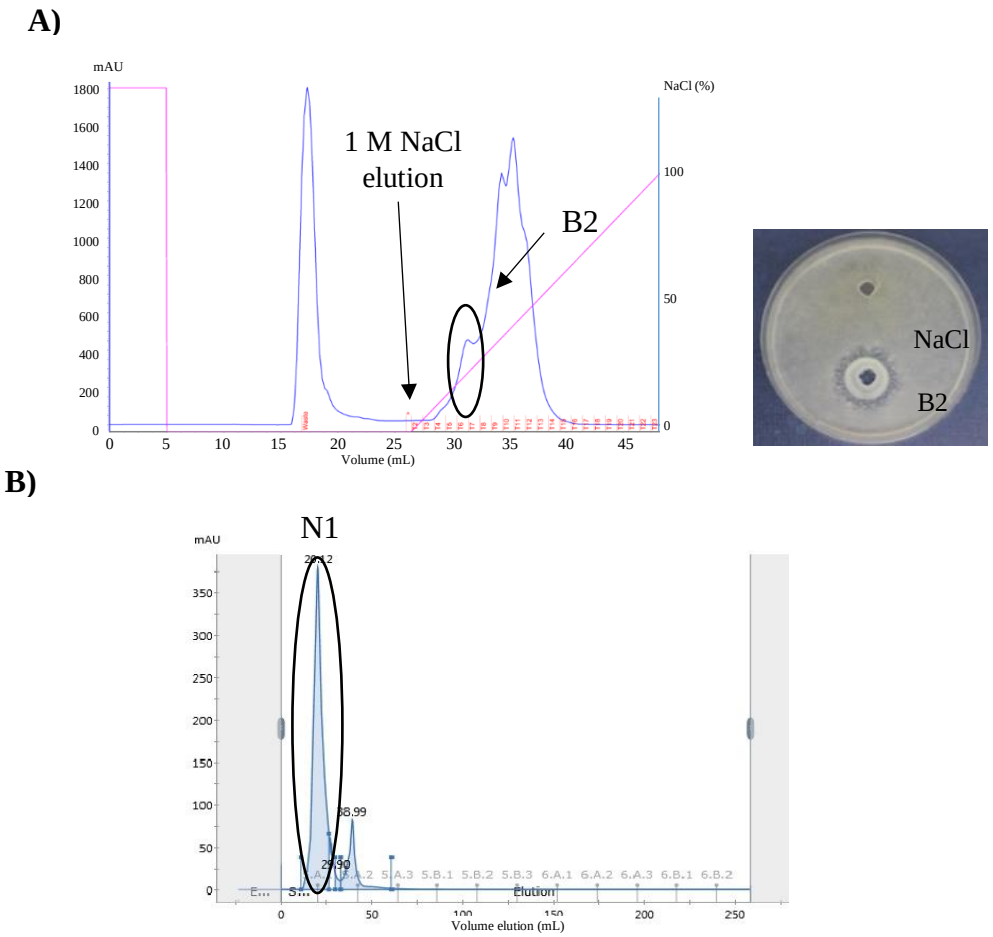


Figure 7.2. Purification of broccoli antifungal compound on a cation-exchange column Sp-Sepharose HP(Fig. 7.2A., blue: A280, pink: NaCl gradient). The fraction was tested for its antifungal activity against *F. culmorum* (adjacent picture). Fraction B2 was then submitted to gel filtration on a Superdex Peptide 10/300 GL Column and fraction N1 was collected (Fig. 7.2B). The graphs were directly exported from the software Unicorn (GE Healthcare).

Identification of the protein

In order to determine the MW of the isolated compound, it was submitted to SDS-PAGE on a 12-20% Criterion™ Tris-Glycine gel (Bio-Rad) with and without reduction with DTT. The peptide showed one band around 15 kDa and after reduction 2 bands at a MW below 10 kDa.

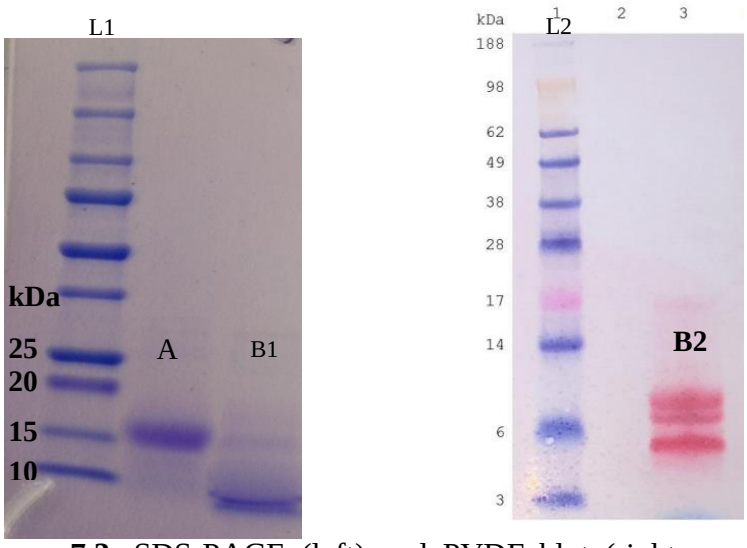


Figure 7.3. SDS-PAGE (left) and PVDF blot (right, provided by Alphalyse) of fraction N1 after gel filtration on a Superdex column. L1 and L2: ladders; A: Native fraction N1; B1: DTT-reduced fraction N1; B2: reduced fraction N1.

A PVDF blot of the protein and the N-terminal sequencing by Edman-degradation of the band with the lowest MW was performed by Alphalyse A/S. (Denmark) and revealed a more precised 3 band-pattern in the reduced fraction (Fig. 7.3)A BLAST comparison of the first 20 residues showed 78% identity to a napin previously isolated from *Brassica napus* napin (Table 7.1) and 90% identity with Chinese Kale (brassica olereacea var..polypeptideThe isolated protein from broccoli seeds was termed BoNap.

Table 7.1. Primary sequence of the small unit of isolated protein

Name	Sequence	%identity
BoNap	PAGPF-IPK(P)—EF(Q/E/F)(Q/F)A--(L)*	-
Brassica napus napin	AGPFRIPKCRKEFQQAQHLKACQQWLHKQAMQSGSG	78
Chinese Kale napin-like polypeptide	PAQPFRIKKRQGPFERP	90

*Hyphens represent undetermined residues and the residues into brackets were impossible to clearly identify conserved amino acids are highlighted in red. This sequence was submitted to a BLAST analysis on Uniprot to determine percentage of identity with existing sequences.

The RNAseq reads of broccoli seed transcriptome (https://trace.ddbj.nig.ac.jp/DRASearch/run?acc=SRR1049400) and the whole genomic data of Big broccoli (https://trace.ddbj.nig.ac.jp/DRASearch/experiment?acc=SRX032205) have been previously indexed. The N-terminal sequence was submitted to BLAST analysis with the genomic and transcriptomic data using Unipro Ugene software to identify the putative primary sequence of the isolated broccoli napin. This sequence shared 94.5% identity with a napin isolated from *Brassica napus* and over 90% sequence similarity with a napin-like protein isolated from *Brassica* species (Table 7.2). The predicted molecular weight of the mature protein was 14.8 kDa (determined with Expasy online predictor). Several napin-like sequences containing the initial amino acids 'PAGP' or similar were also identified, indicating the possible presence of several napin-like proteins in the broccoli genome (Table 7.3). The high percentage of identity between the different species highlighted the conserved nature of the protein.

Table 7.2. Homology between broccoli napin (BoNap) and napin-like proteins isolated from other *Brassica* species [a]

Species	Full sequence	% identity (with first sequence)[b]	Accession number
Brassica oleracea var. *italica*	[1]MANKLFLVSATLAFFFLLTNASVYRTVVEVDEDDATNPAGPFRIPKCRKEFQQAQHLRAC[60] [61]QQWLHKQAMQSGSGPSWTLDGEFDFEDDMENPQGPQQRPPLLQQCCNELHQEEPLCVCPT[120] [121]LKGASKAVKQQVRQQGQQQGQQGQQLQQVISRIYQTATHLPRVCNIPQVSICPFQKTTPG[180] [181]PYY[183]	-	-
Brassica oleracea var. *oleracea*	[1]MANKLFLVSATLAFFFLLTNASVYRTVVEVDEDDATNPAGPFRIPKCRKEFQQAQHLRAC[60] [61]QQWLHKQAMQSGSGPSWTLDGEFDFEDDMENPQGPQQRPPLLQQCCNELHQEEPLCVCPT[120] [121]LKGASKAVKQQVRQQGQQQGQQGQQLQQVISRIYQTATHLPRVCNIPQVSICPFQKTTPG[180] [181]PYY[183]	100%	A0A0D3A6U8
Brassica rapa subsp. pekinensis	[1]MANKLFLVSATLAFFFLLTNASVYRTVVEVDEDATNPAGPFRIPKCRKEFQQAQHLKAC[60] [61]QQWLHKQAMQSGSGPSWTLDGEFDFDDDMENPQGPQQRPPLLQQCCNELHQEEPLCVCPT[120] [121]LKGASKAVKQQVRQQGGQQGQQGQQLQQVISRIYQTATHLPKVCNIPQVSICPFQKTMPG[180] [181]PSY[183]	96%	M4EC64
B. napus	[1]MANKLFLVSATLAFFFLLTNASIYRTIVEVDEDDATNPAGPFRIPKCRKEFQQAQHLKAC[60] [61]QQWLHKQAMQSGSGPSWTLDGEFDFEDDMENPQGPQQRPPLLQQCCNELHQEEPLCVCPT[120] [121]LKGASKAVKQQVRQQQGQQGQQLQQVISRIYQTATHLPKV CNIPQVSVCPFQKTMPG[180] [181]PSY[183]	94.5%	**P17333**

a The highlighted sequences represent the small and big subunits of the napin. The sequence WTLDGEFDFEDDMENPQG is thought to be the pro-protein degraded during post-translational modification; b percentage of identity was determined with BLAST (Uniprot)

Table 7.3. Primary sequences of napin-like proteins identified in broccoli assembled genome. Difference in residues is highlighted in red. Sequences highlighted in yellow represent the BoNap subunits. Hyphens are used for optimized alignment.

Protein name	Sequence
BoNap1	[1]MANKLFLVSATLAFFFLLTNASVYRTVVEVDEDDATNPAGPFRIPKCRKEFQQAQHLRACQQWLHKQAMQSGSGPSWTLDGEFDFEDDMEN[91]
	[92]PQGPQQRPPLLQQCCNELHQEEPLCVCPTLKGASKAVKQQVRQQGQQQGQQGQQLQQVISRIYQTATHLPRVCNIPQVSICPFQKTTPGPYY[183]
BoNap2	[1]MANKLFLVSATLALFFLLTNASIYRTVVEVEEDDATNPAGPFRIPKCRKEFQQAQHLRACQQWLHKQAMQSGSGPSWTLDGEFDFEDDMEN[91]
	[92]PQSPQQRPPLLQQCCNELHQEEPLCVLPNLERSIQSR*TTGSTTARTAGTAAAASN*SYLPDCYALT*SLXXXXFLVVFVLGSWFLLNY*RIYY[183]
BoNap3	[1]MANKLFLVSATLAFFFLLTNASIYRTVVEFDEDDATNSAGPFRIPKCRKEFQQAQHLRACQQWLHKQAMQSGGGPSWTLDGEFDFEDDMEN[91]
	[92]PQGPQQRPPLLQQCCNELHQEEPLCVCPTLKGASKAVKQQIQQQGQQQGKQQ---------MV-SRIYQTATHLPKVCNIPQVSVCPFQKTMPGPSY[183]

Second method of extraction of napin-like protein

Perera et al. (2016) extracted and isolated rapeseed napin with a simpler method which only requires low pH buffer and hydrophobic chromatography. Based on this work, 100 g of seeds were milled and defatted, and extraction of the napin was performed at pH 3.0. Following extraction, the pooled solution was filtered, dialyzed and submitted to hydrophobic interaction chromatography using a Hi-Trap Phenyl Sepharose™ column (Fig. 7.5A). The whole fraction isolated after elution (with low salt buffer) was desalted, dialyzed and freeze-dried. A SDS-PAGE electrophoresis (20 µl, 1 mg/ml) revealed the presence of one band in the range 14.4-17 kDa and two bands after reduction with DTT (Fig. 7.5B).

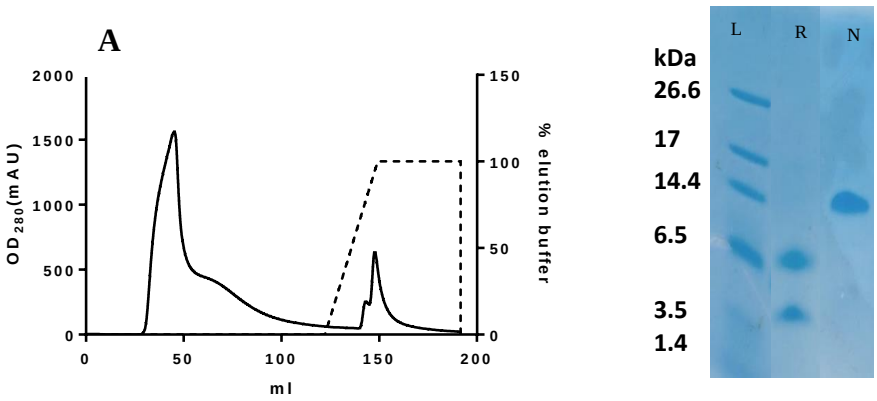


Figure 7.4. Isolation of BoNap submitted to hydrophobic interaction chromatography (HIC) (**A**) and SDS-PAGE electrophoresis 10 ul protein fraction (**B**). Based on the work of Perera et al., the eluted fraction was collected, submitted to desalting column, dialyzed overnight in 2,000 Da cut-off and freeze-dried overnight before use (L: ladder; R: DTT-reduced fraction; N: native fraction). Repeated lanes have been omitted for readability reasons.

After protein transfer onto PVDF membranes (Fig. 7.6), N-terminal sequencing of the 10 first residues of the highest molecular weight subunit confirmed the napin sequence. Two bands of a size below 15 kDa were identified and the N-terminal sequencing of the first 10 residues of the peptide with the highest molecular weight were identified as 1. P; 2. Q, (A); 3. G, (S); 4. P; 5. Q, (F); 6. Q; 7. (R, I), (Q); 8. P, (R); 9. P; 10. L, (P). The identification of the residues in brackets was unclear. Contrary to the first method of extraction, only two bands were shown on the SDS-PAGE, which could indicate the absence of residual groups.

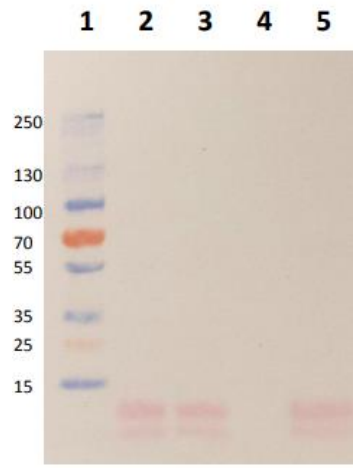


Figure 7.5. PVDF blot (provided by LakePharma, Inc.) of the eluted fraction after reduction (red bands). The two red bands represent the subunits of BoNap. The different lanes represent repeats at different concentrations of sample. 1: Ladder;2: BoNap 5ug; 3 BoNap: 5ug; 4: 1X SDS; 5 : BoNap:10 ug.

Table 7.4. Sequence comparisons between large band of newly isolated protein and indexed napins

Protein name	Amino acid sequence
Large band	$^{1}PQ_{(A)}G_{(S)}PQ_{(F)}Q(R, I,Q)P_{(R)}PL_{(P)}{}^{10}$
BoNap1 large subunit	^{1}PQGPQQRPPLL^{10}
B. napus large subunit	^{1}PQGPQQRPPLL^{10}

Antifungal activity

The antifungal potency of the broccoli napin-like protein was tested against the fungi *F. culmorum, A. niger* and *P. expansum*, in a 2-fold dilution series. After 120 h of incubation, BoNap inhibited conidial germination of *F. culmorum* and *P. expansum* with a MIC between 1.15-4.6 uM. Within the range of concentration tested, BoNap did not inhibit the germination of *A. niger* microconidia (Table 7.3). However, the germination of *A. niger* was delayed by BoNap at a concentration of 18.5 µM (Fig. 7.7). The conidial germination in presence or absence of BoNap was observed under microscope (Fig. 7.8). *F. culmorum* is one of the major contaminants of cereal crops in the field and was chosen as the target organism for the subsequent studies.

Antibacterial activity

The growth of representatives of Gram-positive and Gram-negative pathogenic bacteria was followed over 24 h in the presence of the protein (0-37 µM) in a microtiter plate assay. All the bacterial species were resistant to BoNap with a MIC > 37 µM (Table 7.3).

Table 7.5. Sensitivity of fungal and bacterial species to BoNap, showing MIC (µM) values

	Minimum inhibitory concentration (µM)					
	½ PDB	Heat treatment	½ PDB + NaCl		½ PDB + $CaCl_2$	
Fungi			50 mM	150 mM	1 mM	5 mM
F. culmorum	1.15-4.6	2.3-9.2				
A. niger	>37	ND	ND	ND	ND	ND
P. expansum	1.15-4.6	ND	ND	ND	ND	ND
Bacteria						
***Salmonella enterica* DSM17058**	>37	ND	ND	ND	ND	ND
***E. coli* DSM300083**	>37	ND	ND	ND	ND	ND
***S. aureus* DSM13661**	>37	ND	ND	ND	ND	ND
***S. aureus* DSM20231**	>37	ND	ND	ND	ND	ND

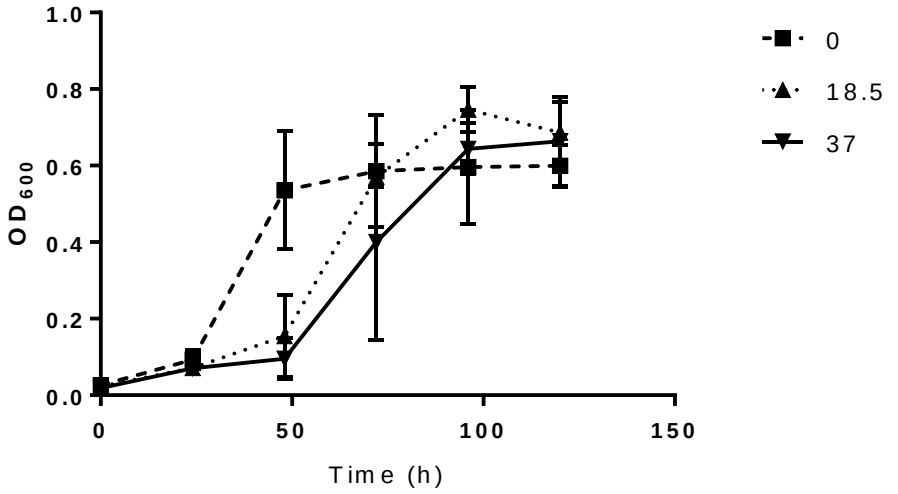


Figure 7.6. Germination of *A. niger* conidia in presence of BoNap**.** Concentrations (in µM) are indicated in the legend. Only concentrations showing significant activity are shown on the graph.

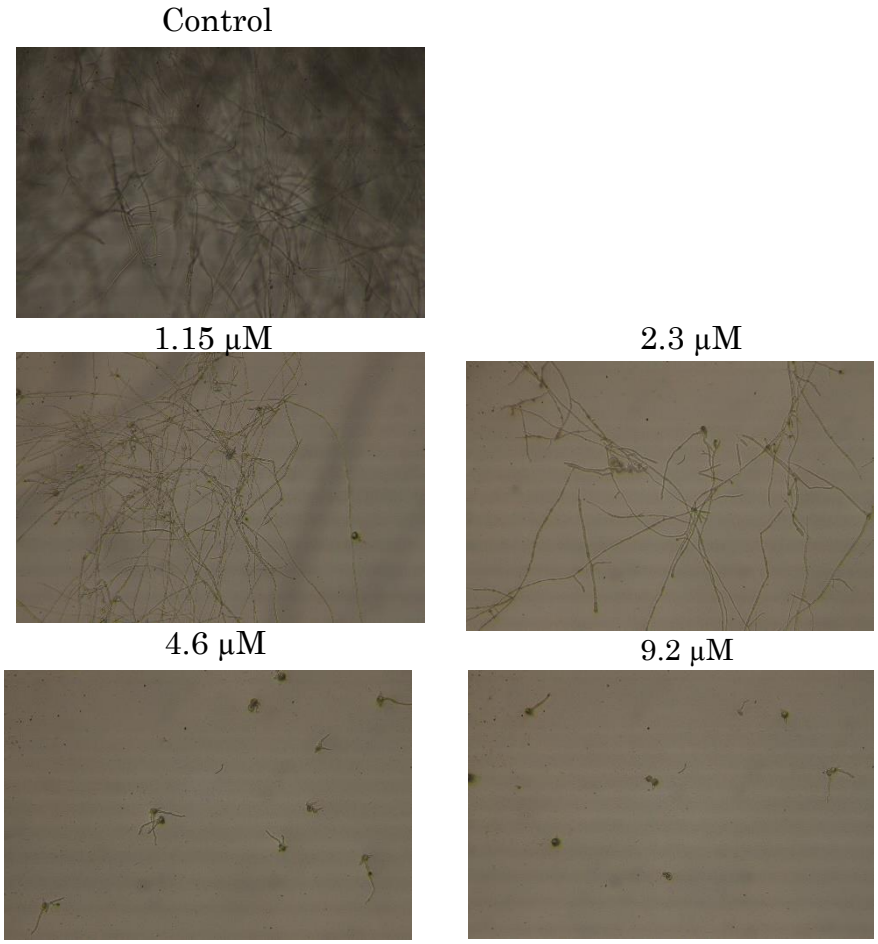


Figure 7.7. Microscopic observation of F. culmorum conidial germination after 120 h incubation in presence of BoNap (0-9.2 µM)

Heat stability and salt sensitivity of BoNap

The heat stability of BoNap was tested at 100ºC for 15 min and monitored by determining if its antifungal activity against *F. culmorum* was retained following heat treatment. The activity of the protein was slightly altered, with a 2-fold increase in MIC against *F. culmorum* (data not shown). However, the inhibitory effect of BoNap towards *F. culmorum* germination was strongly reduced to the presence of monovalent and divalent cations, with at least a 4-fold increase in its MIC, when incubated in NaCl or $CaCl_2$ solutions (Table 7.3).

Structural analysis of BoNap

The CD spectrum of the napin was determined in DIW and in 20 mM SDS micelles as a membrane-mimicking environment. In DIW, the CD spectrum of the broccoli napin presented a positive peak at ~190 nm and two negative peaks at 205 and 225 nm (Fig. 7.9). This spectrum is commonly associated with structures which contain a high number of helical features. This characteristic spectrum was slightly more defined in the membrane-mimicking SDS environment, with a higher positive value of angle deviation.

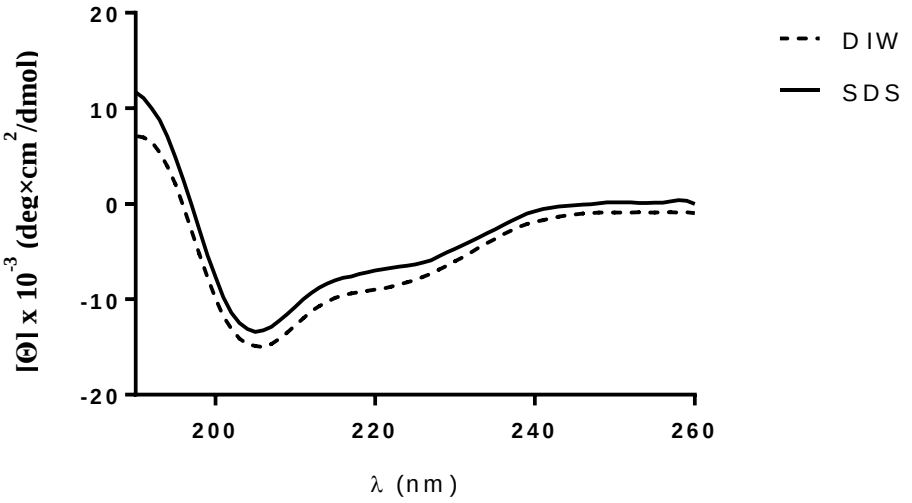


Figure 7.8. Circular dichroism spectra of BoNap in DIW (dashed) and 20 mM SDS micelles (solid). In both cases, the spectrum is characteristic of a high content in helical structures.

Mechanism of action

The ability of napin to disturb the fungal plasma membrane permeability was determined via the uptake of the nucleic acid-binding dye, propidium iodide. The uptake of PI in fungal hyphae was observed with confocal microscopy (Fig. 7.9). After 2 h of incubation of *F. culmorum* hyphae with BoNap, the protein induced an increase in membrane permeability in a dose-dependent manner.

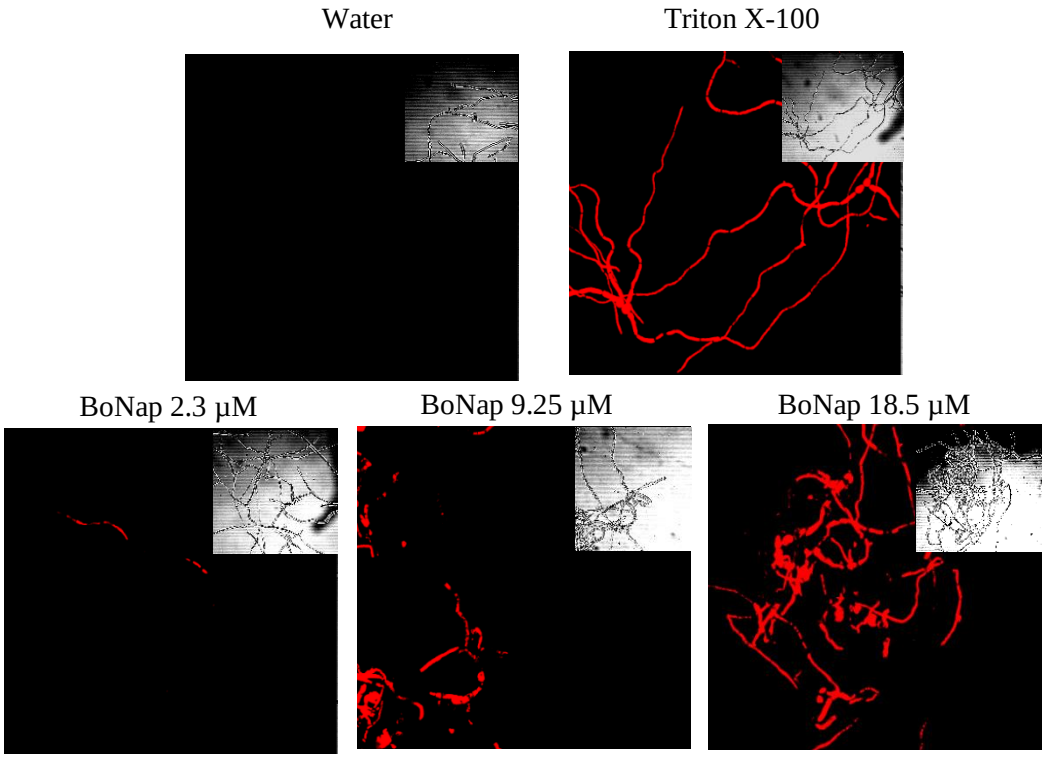


Figure 7.9. Permeabilization of *F. culmorum* hyphae observed under confocal microscopy after 2 h incubation with BoNap (2.31-18.5 µM). Insets show the transmitted light picture of *F. culmorum* hyphae.

The presence of protease inhibitors in plants is an efficient protection mechanism against insects and phytopathogens. The isolated broccoli protein inhibited the activity of the protease trypsin in a paranitroaniline release assay, with an IC_{50} of 9.25 µM and a complete inhibition of trypsin at 18.5 µM (Table 7.4, Fig. 7.10).

Table 7.6. Trypsin-inhibitory activities of broccoli protein

Protein concentration (µM)	% of trypsin inhibition
18.5	98.9 ± 0.51
9.25	50.2 ±-5.1
4.62	22.1±-5.2
2.31	13.3 ±-8.5

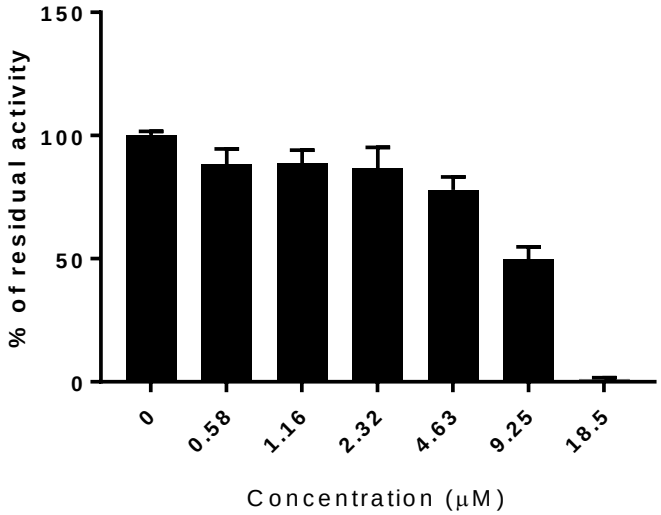


Figure 7.10. Residual trypsin activity after incubation with BoNap via monitoring paranitroalinine

Haemolytic and cytotoxicity activities

The safety of BoNap for food applications was determined via a haemolytic and a cytotoxicity assay towards fresh sheep erythrocytes and colonic Caco-2 cells, respectively. The broccoli protein did not display significant haemolytic or cytotoxic activity at concentrations up to 18.5 µM (Fig.7.11).

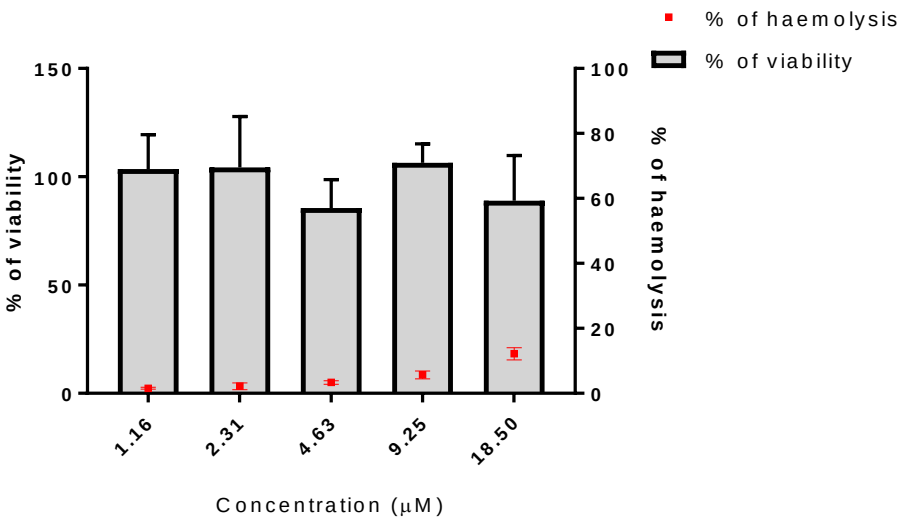


Figure 7.11. Haemolytic and cytotoxic activities of BoNap

Barley grains protection

The application of an aqueous extract (pH 3.0) of broccoli seeds to reduce fungal spoilage of ingredients was performed on barley grains. Grains were contaminated with *F. culmorum* spores and different concentrations of broccoli seed extracts were spread over the grains. Following a 1 week-incubation period, the ergosterol concentration on grain samples was determined, as a marker of fungal contamination. As shown on the Petri dishes presented in Fig. 7.12, fungal growth occurred heterogeneously between the samples. However, a highly concentrated broccoli seed extract (60 mg/ml) completely prevented fungal spoilage of grains (Figs. 7.12 and 7.13).

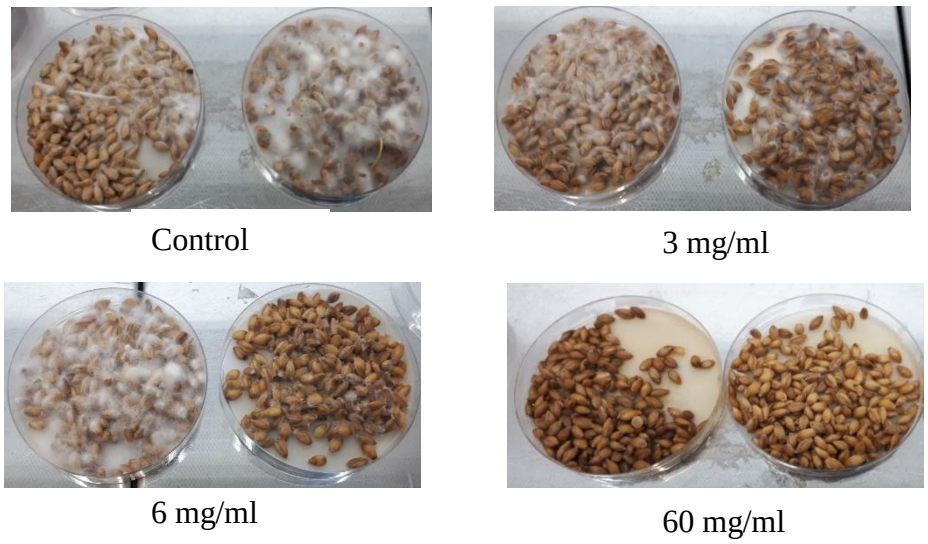


Figure 7.12. Fungal spoilage of grains after 1-week incubation. Two dishes are presented to highlight the heterogeneity of fungal spoilage and the broccoli extract efficacy.

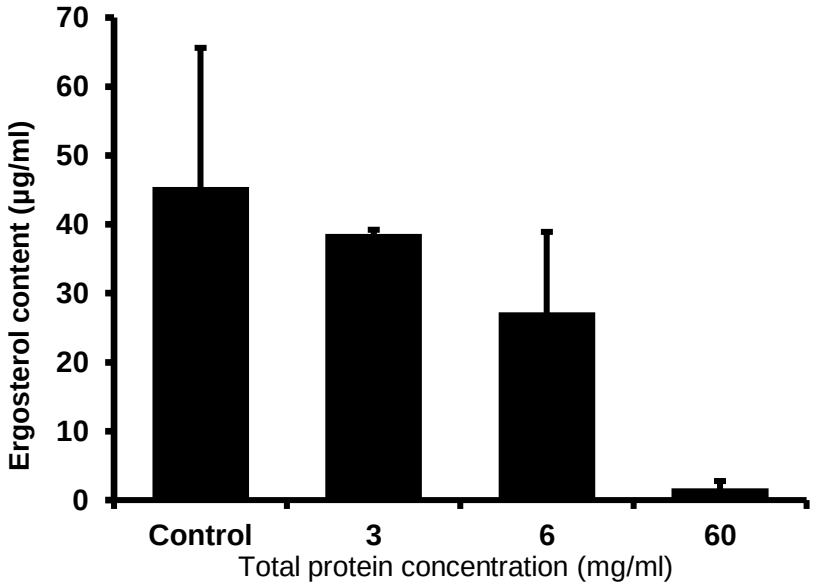


Figure 7.13. Ergosterol content of infected grain samples submitted to treatment with broccoli seed extract

7.4 Discussion

Preventing fungal contamination of food to reduce global food waste is an urgent matter. Foodborne illnesses caused by microbial contamination raises necessities to find alternative sources that are safe to both human health and the environment. Although current chemical preservatives are generally efficient, they possess several limitations and are increasingly rejected by consumers (Oliveira et al. 2014). Thus, research into novel alternative preservatives remains substantial. Water-soluble extracts of certain plants are a promising solution, due to the plants potent immune system which includes several classes of antifungal proteins (Hegedüs & Marx 2013, Kim et al. 2009). Plants of *Brassicaceae* family, including *Brassica oleracea* var. *italica* (broccoli), are a rich source of PR proteins, including defensins (de Beer & Vivier 2011, Terras et al. 1992a), lipid transfer proteins (ns-LTPs) (Lin et al. 2007) and 2S albumins (Agizzio et al. 2006, Terras et al. 1992b). Broccoli extract and its phytoconstituents have also been demonstrated to inhibit and reduce involved in the reduction of foodborne pathogens including *S. aureus* and *E. coli* (Sibi et al. 2013).

In this study, water- and buffer-soluble extracts of broccoli seeds showed antifungal activities against the cereal contaminants, *F. culmorum*, *A. niger* and *P. expansum*. In the research of antifungal compounds, a protein presenting high similarity in primary sequence to a napin from *Brassica napus* was isolated (Muren et al. 1995). Genome assembly revealed the full sequence of the protein comprising 183 residues, and an estimated molecular weight of 14.8 kDa of the mature protein (Expasy ProtParam tool). When submitted to SDS-PAGE electrophoresis, a band around 15 kDa was revealed. Plants express napins in the developing embryo and the signal peptide and additional peptides are removed during maturation and the transfer into the endoplasmic reticulum. Napins are present in seeds as pro-napin proteins of about 180 amino acid residues and are used during seed development as a nutrient (Barciszewski et al. 2000). They represent around 20% of total protein in canola (*B. napus*) seeds, but higher concentrations have been reported in germinated seeds and during seedling growth. Napins have been isolated from many plant species, including *Brassica* species, such as mustard and canola, and non-*Brassica* species, such as chili pepper (Jyothi et al. 2007, Ngai & Ng 2004a, Perera et al. 2016, Ribeiro et al. 2007). The broccoli napin belongs to the 2S storage albumin family, as highlighted by the

sequence homology between napin isolated from *B. napus* (PFRIPKCRKEFQQAQHLR–ACQQ) or Chinese kale (PFRIKKRQGPFERP) and 2S albumin isolated from radish seed (P–R–QRCQKEFQQSQHLR–ACQR) or chili pepper (P–QCPRCSQQFQQAKQLRCSCQS). First, broccoli napin was extracted according to the extraction of AMPs from seeds as described by Terras (Terras et al. 1992a), involving ion exchange and size exclusion chromatography. Initial SDS-PAGE electrophoresis of the isolated fraction revealed the presence of 3 bands, including 2 subunits and an additional band, possibly due the presence of the propeptide or residual acid groups. An extremely high degree of homology among napin variants also makes their separation and purification very difficult (Barciszewski et al. 2000). The second extraction method, described by Perera et al. (Perera et al. 2016), was based on low pH water-solubility of the napin and thus facilitated the extraction and partial purification. SDS-PAGE electrophoresis highlighted the 2 subunits of the protein after reduction. The signal sequence and additional peptides are commonly eliminated during maturation of the peptide and mature napins are made of 2 dissimilar subunits that are derived from proteolytic cleavage of a precursor. The small subunit of napin has a molecular mass of approximately 4.5 kDa and is composed of about 40 amino acids containing two cysteine residues. The large subunit is a 9-10 kDa peptide of about 90 amino acids and six cysteines. The two subunits are connected by two inter-chain disulfide bonds. Some napin-like proteins have, however, been reported to be single-chain proteins. For instance, charantin, a ribosome-inactivating protein isolated from balsam pear, is a single-chain peptide and structurally related to the large chain of the napin-like proteins (Parkash et al. 2002). Interestingly, the sequence of napin small subunit shares sequence homology with the mammalian AMP, cathelicidin, and the large subunit with a trypsin inhibitor from field mustard suggesting specific activities of the subunits (Ngai & Ng 2004a).

The broccoli napin, termed BoNap, presented a prevailing helical conformation, as revealed by CD spectroscopy. Albumins tend to adopt a compact three-dimensional structural scaffold comprising a bundle of five α-helices (2 in the small subunit and 3 in the large subunit), representing over 50% of structural elements, and a C-terminal loop folded in a right-handed superhelix stabilized by four conserved disulfide bonds (Moreno & Clemente 2008, Pfeifer et al. 2015). However, these results appear to differ between several studies and the spectral intervals used during prediction. Thus,

deconvolution of the far-UV CD spectrum of napin revealed about 25% α-helix and 38% β-sheet structure at neutral and slightly acid pH. Napin isolated from *B. napus* has a helical content of 26%, whereas a *B. juncea* napin has 40% helical content (Jyothi et al. 2007).

The amphipathicity of napin and 2S albumins helices is thought to be the determinant of their antifungal activity and their ability to permeabilize the hyphal membrane of fungi (Terras et al. 1993b). Thus, the incubation of *F. culmorum* hyphae with BoNap caused the permeabilization of the fungal plasmalemma, as observed for mustard napin that strongly interacts with phospholipid bilayers. The main role of napin, 2S albumins or other glutamine-rich proteins is nutrient storage for plant development. However, these storage proteins have demonstrated antifungal and antibacterial properties against phytopathogens, including *Fusarium* spp. or *Aspergillus* spp (Agizzio et al. 2006, Wong et al. 2017). Synergistic activity between plant thionins and storage proteins has also been observed to damage fungal membranes (Barciszewski et al. 2000). Thus, the combination of barley or wheat thionins with radish 2S albumins or Bowman-Birk-type inhibitors, improved synergistically by 2- to 55- fold the activity of thionins against filamentous fungi (Terras et al. 1993a). *Brassica napus* 2S albumins (Bn-2S) have been shown to inhibit *F. culmorum* and *F. oxysporum* with IC_{50} values of 40 and 100 µg/ml, respectively (Terras et al. 1993b). Passion fruit albumins can inhibit *Fusarium graminearum* or yeast contaminants, such as *Candida. tropicalis* and *Saccharomyces cerevisiae* (Meneguetti et al. 2017, Pelegrini et al. 2006). The BoNap activity was limited to antifungal inhibition and was inactive against *S. aureus* or *E. coli*, as it has also been observed with napin-like proteins isolated from *B. campestris* or *B. chinensis* cv dwarf. However, *B. chinensis* napin shows inhibitory activity against *P. fluorescens* and*Bacillus* spp. (Ngai & Ng 2004b).

In addition to membrane permeabilization, BoNap inhibited trypsin activity *in vitro* with an IC_{50} of 9.25 µM. Trypsin-inhibition has been reported previously for *B. chinensis* or rape seed napins with IC_{50} values of 8.56 and 3.0 µM, respectively (Ngai & Ng 2004b). It is an important mechanism of action used by plants to protect against insect and fungal pathogens, that express proteinases to disrupt protein and cellular machinary of plants (Habib & Fazili 2007). In contrast to antifungal activity, a trypsin inhibitory activity has been shown not to be affected by the presence of salts and to be increased markedly after heat treatment (Pesoti et al. 2015). Several plant defensins

and thionins also possess this protease inhibitor activity, to inhibit fungal and bacteria growth (Habib & Fazili 2007). Thus, a corn-trypsin inhibitor inhibited the production of extracellular α-amylase of *A. flavus* (Chen et al. 1999). The remarkable large spectrum of action and the resistance to proteolytic degradation stimulates the evaluation of these protease inhibitors.

The BoNap was only tested for trypsin-inhibitory and membrane permeabilization properties, but the antimicrobial properties of 2S albumins have also been attributed to other mechanisms. Thus, the 2S albumin, Ca-Alb, isolated from chili pepper also inhibits fungal growth by abolishing the glucose-stimulated acidification of the medium and by inducing nitrous oxide production in yeasts species (Agizzio et al. 2006, Ribeiro et al. 2007). The napin complexes and the isolated small subunits are also antagonists of the Ca^{2+}-binding protein, calmodulin (CaM), which mediates many of the cellular actions of Ca^{2+}. This is probably due to the fact that CaM and the small subunit of napin contain a similar α-helix-hinge-α-helix motif in their structures, involving competitive inhibition due to the binding of the small subunit of napin to kinases (Neumann et al. 1996). The serine residues at the C-termini of napin subunits can be phosphorylated by calmodulin-dependent plant kinases (CDPKs) (Barciszewski et al. 2000). These enzymes have multiple roles in plant development but are also key components of the innate immunity (Lehti-Shiu & Shiu 2012). Their phosphorylation is involved in the inhibition of tryptic digestion, as demonstrated *in vivo* (Neumann et al. 1996). Medium acidification and CaM activity have been shown to be essential for hyphal proliferation. Interestingly, the CaM antagonist activity of radish seeds, together with napins, disappears during seed germination and might reduce the antifungal potency (Polya et al. 1993) .

The broccoli napin retained its antifungal activity after heat treatment at 100°C. Heat-stability of 2S albumins has been attributed to the presence of a well-conserved skeleton of 8 cysteine residues in the α-helix-rich structure. Indeed, 2S albumins mostly retain their secondary structures after heating to 95°C (Moreno & Clemente 2008). In these albumins, a partial and reversible loss of helical structure appeared at this temperature, which may explain the slight loss of antifungal activity after heating at 100°C, but conserved activity at lower temperatures. Similarly, napins isolated from canola showed denaturation only when heated at 110°C (Perera et al. 2016). However, this stability appears to be plant dependent since a 50% heat-induced unfolding has

also been observed at 60-67°C for rapeseed napin (Folawiyo & Apenten 1997). The facilitated solubility of broccoli napin at pH 3.0 indicated its stability in low pH solution. Previous studies of napin and its precursor show that very little or no structural changes occur at different pH (Krzyzaniak et al. 1998). In addition, the translation-inhibitory activity of a napin-like protein from *B. chinesis* is conserved over a pH range of 4-11 (Ngai & Ng 2004a). However another study demonstrated acid-induced structural modification of rapeseed napin (Folawiyo & Apenten 1997). In addition, pH variation might affect the thermal stability of the napin as shown for rapeseed napin whose denaturation temperature was of 101°C and 88°C at pH 6.0 and 3.0, respectively. At these temperatures, the denaturation is irreversible due to hydrophobic aggregation of the unfolded protein (Krzyzaniak et al. 1998). Thus, although heat stability at 100°C is a good asset for food applications, the acidic profile of the product has to be considered. Despite heat- and pH-stability, the food application of BoNap may suffer a common drawback that is salt sensitivity. The MIC of BoNap to *F. culmorum* increased at least 4-fold in the presence of NaCl or $CaCl_2$. Salt sensitivity is known to be fungus-dependent and combination of mono and divalent cations is even worse against AFPs. The sensitivity of 2S albumins to salts has been attributed either to the interaction with the salts, leading to modification of their structure and hydrophobicity reduction, or to a decrease in electrostatic interactions (Terras et al. 1992b, Vassiliou et al. 1998). Despite a severe salt sensitivity, the synergism between 2S albumins and thionins in high ionic strength media might be involved in a defensive role *in planta* (Terras et al. 1992b).

Like several antifungal peptides/proteins of plant sources, including 2S albumins, *BoNap* did not show haemolytic activity or cytotoxic activity on colonic Caco-2 cells. However, the toxicity of broccoli extracts has been demonstrated against rats and napin isolated from *B. campestris* showed activity against the tumor cell lines MCF7 and HepG2, and inhibits the activity of HIV-1 reverse transcriptase (Lin et al. 2007, Vassiliou et al. 1998). This anti-proliferative activity has been attributed to their protease inhibitory properties. Despite low toxicity to gut cells, the allerginicity of previously isolated napins, their resistance to heat treatment and to trypsin digestion might be a limitation to their application, especially as food preservatives (Moreno & Clemente 2008). The allergenicity of the broccoli napin was not tested due to the absence of human sera, but this is an essential property to investigate.

Just like lipid-based formulations or lemongrass essential oil, our study highlighted the potential of a low pH aqueous extract of broccoli seed in reducing fungal contamination of raw ingredients and suggested its potential application as a new food preservative (Coda et al. 2008). However, the protein concentration required to reduce significantly fungal growth was much higher than the MIC of purified BoNap observed in in vitro assays. As reported for many antimicrobials, their efficacy in food matrices can be hampered by the interaction with other food components, such as carbohydrates or proteins (Davidson et al. 2005), and physiological salt concentrations. The potential of broccoli is not limited to food applications but also holds promises for crop applications. Demonstrating such an application, a defensin gene (http://www.uniprot.org/uniprot/J7ENB9) from *Brassica oleracea* var *italica* has been expressed and shown to delay the growth of mould mildew. Interestingly, genome analysis of broccoli seeds allowed us to identify the presence of defensin-like peptides, with sequences with high homology with defensins from other Brassica species, which could be applied in crop protection. In conclusion, the reduction of food and ingredient losses faces many challenges, including changes in people's behaviour and better preservation methods. In conclusion, a novel 2S albumin napin was isolated from a water-extract of broccoli seeds and structurally and functionally characterized. The antifungal potency, the stability and non-cytotoxic nature of this protein make it a candidate with great potential to respond to the growing need of novel and natural food preservatives. In comparison to previously used water-soluble plant extracts, the more selective isolation procedure, as shown with the preferential low pH solubility of BoNap, offers the possibility to standardize the extraction and to reduce the drawbacks associated with full plant extracts. However, cost-effective procedures for the isolation of natural preservatives remain a major challenge.

7.5 Acknowledgements

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Appendix: Supplementary material

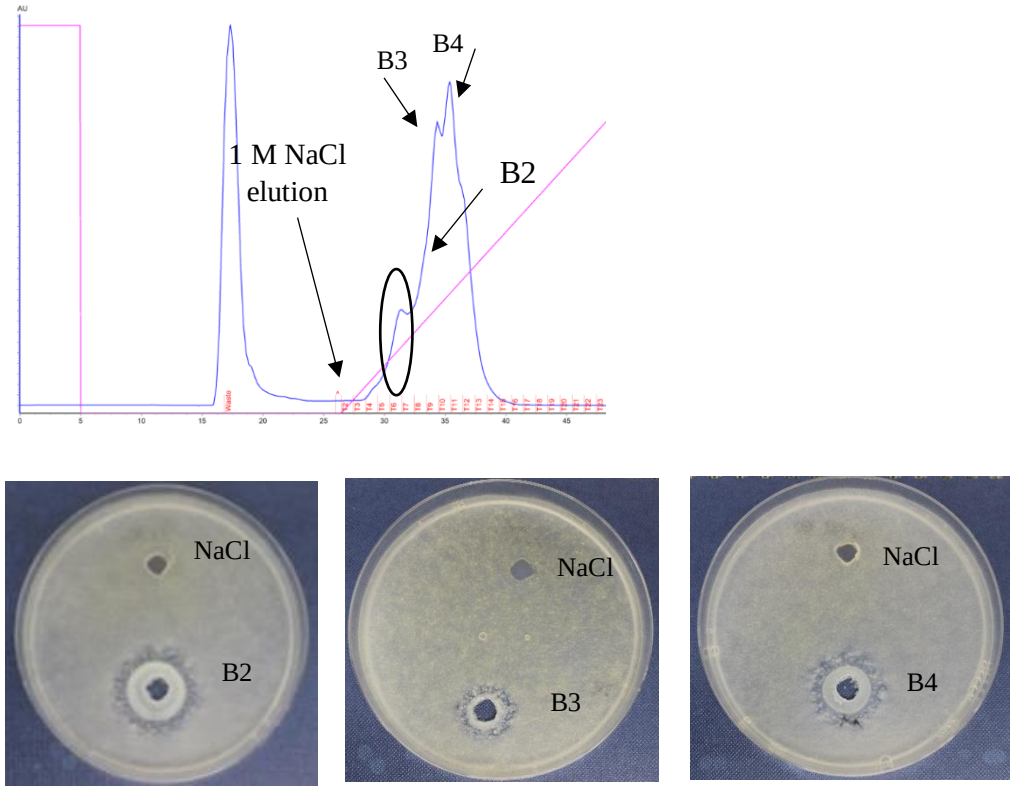


Figure 7.S1. Antifungal activity of fractions purified with cation-exchange chromatography on Sp-Sepharose column. The activity was tested against *F. culmorum* in a radial diffusion assay

Chapter 8
General Discussion

In 2015, the FAO estimated that about 10.7% of the world's population was suffering from chronic undernourishment, with a large majority living in lower-middle-income countries. In 1985, Band Aid was singing "Feed the world" during Live Aid at Wembley stadium. The year 2015 was also marking the date of the first report of two major programs that were set up to reduce hunger worldwide. Despite the progress that permitted a 42% reduction in the prevalence of undernourished people between 1990–92 and 2012–14, it was estimated that undernutrition was the cause of 3.1 million child deaths annually (UNICEF / WHO / World Bank Group 2018). Between the 1960s and 2017, the world produced enough food to feed everyone on the planet with an increase of caloric availability and food diversity (FAO [Food and Agriculture Organization of the United Nations] 2017), along with improved access to food. As a proof of this success, the Sustainable Development Goals program aimed at ending all forms of malnutrition by 2030 (FAO [Food and Agriculture Organization of the United Nations] 2017). A principal problem was and is still that many people in the world do not have sufficient income to purchase (or land to grow) enough food or access nutritious food. Throughout the last 50 years, natural disasters and unstable weather patterns, accentuated by climate change, have become increasingly common, leading either to drought or, on the other extreme, stormy seasons and flooding, causing major damage. The ecological effects of climate change affected the emergence of diseases on a larger scale, which ultimately could prolong the cycle of malnutrition (FAO [Food and Agriculture Organization of the United Nations] 2017).

Food loss and waste affected local and global environments at both production and consumption levels, and with an impact on climate deregulation. The

protection of foods and ingredients was an urgency, which would reduce, at least partially, the crisis of undernutrition. The use of natural antimicrobials for food preservation, either as an ingredient or combined with traditional techniques (Malhotra et al. 2015, Nielsen & Rios 2000), was not a new concept. As a first line of defence and before the evolution of adaptive immunity, nature has placed a complex mixture of compounds with antimicrobial properties on the regions that are most exposed to invading pathogens. This innate immunity represents the principal defence system for most living organisms. Amongst the arsenal, AMPs had gathered a great interest, due to their broad spectrum of activities and multiple mechanisms of action, not only for therapeutic applications but also, as novel food preservatives. However, the research involving natural AMPs was mostly focused on food-borne pathogens and in spite of many reports of the use of natural (Lucera et al. 2012, Rai et al. 2016, Rydlo et al. 2006) and synthetic (Appendini & Hotchkiss 2000, Thery et al. 2018) AMPs against food spoilage, only a few were accepted by the FDA (Keymanesh et al. 2009, Rai et al. 2016).

Natural AFPs, which had been reported to inhibit food fungal (moulds and yeasts) spoilage, were presented in **Chapter 2**. The antifungal spectrum, the structural properties, and the mechanisms of action of antifungal peptide-based compounds from living organisms of all domains were listed. With one of the richest diversities of peptide-based compounds with antifungal properties, plants were of particular interest. The knowledge gained with the continuous identification of new AFPs, the identification of structural and sequences determinants and the development of bioinformatic tools enabled the generation and prediction of active antifungal peptides. The drawbacks associated with the use of identified AFPs against food spoilage, including in the food environment, were investigated. Using a synthetic, a recombinant and a natural approach, the aim of this work was to study the activity of novel AFPs against fungal spoilage, the underlying mechanisms of the antifungal activity and the potential applications of the peptides as a new generation of food preservatives.

From the 1960s and the development of solid-phase technology, pioneered by Du Vigneaud and Merrifield, the synthesis of peptides had been continually facilitated (Merrifield 1963). A first objective of this research was to test the antifungal activity

of synthetic AMPs against fungal contaminants of foods. The research carried out with synthetic versions of the human β-defensin 3 (HBD-3), and the cowpea defensin Cp-thionin II (here, termed KT43C), was presented in **Chapter 3** and **Chapter 4**, respectively. The two peptides inhibited the germination of conidia of the fungal species *F. culmorum*, *A. niger* and *P. expansum*, at a micromolar concentration. Despite the absence of native disulfide bonds, the antifungal activity of the synthetic peptides was not impaired, as highlighted for other linear AMPs (Wu & Hancock 1999) (Liu et al. 2008). In addition, the random coil structure, highlighted by CD spectroscopy, and the flexibility of these disulfide-bond-lacking peptides are thought to explain their heat stability. Interestingly, similar behaviours in terms of structures and mechanisms of action, such as membrane permeabilization, were observed between the natural and synthetic versions of the peptides (Van Der Weerden et al. 2010), (Mason et al. 2007). However, the pattern and the full mechanisms of antifungal activity required deeper investigation (Thevissen et al. 2004). KT43C did not show haemolytic activity towards sheep erythrocytes, up to 200 µg/ml. Mammalian cells were generally known to be less sensitive than fungal or bacterial cells to cationic AMPs, especially due to the presence of cholesterol in their membrane (Mason et al. 2007). However, the lack of hemotoxicity could also be explained by the presence of physiological salt concentrations, salt sensitivity being a common feature of defensin-like peptides (Adem Bahar & Ren 2013, Vriens et al. 2014).

The high purity of synthetic compounds and the numerous possible modifications are major advantages for the development of optimized peptides, with improved activity and stability. However, these advantages have to be put into perspective, given the high cost of synthesis of long peptides (€100-600/gram, depending on the length and the chemistry used for synthesis), although this cost tended to decrease and the fact that in the global food market, of 30 billion dollars, only 5% of additives aims to fight bacterial spoilage and rapid deterioration.

This drawback was even more startling for food applications for which a high amount is generally required. Thus, the amidated ultrashort peptide O3TR (Orn-Orn-Trp-Trp-NH_2) and its ester C12-depsipeptide (lauric acid at the N-terminus) described previously by Laverty and al. (Laverty et al. 2010) were tested against yeasts and filamentous fungi. The study, presented in **Chapter 5**, highlighted the strong

antifungal activity of the two peptides, which inhibited all fungal species in a MIC range of 6.25-50 μg/ml. As observed against bacterial pathogens, the lipidation at the N-terminus enhanced the antifungal activity against the food spoilage fungi 4-fold (Chu-Kung et al. 2004, Laverty et al. 2010, Malina & Shai 2005). The choice of lauric acid was based on previous work by Laverty et al., showing that longer chains could result in oligomerization, which would reduce the antimicrobial activity (Laverty et al. 2010) (Matsuzaki et al. 1997). This modification also improved the stability of O3TR in salt solutions and to proteolytic digestion. As revealed by a permeabilization assay and CD spectroscopy, the esterification, and the subsequent increase in peptide helicity and hydrophobicity, also favoured penetration into the conidia and the fungal cytoplasm. However, the price to pay for such improvements was an increase of hemotoxicity and toxicity towards gastrointestinal cell line. Research into the optimal therapeutic index (TI, generally defined as the ratio of antimicrobial activity (IC_{50}) to toxicity (EC_{50})) is the challenge and the objective of most peptide researches. Interestingly, the potential of fatty acids was studied beyond their antifungal properties, including antiparasital and antiviral activities (Carballeira 2008). They also were considered for food preservation but their application was limited due to their strong impact on organoleptic properties. Despite their salt sensitivity, the application of HBD-3 and KT43C in baked goods or O3TR in beverages delayed the development of fungal growth and thus extended the shelf life of these products. However, the presence of ingredients (carbohydrates, fat and proteins) and the heterogeneity of food matrices may affect the antifungal activity of the peptides, explaining the limited improvement (several days) of food preservation (Davidson et al. 2005, Skovgaard 2004). USPs are economically more realistic than synthetic defensins but showed poorer stability. In **Chapter 5**, the combination of O3TR with lauric acid induced a problematic toxicity on gastrointestinal cells lines. Although lipidation methods for USPs needed further investigation, such conjugation offered many promises as shown with the lipopeptides gageotetrins. Indeed, these natural compounds, made of 2 to 4 residues and a new fatty acid 3-hydroxy-8,10-dimethyldodecanoic acid (HDDA), isolated from a marine *Bacillus subtilis* showed strong antimicrobial activities, with no cytotoxicity (Tareq et al. 2014). Just like an explorer searching for a relic, further research focused on a novel optimized peptide, being both short (<15 residues), stable and safe.

Based on rational design, a novel short peptide (14-mer) was constructed using the key determinants of antifungal activity and the known sequences indexed in databases. The comparison of the different peptides and online prediction tools led to the design of KK14, with the sequence KKFFRAWWAPRFLK-NH_2, and several analogues. As predicted via online tools, the antimicrobial nature of the parental peptide was presented in **Chapter 6**, this peptide displaying antifungal activity against fungal contaminants of cereals. Among the different modifications, D-enantiomerization, known to confer protease resistance to many peptides, prevented the proteolytic degradation of KK14 by tryspin (Mohamed et al. 2017, Zhao et al. 2016). Proteolytic stability is of major importance in drug development using HDPs, which are known to have a short half-life. Another option is the improvement of the entire formulation, with the use, for example, of nanoparticles or polymers (Jebali et al. 2014, Liu et al. 2009, Raman et al. 2015). The substitution of Pro_{10} with Arg induced a high increase in antifungal activity, whereas, the insertion of the unnatural bulky residue β-β diphenylalanine (Dip) was not a major improvement. However, the Proline-substituted peptide showed a higher cytotoxicity, highlighting the need of further research on this peptide. In addition to residue substitutions, toxicity can be reduced by modifications such as the use of the prodrug methanesulfonate (Huttunen et al. 2011). Despite an overall low cost and the numerous possibilities to optimize their properties, synthetic compounds were rarely to be approved by regulatory authorities for food applications.

Antimicrobials will undoubtedly continue to be needed to provide the food supply that will be demanded in the future but the food industry will always be reluctant to expand the use of food preservatives, however, because of the unknown problems that may result from an increased consumption of such compounds in combination with other additives and food components. The majority of the antimicrobials now being used in food products have been extensively tested for toxicologic safety, and although questions will continue to be asked, an evaluation of the risks versus the benefits indicates that approved antimicrobials are acceptable.

Natural extracts are more likely to be approved such as thaumatin, and strong clinical studies are needed for new products. It was a long time before the launch of new preservatives and the research against fungal food contaminants was at its

beginnings. Only a few novel preservatives were expected to be approved, which implied a collaborative work between the optimization of novel peptides and the reduction of the cost of synthesis. Peptide synthesis seems infinite and more and more complex methods had been considered to improve the stability and the efficacy of AMPs. The non-exhaustive list included the use of dendrimers, cryptides, polymers, peptoids or the methods of delivery, such as liposomes or cyclic lipopeptides.

An alternative to alleviate the cost of synthesis was the use of recombinant peptides, expressed and produced by genetically modified (GM) yeasts, as presented in **Chapter 3**. In spite of a certain critical public perception, the demand for suitable GM strains for food or biotechnological industries was continuously growing. The unlocking of genetic complexity contributed to the genetic make-up of novel recombinant yeast strains with successful applications, especially in the winemaking industry (Schuller & Casal 2005). In **Chapter 3**, the use of the recombinant *Saccharomyces pastorianus* strain, CM-INT-51, expressing a recombinant HBD-3, increased the shelf life of bread compared to a bread prepared with a *S. pastorianus* wild strain. The limited effects on the final product characteristics highlighted the potential of this GM strain, used in a previous study, to protect beer from microbial spoilage (James et al. 2014). The recombinant expression of AMPs by bacterial vectors was commonly used in a cost effective way, mostly for fundamental studies of the peptides. However, the use of eukaryotic organisms for the expression of AMPs was a requirement to obtain the same configuration as the native peptide, especially via the insertion of PTMs. Due to a readily available and cost-effective raw material, GMM have been used to produce AMPs, leading to a high yield at low cost, but several drawbacks were recognized. The first one is the potential direct antimicrobial nature of peptides on the host organism. The latter is also susceptible to producing proteases that could destroy the peptide, a limitation that could be overcome by the fusion of the peptide to larger protein (Chen et al. 2006). Moreover, the use of such organisms and the associated genetic engineering require extensive quality management and regulatory control. Despite a relatively low cost, the price of manufacturing using fermentation of a recombinant organism was not expected to be more beneficial than chemical synthesis in the future and this would involve the need for GMP-grading for industries. The applications of GMM were especially considered in the wine and beer

industry, to improve technological properties. These "bioengineered foods", as termed by the FDA, were not required to carry the GM label as long as the uniformity with traditional product was conserved. However, in the case of the introduction of a gene that encodes for a new compound (not found traditionally), the required evaluation and approval would impair their use. Some companies had taken up the challenge. Thus, using an FDA-approved microbial strain and food-grade chemicals, a recombinant brazzein, an antibacterial peptide sweetener, was produced to similar quantities as found in nature.

Moreover, the novel compounds must not have adverse effects on the environment. Indeed, during food process, recombinant proteins might be released are released or DNA is potentially transferred between yeast species, The last issue is of public concern against the use of GM. Although GM applications in foods were not associated with a high ethical concern, they were not considered to have strong benefits, in contrast to pharmaceutical or certain crops (Frewer 2003). Thus, the GM approach had been successfully used to reducing phytopathogenic threats on several vegetable/cereal crops expressing AFPs. Despite commonly low yield, the insertion of several copies of the genetic material into the GM organisms and/or the optimization of growth conditions can increase the expression of the desired peptide. Challenge with abiotic or biotic stresses could stimulate the expression of AMPs (Belda et al. 2017, Fleet 2007). Thus, when challenged with other fungi, *Aspergillus giganteus* increases its production of an antifungal protein called AFP, which is effective against fungal cereal spoilage (Meyer 2008). The application of such peptide-rich growth medium appeared a promising alternative.

The use of natural antifungal peptides with a limited use of chemical processes appeared as the most economically viable and consumer friendly approach to satisfy nutritional, socio-economic and quality requirements. Lack of knowledge in the population toward food additives also affects their acceptance. Various surveys indicate that some consumers are alarmed about food additives and do not feel well informed regarding their role in food and highlight a distrust to food safety agencies

Although the natural additives do not always represent a benefit compared to chemical ones, in most cases they are believed to be healthier, can carry out various functions in the food, and confer added value (bioactivity, nutraceutical). In order to

characterize the antifungal compounds involved in the antifungal activity of broccoli seed extract, a new protein was isolated by ion exchange and size-exclusion chromatography. Following N-terminal sequencing and bioinformatic analysis, the protein was identified as a napin-like protein, a member of the 2S albumins family, and its full sequence revealed. The first napin was identified in rapeseed (*Brassica napus*)(Muren et al. 1995) and other napin-like proteins have been identified in other *Brassica* and non-*Brassica* species. As described previously by Perera, due to the preferential solubility of napins at low pH, the broccoli napin was successfully extracted with a simple pH 3.0 buffer (Perera et al. 2016). An in depth characterization highlighted similarities with previously studied napins, in terms of structure, size of subunits, and mechanisms of action against phytopathogens (Terras et al. 1993). Thus, BoNap caused the permeabilization of *F. culmorum* plasmalemma and inhibited the action of trypsin, a protease used by insects to degrade plant proteins. However, napins are known to possess a large range of mechanisms, including a CaM antagonist activity (Neumann et al. 1996). In addition to their role in nutrient storage, many 2S albumins have demonstrated antifungal and antibacterial properties against phytopathogens. However, BoNap was inactive against the bacterial pathogens *S. aureus* and *E. coli*, as observed with napin-like proteins isolated from *B. campestris* or *B. chinensis* cv dwarf. Like other 2S albumins, BoNap presented heat-stability, a characteristic attributed to the presence of several disulfide bonds in its skeleton (Krzyzaniak et al. 1998). Two potential drawbacks may limit its application in foods. One one hand, as revealed by the increase of MIC against *F. culmorum*, BoNap suffered a certain salt-sensitivity. On the other hand, a potential allergenicity has been shown for 2S albumins (Moreno & Clemente 2008). Interestingly, analysis of broccoli genomic data enabled the identification of defensin-like peptides, with sequences with high homology to defensins from other *Brassica* species. The potential of broccoli is not limited to food applications but also holds promise for crop application, as reported for other plant defensins.

Several technologies using plant extracts had shown their efficacy for food applications without inducing sensory modifications in final products, including in baking applications (Burris et al. 2012, Hyldgaard et al. 2012, Negi 2012, Nielsen & Rios 2000, Tserennadmid et al. 2011). However, the preferential solubility of BoNap at low pH offered the possibility to standardize the extraction and to reduce the

potential drawbacks associated with full plant extracts, such a poor purity and flavour modifications. In addition, such extract would be easier to apply than synthetic peptides during the food production process (pre and post-harvest contamination). For obvious economic concerns, cost-effective procedures for the isolation of natural preservatives remained a major challenge, and the use of cheaper raw materials, such as by-products, appeared as a more valuable option. Thus, the company Apeel developed a spraying process of a lipid extract of plant skins and stems to create a layer on fruits and vegetables, and protect them from not only microbial spoilage.

As AMPs can act on different targets, the synergistic activity of AFPs is also an important parameter to consider for food applications (Cruz et al. 2010, Kraszewska et al. 2016). Synergistic effects have also been highlighted between traditional antibiotics/antimycotics and AMPs, in combination therapy. This synergy, attributed to the increased uptake of the peptide resulting from the action of the antimycotic on the fungal membrane, would then limit the risk of development of resistant fungal strains. However, in 2015, fungi were not considered as the most dangerous super-villains of the microbial world, and despite the menacing increase in fungal-related diseases, only a few AMPs were approved by the FDA (Gordon et al. 2005). Lastly, the synergistic effect of HDPs seemed to have particularly a major role in the regulation of the immune response. Beyond antimicrobial treatments or food preservatives, the role of AMPs seemed to extend towards multiple horizons.

In conclusion, in the years 2010s, innovative solutions were studied to overcome the food loss and waste. Food preservatives will definitely change throughout the next decades, either tending to natural extracts that need to be thoroughly studied for interactions or to the synthetic chemical ones that will continue to scare the consumers. Careful studies regarding natural additives must be carried on these new technologies and approved by the scientific community, but also by food companies for economic feasibility. A soft transition is expected to occur with a strict control of legislation leading to a simultaneous reduction of additives altogether, relying on new technologies to carry out the same effects.

Beyond their therapeutic applications, antifungal peptides, either synthetic, recombinant or natural were shown to be potential candidates to reduce fungal spoilage of foods. An understanding of the antifungal mechanisms, the limitations and the

advantages of each approach enabled the generation of continuously improved peptides, aiming at saving humanity from hunger.

Luckily, 2050 is not here yet and let us hope that the scenario presented here will remain fiction and could make a great science-fiction movie.

And since every great movie contains momentous quotes, why not quoting the scientist at the origin of the understanding of the microbial world:

"If it is a terrifying thought that life is at the mercy of the multiplication of these minute bodies, it is a consoling hope that Science will not always remain powerless before such enemies", Louis Pasteur, before the Academy of Sciences, April 29, 1878.

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Appendix I

Novel peptide conjugates: characterisation of the candidacidal activity, immunomodulation and efficacy as topical treatment for oral candidiasis

A preliminary study

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Abstract & relevance

Candida albicans is a common member of the human gut flora, but, following alterations of host immunity, yeast overgrowth can be responsible of severe infections. Other species belonging to the genus *Candida* such as *Candida tropicalis* have also been reported as food contaminants. Previous research on the peptide KK14 and, especially, its D-enantiomer highlighted its potency as a novel antifungal drug. Thanks to the promising results obtained against filamentous fungi and yeast contaminants, a KK14 derivative, named kk14r and made of the sequence Ac-kkffrawwarflk-NH2, was tested against *C. albicans*. The novel peptide showed candidacidal activity with a MIC of 25 µg/ml. In spite of a certain efficacy in repeated topical treatment to limit fungal growth on murine tongues in an *ex vivo* assay, its therapeutic application would be limited by a significant cytotoxicity. The short half-life is another common drawback associated with therapeutic use of small peptides. A strategy of improvement consists in the conjugation of peptides by different types of linkers, to increase the specificity of targets or to reduce their degradation. Furthermore, the absence of antifungal vaccines has emphasized the need of a novel effective way for the prevention and the reduction of disseminated candidiasis. Then, the candidacidal and cytotoxic activities of kk14r conjugates comprising an immunogenic moiety were determined. The conjugation of kk14r with a *C. albicans* cell-wall epitope, via a specific cleavable linker, led to the reduction of the cytotoxicity and the stimulation of the immune response of macrophages. However, deeper investigations are necessary to study the stability of the conjugate, the conditions of cleavage and to improve both the antifungal activity and the immunogenic properties.

This preliminary study is an opening to the multiple possibilities of peptide design, beyond food applications. Here, a novel peptide-based strategy was studied for the direct treatment but also the modulation of the immune response to fight fungal pathogens.

A1.1 Introduction

Fungal infections are estimated to cause 1.6 million deaths in 2050. Nowadays, the mortality rate associated with these infections is substantial (15-35% for adults) and the treatment of invasive fungal infections faces several hurdles (Guinea 2014, Minnebruggen 2010). *Candida albicans* is the most frequently isolated fungal pathogen in humans, representing the fourth cause of nosocomial infection. Although the human *C. albicans* is a commensal fungus commonly colonizing human mucosal surfaces, it can become an opportunistic pathogen under conditions of immune dysfunction (Harris 2010). Non-*C. albicans* species such as *Candida glabrata* are also being increasingly isolated as causal agents (Pfaller & Diekema 2007). Patients treated with invasive procedures or infected by HIV are thus particularly subject to candida infections of mucosa, which occurs particularly in the oral cavity (Peters et al. 2010). Treatment of invasive mycoses by some of antimycotics is hampered by the limited activity spectrum, resistance development, and fungistatic rather than fungicidal activities. The most common treatment of fungal infections is based on azole or polyene compounds. These compounds bind to the yeast membrane component ergosterol, thus limiting negative effects on mammalian cells. When administered via the gastrointestinal tract, these agents have few side effects, in contrast to systemic antifungal treatments. When administered intravenously, common side effects include fever, headaches or nephrotoxicity. Current international guidelines suggest the use of echinocandins for the primary treatment of invasive candidiasis because of their fungicidal activity, safety profile, and better clinical outcomes compared with those of fluconazole and amphotericin B (Pappas et al. 2015). Another obstacle is the development of resistant pathogenic fungal strains due to the overuse of antibiotics or antifungal products. To date, resistance exists to all of the currently available classes of antifungal agent, including the new class of echinocandins. This trend is more prevalent with the development of bacterial superbugs but more and more resistant pathogenic fungal strains have been reported, including food-related fungi. The latency before the beginning of the treatment is also a major obstacle to successful treatments. Novel strategies, such as antibody-derived “killer peptides” or sphingolipid pathway inhibitors are, nowadays, suggested (Magliani et al. 2012). In the last decades, the number of studies about natural and synthetic peptides with candidicadial activity

has increased exponentially. The buccal environment is a primary target for opportunistic infections, although, in healthy people, the oral mucosa is protected by a complex mix of fluids (Rayhan et al. 1992, Ruissen et al. 2001, Situ et al. 2003). Saliva and oral mucosa are particularly rich in antimicrobial peptides with anticandida activity, such as histatins, defensins or mucins. However, their activity appears to be inhibited by candida proteases, such as secreted aspartic proteases (Sap) (Peters et al. 2010, Vilanova et al. 2004). Natural and synthetic AMPs can be potentially promising candidates for development of novel therapeutic agents against pathogens, and several are either under clinical trial or FDA-approved, such as omiganan or iseganan (Desbois et al. 2011). We previously developed several peptides based on the key determinants of the antifungal activity. The peptide KKFFRAWWARRFLK showed strong antifungal activity against yeast contaminants (data not published). In this study, the antifungal activity of a D-enantiomer of this peptide, termed kk14r, was tested against *C. albicans* in *in vitro* assays and in an *ex vivo* model on murine tongues.

Either in the prevention of specific fungal pathogens or for the treatment of mycoses, a growing number of research has focused on the utility of vaccines and/or immunotherapy. To date, there is no approved antifungal vaccine for use in humans and significant therapeutic challenges remain, which emphasizes the need for effective preventative strategies. Whereas some vaccines are constructed to induce cell-mediated immunity, others favour protective antibody responses. The role of antibody immunity against fungal disease has been controversial, especially due to the less understood phenomenon of immunoglobulins and potential relationship between allergic hypersensitivity and recurrent candidiasis (Magliani et al. 2012). Classical vaccination using whole organisms is still common, with successful long lasting immunity. However, it may cause autoimmune or strong allergic responses. Attenuation or inactivation of such *C. albicans* vaccines might not be perfect, due to the complexity of the antigen spectrum and the remaining risk that the pathogen returns to its virulent state. A poor vaccine stability and the need for a “cold chain” are other major difficulties of classical vaccines (Wang et al. 2015). The use of vaccines comprising only a subunit of the whole pathogen is more controllable and can be produced with only the use of recombinant proteins. Therefore, the use of only minimal antigenic epitopes that can trigger the desired immune responses appears to be a smart approach to develop safe vaccines. Fungal cell-wall polysaccharide or

proteins have then been investigated as vaccine targets. Thus, Xin et al. developed a vaccine based on the peptide YGKDVKDLFDYAQE, which is derived from the cell wall protein fructose-bisphosphate aldolase (Fba), present on *C. albicans* cell wall (Xin et al. 2008). In our study, kk14r was conjugated to the Fba epitope either with a flexible or cleavable linker. The first objective of conjugating Fba was to obtain an immune response upon exposure to the peptides while maintaining an antifungal aspect. Conjugation is commonly used to reduce the potential toxicity of peptides to mammalian cells and increase their stability. The antifungal activity, the cytotoxicity of the conjugates and the stimulation of the immune response of macrophages upon exposure to these conjugates will be determined. The overall objective was to set up a novel peptide-based strategy that offers a quick antifungal treatment while eliciting an immune response for a long lasting immunity.

A1.2 Material and Methods

Peptide design

The peptide KKFFRAWWARRFLK-NH_2 has been used before in our group and showed activity against filamentous fungi and yeast strains (Thery et al., 2018, in press). A D-enantiomer of this peptide, termed kk14r (lowercases are used for d-residues), was acetylated at the N- terminus, to improve its salt and serum resistance (peptide 1). An analogous peptide, comprising 3 unnatural residues d-β-naphthylalanine (Nal) at the C-terminus was also designed (peptide 2). This modification has been used before to improve the salt resistance (Wang et al. 2009). The peptides were synthesized by Fmoc-chemistry and showed a purity > 90%, according to manufacturer. Upon reception, the peptides were dissolved in deionized water at a concentration of 4 mg/ml, except for kkffrawwarrflkNalNalNal which was solubilized at 4 mg/ml after ultrasonication, due to a lower solubility. The peptide YGKDVKDLFDYAQE (peptide 3) is derived from the cell wall protein fructose-bisphosphate aldolase (Fba), present on *C. albicans* cell wall and used previously (Xin 2014). The peptide kkffrawwarrflk was conjugated to a carrier Fba peptide with either a flexible linker (L1: AEAAAKEAAAKA) (peptide 4) or a cleavable linker (L2: PLGLWA) (peptide 5). The nature of the linkers has been described previously: L1 is a helical rigid linker and L2 is signal peptide, cleavable by the enzymes Matrix Metalloproteinases (MMPs) 2 and 9 (Klein et al. 2014).

Table A1.1. Characteristics of the peptides used in this study

Peptide	Name	Primary Sequence	Length	pI [a]	Net charge	GRAVY[b]
1	kk14r	Ac-kkffrawwarrflk-NH_2	14	14	+6	-0.80
2	kk14rNal	Ac-kkffrawwarrflkNalNalNal-NH_2	14	ND	+6	ND
3	Fba	YGKDVKDLFDYAQE	14	3.8	-2	-1.12
4	Fba_L1_ kk14r	YGKDVKDLFDYAQE-AEAAAKEAAAKA-kkffrawwarrflk	40	10.2	+4	-0.68
5	Fba_L2_ kk14r	YGKDVKDLFDYAQE-PLGLWA-kkffrawwarrflk	36	10.4	+3	-0.61

* parameters estimated with online tool PepCal.com (a) and Expasy/ProtParam(b)

Candida strain and culture conditions

The *C. albicans* strain was originated from Cork Institute of Technology (Cork, Ireland) culture collection and was used for subsequent experiments. Yeast stock solution was spread on YPD agar plates and cultured at 30°C for 24 h. Single colonies were inoculated into 10 ml of YPD medium and grown overnight at 30°C.

Antifungal activity assays

Candidacidal assays were performed using the microdilution plate method, as previously described (Tati et al. 2014). Overnight-grown cells were washed twice with 10 mM sodium phosphate buffer (NaPB, pH 7.4), and resuspended in RPMI-1640 medium with L-glutamine and sodium bicarbonate (Sigma-Aldrich). Washed cells ($1x10^6$ cfu/ml) were inoculated in RPMI-1640 containing peptides (0-400 µg/ml) at 30°C for 30 min. Due to the presence of salt in RPMI-1640 medium, a modified microdilution method was also used in this study. In this modified method, washed cells were inoculated in 10 mM NaPB and mixed with peptides (0-400 µg/ml) at 30°C for 30 min. Following incubation, yeast solutions (either incubated in RPMI or in NaPB) (20 µl) were drop-inoculated onto YPD agar plates and incubated for 24 h at 30°C, until colonies could be visualized.

Aliquots of 500 cells (only subjected to modified method) were also spread onto YPD agar plates and incubated as described above. Cell survival was expressed as a percentage compared with that of untreated control cells.

Ex vivo model of oral mucosa infection

A murine *ex vivo* model of oral infection was performed as described previously (Peters et al. 2010, Tati et al. 2014) in order to demonstrate the anticandidal potency of the peptides on host oral tissue. BALB/c mice, 6 to 8 weeks old, were used in all animal experiments (performed under the approval of Health Products Regulatory Authority). Tongues and adjacent hypoglossal tissues were excised from mice, euthanized by cervical dislocation beforehand, and placed in the wells of a 6-well tissue culture plates with RPMI-1640 (containing L-glutamine, sodium bicarbonate and penicillin/streptomycin (100 µg/ml). Tongues were infected in 1mL PBS,

containing $1x10^6$ cells/mL of *C. albicans*, in the presence of peptides 1 and 5 (200 and 400 µg/ml). Control consisted in uninfected tongues in PBS (without peptides). Plates were incubated at 37°C for 30 min under gentle rotation. Following washing twice with PBS, to remove non-adhering yeast cells, tongues were further incubated for 3 h in fresh RPMI-1640 (supplemented with glutamine and sodium bicarbonate) to allow germination and hyphal production by adhering yeast cells. The effect of a long exposure was studied by adding peptides 1 and 5 (400 µg/ml) in RPMI media for a total exposure time of 3.5 h. Following incubation in RPMI, tongues were either weighed and homogenized in PBS for quantification of infection level or resuspended in RPMI. Infection levels were quantified on YPD, supplemented with ampicillin 20 mg/ml (incubated 24 h at 30°C), by calculating cfu/g tongue tissue weight. Half of the resuspended tongues were incubated for 3 days (with fresh RPMI every 24 h) a 37°C without further treatment. Following incubation, infection levels were quantified as described above and tissue was kept for histopathology analysis. The other half was treated with peptides (10 µl, 400 µg/ml) every 24 h and tongues observed after 3 days of incubation.

Tissue histopathology analyses

Following incubation, tongues were fixed in formalin (Sigma-Aldrich), followed by dehydration in a gradient ethanol series, cleared with xylene and embedded in paraffin (Sigma-Aldrich). Tongues were processed for histopathological analysis as described previously (Tati et al. 2014). Thin sections (4 µm) were cut using a microtome (HM355S, ThermoScientific) and stained with periodic acid-Schiff stain, as described by manufacturer (Sigma-Aldrich). Tongue section was examined by light microscopy and evaluated based on the presence and extent of adhering yeast cells and invasive hyphae.

Cytotoxicity assays

The cytotoxicity of the peptides was determined towards mouse macrophages RAW 264.7 cell line ($1x10^5$ cells/ml) by measuring cell viability using an MTT cell viability kit (Roche, Switzerland) as described previously (Thery et al. 2018). Cells were

cultured in a 96-well microtiter plate in Dulbecco's modified Eagle's medium (DMEM) and FBS (10%), at 37°C in 5% CO_2. Cells were allowed to adhere overnight, medium was removed and 200 µl of fresh medium (with 2,5% FBS) supplemented with peptides 1-5 (0-400 µg/ml) was added to the wells and incubated for 24 h at 37°C. After removal of the medium, fresh medium (100 µl) and 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (10 µl) (Cell viability kit, Roche) was added to each well and incubated for 4 h at 37°C. The solubilisation solution (100 µl) was added to each well and the plate was incubated overnight at 37°C. Cell viability was measured spectrophotometrically at 570 nm with a background reading at 690 nm. The cytotoxicity of each peptide was determined in 4 wells for 3 biological replicates.

Cytokine Secretion

The effect of the peptides on the production of the pro-inflammatory cytokine tumor necrosis factor TNF-α were determined by specific enzyme-linked immunosorbent assay (ELISA) techniques using a TNF-α Mouse Uncoated ELISA Kit (Invitrogen, USA). TNF- is a cytokine produced primarily by macrophages and monocytes with multiple biological functions in addition to tumor killing. Mouse macrophages RAW 264.7 cell line ($5x10^5$ cells/ml) were cultured in a 96-well microtiter plate in Dulbecco's modified Eagle's medium (DMEM, ThermoFischer Scientific) and Foetal Bovine Serum (FBS, ThermoFischer Scientific, 10%), at 37°C in 5% CO_2. After adhesion overnight, medium was removed and 200 µl of fresh medium (with 2,5% FBS) supplemented with peptides 1-5 (0-400 µg/ml) was added to the wells and incubated for 24 h at 37°C. Following incubation, the concentration of TNF-α in growth medium was determined according to manufacturer's instruction. The concentration of cytokine was determined spectrophotometrically at 450 nm with a background reading at 570 nm, and calculated via standard curves.

MMP-specific peptide cleavage

Candida infection is associated with changes in concentrations of the enzymes MMPs, involved in multiple physiological and pathologic processes. Increased levels of the MMP-9 have been observed for instance during corneal fungal infection. Peptide 5 was the conjugate of Fba and kk14r linked by a cleavable linker PLGLWA, which has

been shown previously to be cleaved by the MMP-2 and MMP-9. Peptide 5 (200 μg/ml) was incubated with recombinant MMP-2 enzyme (catalytic domain, Enzo Life Sciences) at a concentration of 2 μg/ml in 50 mM Tris, 0.15 M NaCl, 10 mM $CaCl_2$, 50 mM $ZnCl_2$, and 0.02% Brij35. The peptide was digested for 1 h at 37°C. Cleavage of the peptide was assessed by resolution in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels using Mini-Protean 16.5% Tris-Tricine gels (Bio-Rad), according to Schägger and von Jagow (Schägger & von Jagow 1987).

A1.3 Results

Candidacidal assay

The five peptides were tested for their activity against *C. albicans* with two different methods. *C. albicans* was inoculated in 10 mM NaPB (pH 7.4) or RPMI-1640 at a concentration of $1x10^6$ cells/ml. After 30 min of incubation in presence of the peptides (0-400 μg/ml), the fungal solution was drop inoculated (20 μl) onto YPD agar and incubated 24 h at 30°C (Fig. A1.1). Peptide 1 (kk14r) showed the highest antifungal activity with a MIC of 25 μg/ml when incubated in 10 mM NaPB and 400 μg/ml in RPMI. The antifungal activity of the peptide 1 was reduced by the addition of Nal residues at the C-terminus of the peptides (peptide 2, MIC > 400 μg/ml in 10 mM NaPB). However, in contrast to peptide 1, the activity of peptide 2 was not impaired in RPMI at 37°C. The epitope Fba, peptide 3, did not show any antifungal activity in the range of concentrations used in this study. In addition, the conjugates peptide 4 and 5 demonstrated a limited antifungal activity with a MIC of 400 μg/ml or higher, in NaPB. These two peptides did not display antifungal activity after incubation in RPMI.

The percentage of *Candida* survival (based on aliquots of 500 cells) after peptide treatment was determined after 30 min incubation in 10 mM NaPB (Fig. A1.2). The peptides 1 and 2 showed the highest antifungal activity, with a complete killing of yeast cells, at 12.5 and 25 μg/ml, respectively. The percentage of viability of *C. albicans* was 10 and 26%, after treatment with peptides 4 and 5 (50 μg/ml), respectively.

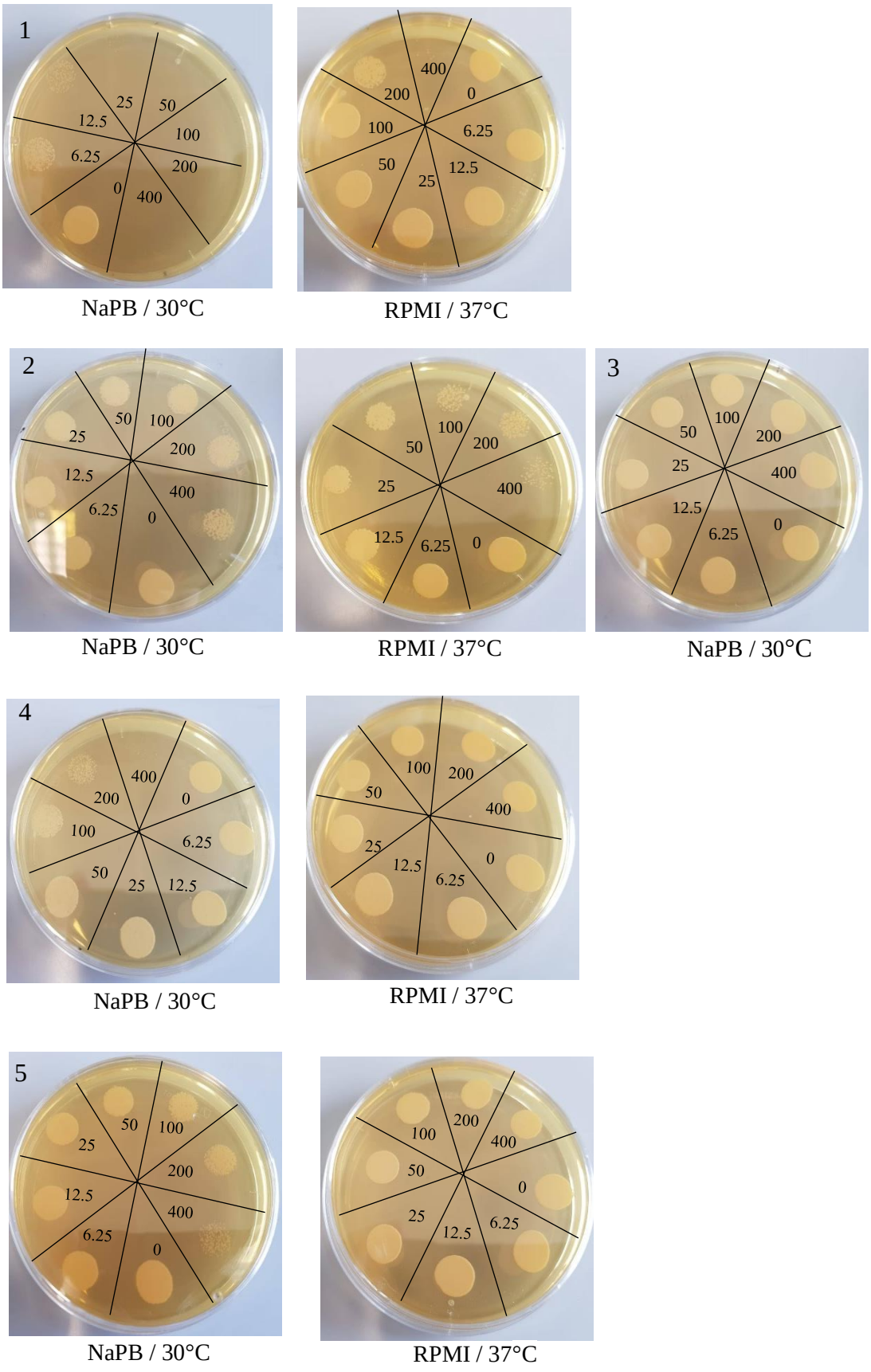


Figure A1.1 Anticandida activity of peptides 1-5 in NaPB and RPMI.

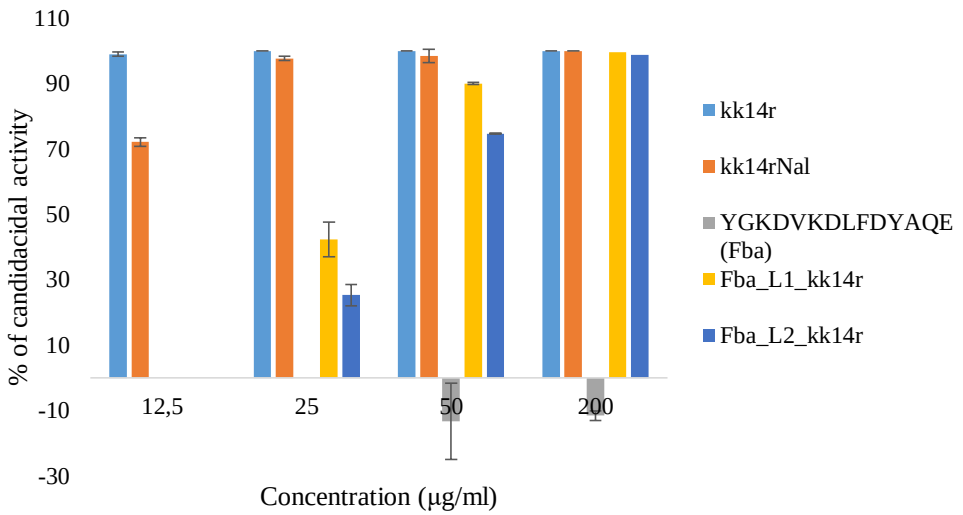


Figure A1.2. Percentage of candidacidal activity after incubation of *C. albicans* with Peptides 1 to 5 in 10 mM NaPB

Cytotoxic activity of Peptides 1-5

The cytotoxicity of the peptides was determined against murine macrophages cell line RAW 264.7. The 50% cytotoxic concentration (CC_{50}) of the parental peptide, peptide 1, was in the range 50-100 µg/ml, whereas peptide 3 did not present any cytotoxic activity (Fig. A1.3). The addition of the Nal residues increased the toxicity of peptide, with peptide 2 presenting a CC_{50} of 25 µg/ml. Lastly, the conjugation of peptide 1 and 3 reduced by over 4-fold the cytotoxic activity, with peptide 5 showing an CC_{50} of 100-200 µg/ml.

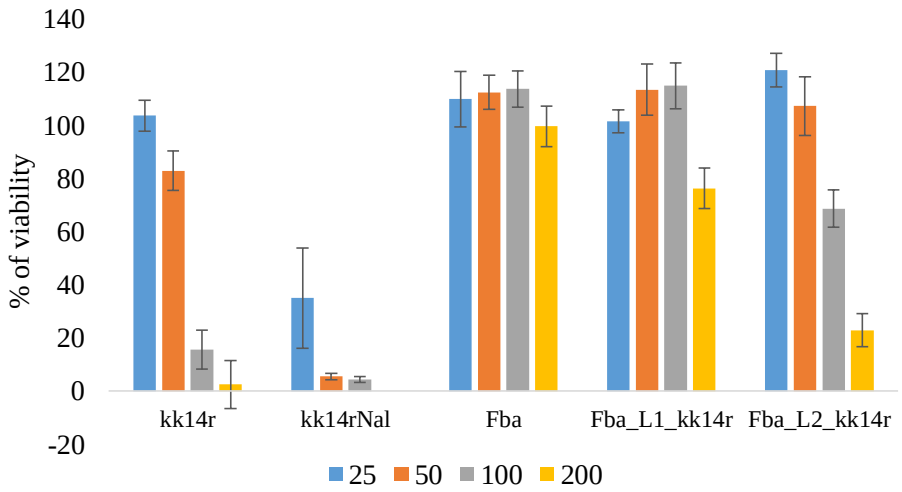


Figure A1.3. Cytotoxicity of Peptides 1-5 towards murine macrophages RAW 264.7. Concentrations indicated below are in µg/ml

Determination of TNF-α

The immunogenic properties of peptides 1-5 were determined by measuring the concentration of TNF-α produced by RAW 264.7 cells upon exposure to the peptides. TNF-α is a multifunctional proinflammatory cytokine that belongs to the tumor necrosis factor (TNF) superfamily. Mainly secreted by macrophages, this cytokine is involved in the regulation of immune cells but also cell proliferation, differentiation, apoptosis and coagulation. The concentration of TNF-α produced by RAW cells was not increased after incubation with peptides 1 to 4 (data not shown). Only peptide 5 (400 µg/ml) induced a significant higher production of TNF-α in cell culture medium with a concentration of 206 pg/ml, compared to a concentration of 101 pg/ml without exposure to peptide (Fig. A1.4).

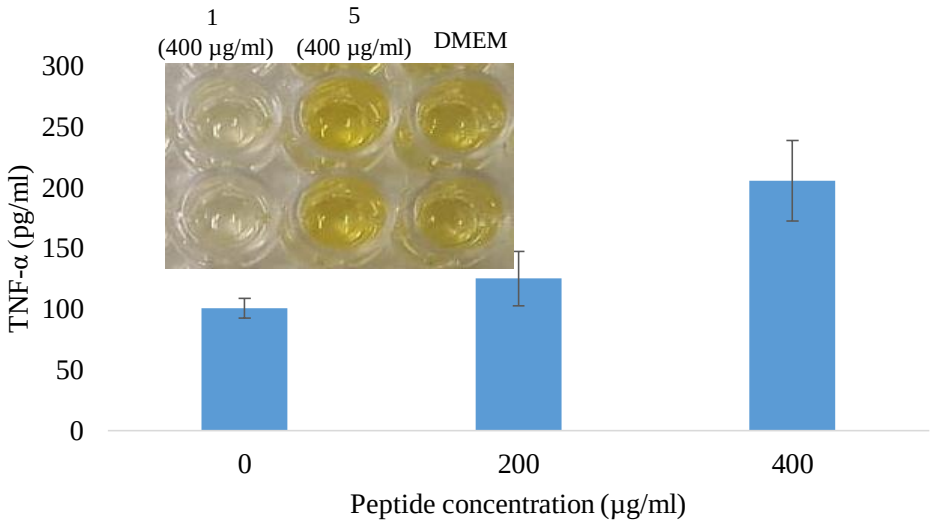


Figure A1.4. Production of the cytokine TNF-α by RAW 264.7 cell line after exposure to Peptide 5. Insert: detection of TNF-α (in yellow) in RAW cells growth medium upon different treatments

Protection of oral mucosa

The peptides 1 and 5 were tested in a murine *ex vivo* model of oral infection as described previously (Peters et al. 2010, Tati et al. 2014). After 30 min of incubation in infected PBS, in presence of peptide 1, the concentration of adhering yeast cells on the tongue was significantly reduced (Fig. A1.5), compared to untreated tongue. However, the treatment with peptide 5 did not reduce the concentration of yeast cells on the epithelium. After 3 days of incubation, untreated and treated tongues showed the same level of contamination.

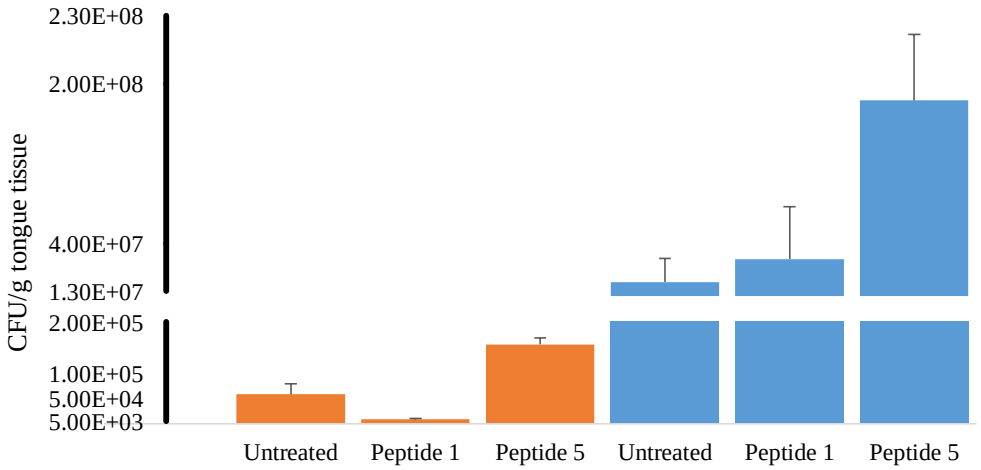


Figure A1.5. Cfu per gram of tongue tissues recovered from infected mice at days 0 (orange) and 3 (blue) post-infection with *C.albicans*. Tongues were treated with single treatment of Peptide 1 or 5 (400 µg/ml) at day 0.

Tissue histopathology analyses

After 3 days of incubation in RPMI, infected tongues sections were examined by light microscopy. The observation revealed the presence of yeasts cells and hyphae on the epithelium of untreated tongue (Fig. A1.6b) and tongue treated with peptide 1 (Fig. A1.6c). However, a topical treatment with peptide 1 (400 µg/ml) performed every 24 h during incubation time, reduced the development of the yeast on the tongues (Fig. A1.7), as revealed by the presence of more extensive yeasts white biofilm at the surface of the untreated tongue.

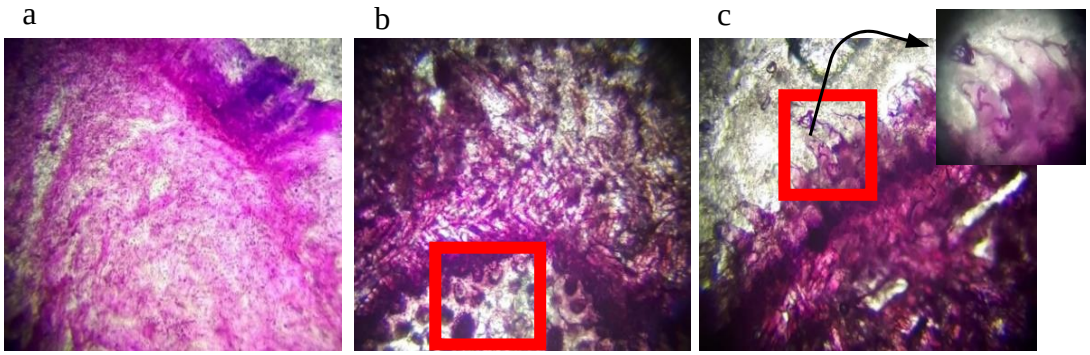


Figure A1. 6. Histopathology analysis of mouse tongues uninfected (a) or infected with *Candida albicans* in the absence (b) and presence (c) of peptide. Representative microscopic images (x100) from PAS-stained tissue demonstrating *C. albicans* invasion of the tongue tissue untreated (b) and treated with peptide 1 (c). Red frames highlight the presence of yeasts and hyphae at the surface of the epithelium.

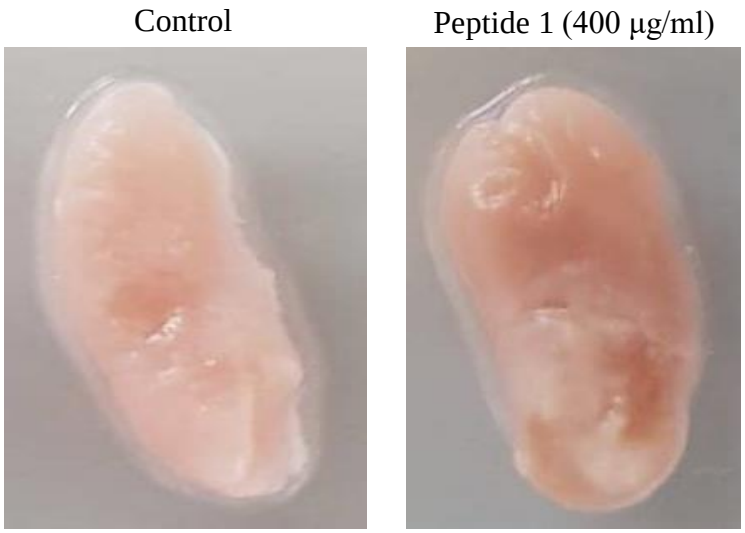


Figure A1.7. Appearance of fungal lesions on animal tongues is shown on day 3 post-infection with *C. albicans*

MMP-specific peptide cleavage

Peptide 5 is a conjugate of Fba and kk14r linked via a cleavable PLGLWA sequence. In infectious environment, the concentrations of MMPs are generally higher than in the rest of the body. A specific cleavage of the linker PLGLWA in the microenvironment of a candida infection would aim to release specifically two peptides, one comprising kk14r and another one with the Fba moiety. After 1 h incubation with MMP-2, the cleavage of peptide 5 was monitored on a SDS-PAGE. Unfortunately, in the conditions tested, the enzyme failed to cleave the peptide (data not shown).

A1.4 Discussion

Candida albicans is a common member of the human gut flora, but, following alterations of host immunity, overgrowth has been observed causing a wide range of infections with detrimental effects, especially on health of immunocompromised patients. In addition to being involved in numerous nosocomial diseases, species belonging to the genus *Candida*, such as *Candida tropicalis*, have been reported as food contaminants (Viljoen et al. 2003). Based on previous work, performed in our group, on the antiyeast activity of the peptide KKFFRAWWARRFLK (unpublished), a series of 5 peptides were tested against *C. albicans*. The parental novel peptide, termed kk14r, inhibited the growth of *C. albicans* after incubation in a low-buffered solution (10 mM NaPB), with a MIC of 25 μg/ml. However, researchers have highlighted the problem of antibiotic testing of AMPs in classical enriched bacteriologic media instead of using media that better resemble normal human body fluids, such as DMEM or RPMI (Nizet 2017). Moreover, *C. albicans* has shown the ability to undergo different forms of growth depending on culture conditions, from yeasts to pseudohyphae, and the formation of biofilms. This mechanism, switching from a unicellular yeast form into various filamentous forms, is thought to be required for virulence (Mayer et al. 2013). While the filamentous growth form allows the fungus to evade the cells of the immune system, the yeast form may be the mode of proliferation in infected tissues. Thus, the anticandida potency of kk14R was also tested in RPMI at 37°C. In these conditions, the antifungal activity of kk14R was strongly reduced, with a MIC of 400 μg/ml, possibly due to the presence of salt in the media and the changes in *C. albicans* growth form. Interestingly, although an overall reduction potency of the peptides, the insertion of Nal at the C-terminus of the kk14r, increased the peptide stability. This feature, observed previously, would be of interest for therapeutic applications (Wang et al. 2009). Despite significant toxicity at high concentrations, kk14r did not reduce the viability of murine macrophages RAW 264.7 at the concentration that inhibited the growth of *C. albicans*.

The increased rate of infections and the absence of antifungal vaccines emphasized the need of a novel effective way for the prevention and the reduction of disseminated candidiasis. The complexity of *C. albicans* antigens requires the need to combine two

or more vaccine candidate antigens to develop multivalent vaccine for the best efficacy. Peptide-based synthetic vaccines offers many advantages to traditional vaccines, such as storage stability, large-scale production, absence of biological contamination, minimal allergic responses, and customization. However, current drawbacks of peptide-based vaccines include poor immunogenicity, instability *in vivo* and limited population effectiveness. Multiple solutions are proposed to overcome these issues. For instance, the immunogenicity of peptide vaccine can be improved by the use of safe adjuvant, the use of nano/microparticles delivery systems, or multi-epitope strategies using chemical ligation (Skwarczynski & Toth 2016). The addition of fungal cell wall peptides, that would both serve as carriers and provide an additional protective epitope, improves the stability of the epitope (Xin 2014). This strategy has been exploited by Xin et al. to design an effective antigen-pulsed dendritic cell (DC)-based vaccine (Xin et al. 2008). Dendritic cells (DCs) are antigen-presenting cells (APCs) that reside in the peripheral regions such as skin, nose mucosa, the respiratory system and the digestive system and in all tissues apart from the brain, testis and eyes. DCs play key roles in the early stages of the infection and in the initiation of adaptive immunity. DCs should be phagocytic, to act as APC and to initiate cellular response against fungi, especially by stimulating undifferentiated T cells. Another aspect of APC activity is the release of proinflammatory cytokines and chemokines, which participates to the attraction of polymorphonuclear leukocytes and to the differentiation of T cells. The type of cytokines secreted also influences the differentiation of T-helper cells (Th cells) as type 1 (Th1) and type 2 (Th2) T-helper cells. Reciprocally, the balance between the two types of reactivity regulates the response to commensalism and to infection and associated pathology. Cytokines produced by Th1 cells are known to activate macrophages and neutrophils to a candidacidal state, whereas cytokines produced Th2 cells inhibit Th1 development, leading to deactivation of phagocytic effector cells and susceptibility to candida infection. (Romani 1999). The role of Th cells secreting the cytokine IL-17 and IL-17-mediated immunity to combat *C. albicans* infections have also been extensively studied (Trautwein-Weidner et al. 2015). The stimulation of APCs with heat-inactivated *C. albicans* also triggers specific immune response (Marcil et al. 2002). In their study, Xin et al. used the cell-wall peptide epitope Fba of *C. albicans* to prevent disseminated candidiasis in murine models, via the production of Fba-specific antibodies (Xin et al. 2008). However, in our study, the exposure of RAW cells to *C.*

albicans cell-wall epitope Fba did not increase the production of TNF-α, highlighting the poor immunogenic response of RAW cells upon exposure to Fba, possibly due to a limited stability in serum. The conditions of the assay (cell number, incubation time) might also influence this response. Thus, the treatment of the cell line RAW 264.7 with the *C. albicans* ethylenediamine cell wall extract results in an immune response, via a significant elevation in the level of TNF-α (Vazquez et al. 1996).

The Fba moiety and kk14r were conjugated either by a rigid (AEAAAKEAAAKA) or by a cleavable (PLGLWA) linker. The conjugations had two objectives, namely the reduction of kk14r cytotoxicity and the protection of Fba epitope by kk14r, in order to stimulate RAW cells immune response. Both conjugations reduced the antifungal activity of kk14r, which could be attributed to a decrease of global net charge, a key parameter of the antifungal potency. The conjugated peptides also showed less toxicity with a CC50 of 200 µg/ml or higher.

The exposure of RAW cells to peptide 4 did not increase TNF-α secretion. The absence of stimulation could be attributed to folding modification or inversely to a lack of protection by kk14r, due to an increased distance. Inversely, peptide 5 (400 µg/ml) induced a significant increase in the production of TNF-α by RAW cells. Based on the knowledge of increased concentrations of MMPs following *Candida* infection, particularly in MMP-2/MMP-9, peptide 5, which comprises a MMP-specific cleavage signal, PLGLWA, was designed. A similar rationale has been used previously to provide specific activity of the peptide, while maintaining safety to human cells. Jang et al. conjugated the toxic peptide buforin IIb with an anionic peptide via a MMP-2/MMP-9-cleavable linker. The anionic peptide neutralizes the positive charge of buforin IIb., thus render it inactive. However, in tumour micro-environment and the associated higher concentration of MMPs, buforin IIb is released and specifically active to tumour cells (Jang et al. 2011). However, the preliminary results carried out with incubation of peptide 5 with the enzyme MMP-2 did not clearly show the release of two peptide units. Further work is necessary to characterize with precision the parameters of cleavage (e.g., enzyme concentration) and evaluate their similarities with *in vivo* conditions. Other conditions, specific to infectious microenvironments, could also be exploited in the design of novel peptides. For instance, pH-selective cytotoxic peptides have been designed to target specifically tumour cells (Burns et al. 2016).

Lastly, the potential application of peptides 1 and 5 in the treatment of oral candidiasis was evaluated in an *ex vivo* experiment on murine tongues. In presence of kk14r, after 30 min incubation in a *C. albicans* solution, the tongues presented a reduced concentration of yeast at their surface. However, after 3 days of incubation at 37°C, untreated and treated tongues similar levels of contamination. After this incubation time, the microscopically observation of the tongue epithelium revealed the presence of yeasts and hyphae on peptide-treated tongues. However, a repeated topical treatment of kk14r reduced effectively the tongue contamination, as shown with topical treatment of murine tongues with the AMP Hst-5 in *in vivo* experiments (Tati et al. 2014).

The main objective of this work was to highlight the potential and the limitations of a series of novel peptides against *C. albicans*. This common microorganism of the gastrointestinal tract, in cases of impaired intestinal mucosal barrier and weak immunity, is able to establish systemic infection within the host. The results presented here are preliminary and further research is necessary, especially focusing on the immune response upon exposure to peptides, the specific cleavage of the peptides and animal experimentation. However, this study offers perspectives in a novel strategy in the design of peptide-based antifungal vaccine, combining rapid antifungal activity and the use of immunogenic properties. A growing body of work has focused on the immunomodulatory properties of antimicrobial peptidess, so-called host defence peptides (HDPs), in particular, on the role of the AMPs human cathelicidin (LL-37) and β-defensins. These AMPs, expressed by the gastrointestinal epithelium, play a role in diseases as diverse as Alzheimer's disease, Crohn's disease or psoriasis. Furthermore, AMPs are recognized as the most efficient molecules produced by resident gut microbiota. An in-depth understanding of the role of the microbiota in the regulation of AMPs, antimicrobial functions and innate immune response would help the treatment of such immune disorders and diseases. This knowledge involves necessarily an expanded characterization of the commensal species, including *C. albicans*. With this in mind, revolutionary method of isolation, such as the i-chip technology (Kim et al. 2016), already proved its worth. This technology allowed the isolation of the AMP teixobacterin, produced by the bacterial species *Eleftheria terrae*, which is active against many multi-drug resistant bacterial strains (Ling et al. 2015).

Since, the possibility to limit the development of resistance of *Candida* species and the high number of fungal infections expected in the future appears tangible.

A1.5 Acknowledgements

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J. Pep. Sci. 25, e3137

Supplementary Information for Chapter 4

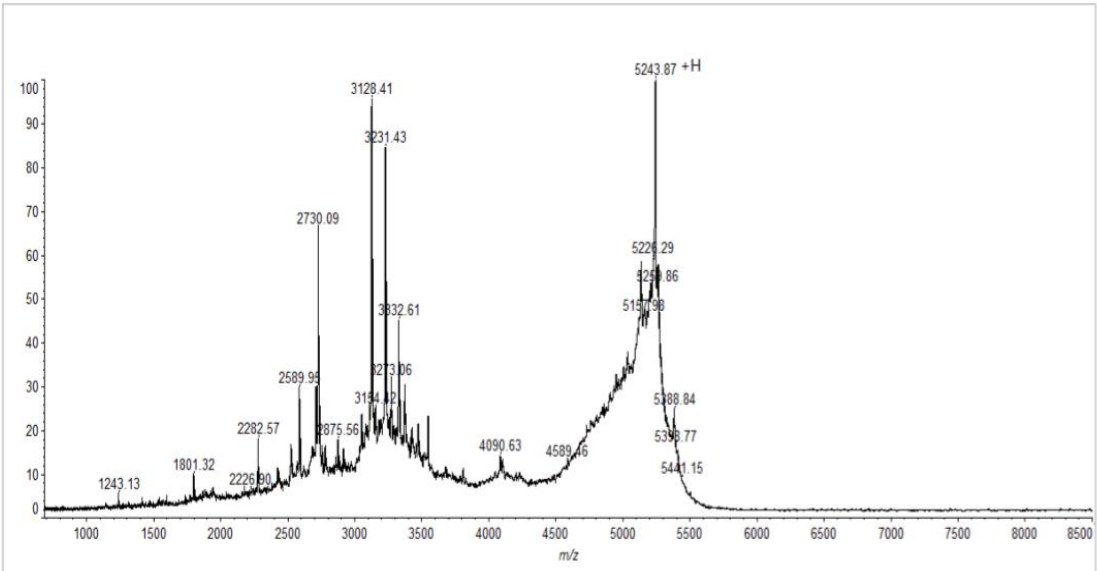


Figure 4.S1. MALDI-TOF report of KT43C provided by GLBiochem, Shanghai, China

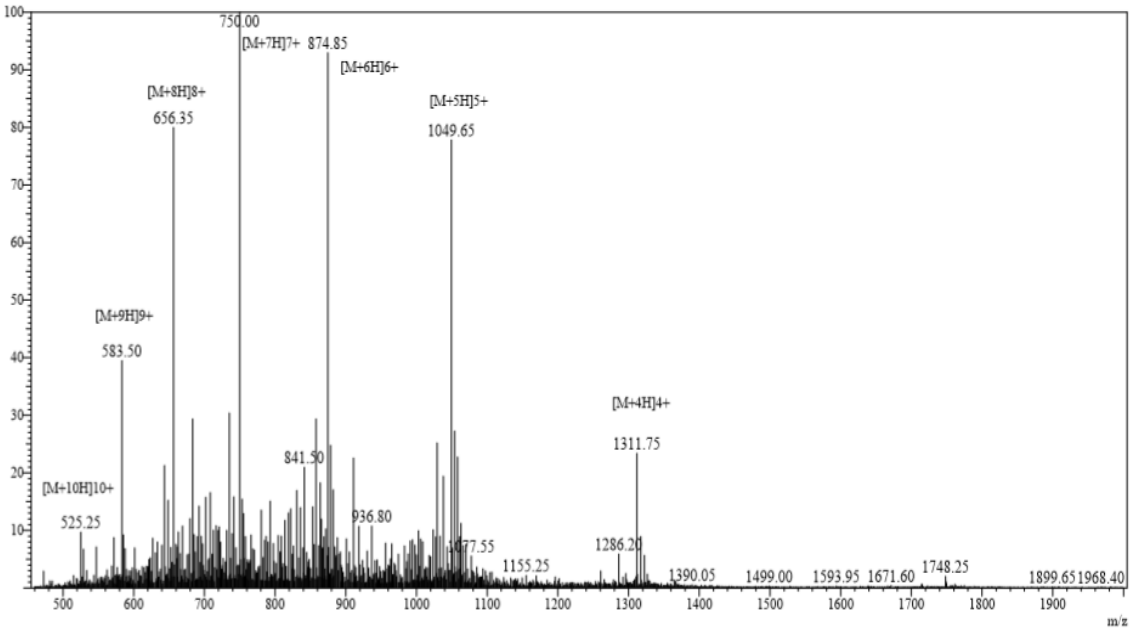


Figure 4.S2. Mass spectrum of KT43C provided by GLBiochem, Shanghai, China

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Antifungal activity of a synthetic human β-defensin 3 and potential applications in cereal-based products

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ABSTRACT

Defensins are cysteine-rich antimicrobial peptides of the innate immune system involved in constitutive and adaptive immunities. The aim of this work was to characterize the antifungal activity of a linear human β-defensin-3 (HBD-3) and test this peptide as a potential food preservative. Antifungal activity of the synthetic HBD-3 was demonstrated against *Fusarium culmorum*, *Penicillium expansum* and *Aspergillus niger*. To determine potential baking applications with the defensin, a parental wild yeast *Saccharomyces pastorianus* strain, called CMBS-33, was transgenically modified. The novel lager yeast strain, called CM-INT-51, was able to express the gene encoding HBD-3. The use of CM-INT-51 to ferment the dough extends the shelf life of bread by three days in comparison to CMBS-33. Furthermore, synthetic HBD-3 (80 $\mu g \cdot ml^{-1}$) delayed fungal growth on bread for >13 days. These results revealed that both the synthetic peptide and the yeast containing HBD-3 have the potential to increase the shelf-life of baked goods.

Industrial relevance: Fungal spoilage is an important cause of economic losses in the baking industry and might be a source of mycotoxins with detrimental effects on human health. Furthermore, the overuse of preservatives is subject to controversy. We showed that the linear analogue of HBD-3 displays antifungal activity against food contaminants *in vitro* and can be used to extend the shelf-life of bread. These results have direct applications to the baking and other related industries.

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1. Introduction

The fungal spoilage of cereal-based products is a source of economic losses and has the potential to cause serious health problems (Legan, 1993; Pitt & Hocking, 2012). The main fungal contaminants of bakery products belong to *Fusarium*, *Aspergillus* and *Penicillium* genera. In the baking industry, heat treatment reduces the risk of microbial contamination but fungal spores survive the baking process. The resulting mould growth is responsible for formation of unpleasant flavours, reduction of the shelf life and has the potential to produce mycotoxins and allergens (Nielsen & Rios, 2000).

A consumer study published by Van Sint Fiet (2015) highlights that the appearance of mould is the first reason for people to discard bread. To satisfy consumers' demands, bread should be a high quality product with extended shelf-life and a limited proportion of chemical preservatives. Before the advent of cultured yeast in baking industry, sourdough used to be the standard method for bread leavening. The development of gluten-free bread and the research for a healthy bread lead to a revival of sourdough. Among the alternative methods to current preservatives, sourdough is the most commonly used and has been proven to be efficient in preserving bread (Giuseppe Rizzello et al., 2009). However, the control of the different parameters of the production of bread with sourdough, like the pH or the starter cultures, can be a challenge for large-scale bread production (Denkova et al., 2014).

Antimicrobial peptides (AMPs) are usually small peptides, between 12 and 57 amino acid residues, with cationic amphipathic properties. Those AMPs are members of the innate immune system of many living organisms. The expression of these antimicrobial peptides can be constitutive or can be inducible by infectious and/or inflammatory stimuli (Jenssen, Hamill, & Hancock, 2006). Natural antimicrobial peptides are found or used in food products, mainly bacteriocins like nisin produced by certain strains of *Lactococcus lactis* (Cleveland, Montville, Nes, & Chikindas, 2001) or lactoferrin, a glycoprotein of milk (Keymanesh, Soltani, & Sardari, 2009).

Among these AMPs, defensins represent the largest family. These are basic and small peptides (between 45 and 54 amino acids residues), with a high proportion of cysteine residues. They have been identified as host defense members of various species of invertebrates, plants and vertebrates. The mammalian defensins are divided into three classes based on disulfide connectivity and their three-dimensional structure. The α-defensins are found only in neutrophils and Paneth cells of humans (Cunliffe, 2003). The θ-defensins are only found in old world monkeys and possess a cyclic structure (Lehrer & Lu, 2012). Human β-

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defensins (HBD) are expressed in different tissues of the human system (Dhople, Krukemeyer, & Ramamoorthy, 2006) and display antimicrobial activity against human pathogens such as *Escherichia coli, Staphylococcus aureus* (Harder, Bartels, Christophers, & Schröder, 2001; Hoover, Wu, Tucker, Lu, & Lubkowski, 2003) or *Candida albicans* (Vylkova, Nayyar, Li, & Edgerton, 2007). Different models have been proposed to explain the mode of action of these peptides. In these models, it is proposed that due to their positive net charge and their amphiphatic nature, the defensins are capable of interacting with the microbial cell wall (Sahl et al., 2005). The ensuing antimicrobial activity would be the result of several processes, *e.g.*, cell leakage, production of reactive oxygen species or induction of cell apoptosis (Guilhelmelli et al., 2013).

Apart their antimicrobial activities, HBDs possess immunomodulatory activity. There is some evidence that these defensins play a role in certain genetic diseases, such as cystic fibrosis, and cancer (Droin, Hendra, Ducoroy, & Solary, 2009; Pazgier, Hoover, Yang, Lu, & Lubkowski, 2006). To date, among the six characterized human β-defensins, HBD-3 is the defensin with the highest net charge (+11 at pH 7). HBD-3 is a peptide of 45 amino acid residues, whose native form includes 6 cysteine residues, bound as *C*1-C5; C2-*C*4; C3-C6. Although these defensins represent an interesting candidate to fight microbial spoilage, no application has been so far conducted in food products (Davidson, Sofos, & Branen, 2005). However, a recombinant HBD-3 has been proven to inhibit the growth of spoilage bacteria in beer (James et al., 2014). In addition, Liu et al. (2008) showed that the linear analogue of HBD-3 has no hemolysis activity towards rabbits erythrocytes nor cytotoxicity on human conjunctival epithelial cells. Furthermore, to our knowledge, HBD-3 or any analogue have never been shown to display allergenic activity. We report here the activity of synthetic HBD-3 as well as the activity of a *S. pastorianus* strain expressing HBD-3 against fungal contaminants isolated from cereal-based products. The potential application of HBD-3 as a preservative agent for bread was also studied. However, given the limited *in vivo* research on defensins and more generally AMPs, no defensin has been approved so far, by safety agencies, for food application (Hintz, Matthews, & Di, 2015).

2. Material and methods

2.1. Defensins

A synthetic linear analogue of the human β-defensin 3 (sHBD-3, 5.1 kDa, purity >70%) was purchased from GLBiochem, Shanghai, China. A crude peptide was synthesized by solid phase peptide synthesis using Fmoc chemistry on a solid support of rink amide MBHA resin. Following a final deprotection with 20% piperidine in dimethylformamide (DMF) and cleavage with a mixture of TFA/water (90:10), crude peptide was purified using reversed phase HPLC on a Gemini-NX 5 μm C18 column (4.6 × 250 mm, 110 Å). The lyophilized peptide was reconstituted in 0.1% acetic acid (pH 3.5) as described by the manufacturer and stored in vials of different concentrations at −20 °C.

2.2. Fungal strains

The filamentous fungi used in this study represent some of the main contaminants of the cereal-based products (Beuchat, 1987). They were *Fusarium culmorum*, *Aspergillus niger* and *Penicillium expansum*. The fungal strains *F. culmorum* FST 4.05, *A. niger* FST4.21, *P. expansum* FST 4.22 originated from the culture collection of School of Food and Nutritional Sciences, University College Cork (Cork, Ireland).

2.3. Antifungal activity assay

The antifungal activity of sHBD-3 was determined using a microtiter plate assay as described previously (Finkina, Shramova, Tagaev, & Ovchinnikova, 2008; Li et al., 2013)with some modifications. Briefly, fungal spores were collected from mature colonies grown for 72 h on PDA (Sigma-Aldrich) plates at 28 °C, counted on a Neubauer chamber haematocytometer and inoculated in synthetic nutrient-poor broth. Fungal spores were then diluted in ½ strength potato broth (1/2PDB) (Sigma-Aldrich) to obtain a final concentration of 10^3–10^4 spores·ml^{-1}. Different concentrations of sHBD-3, ranging from 5 μg·ml^{-1} to 150 μg·ml^{-1} (final concentration), were added into the aliquot of fungal cells and the growth inhibition was assessed by measuring the solution absorbance at 620 nm for 96 h at 28 °C on a 96-well microplate reader (Multiskan TM, Thermo Scientific). As negative control, 0.1% acetic acid in ½ PDB (pH = 5.0) was used instead of sHBD-3. The inhibition of the fungal growth by the presence of sHBD-3 was then examined on PDA plates. The same solutions were prepared as described above and after 24 h of incubation at 28 °C, 50 μl were spot-inoculated on PDA plates. Pictures of the plates were taken every 24 h for a period of three days.

The inhibition of *F. culmorum* by sHBD-3 peptide was observed using a phase contrast microscope. An aliquot of 1/2PDB inoculated with a concentration of 10^4 spores·ml^{-1} of *F. culmorum* and 10 μg·ml^{-1} of sHBD-3 was incubated at 28 °C, and observed over a 24-h incubation time. The experiments were performed in triplicate.

2.4. MIC/IC_{50} determination

After 96 h of incubation at 28 °C, the MIC was read as the lowest concentration of sHBD-3 resulting in complete inhibition of fungal growth (Fig. 1). The required concentration to inhibit 50% of the fungal growth (IC_{50}) was determined by non-linear regression, using the software Graph PRISM (GraphPad Software, Inc., La Jolla, CA) with these data.

2.5. Effect of NaCl on antifungal activity of HBD3

HDB-3 has already been reported to be the most stable defensin in salt solutions (Jenssen et al., 2006). Indeed, the peptide retains its antimicrobial activity against *E. coli* and *S. aureus* in solutions containing 100 mM NaCl (Hoover et al., 2003). Here, the activity of sHBD-3 was studied against *F. culmorum*. Fungal spores (10^3–10^4 cells·ml^{-1}) were inoculated into 1/2PDB solutions containing either 50 mM or 150 mM of NaCl. Reconstituted sHBD-3 (final concentration = 15 μg·ml^{-1}) was added into the two spore solutions and the antifungal activity was determined as described above. The effect of NaCl on the antifungal activity of sHBD-3 was measured as the difference of percentage of inhibition of the fungal growth between the non-salted and the salted solutions, based on the microtiter plate results.

2.6. Thermal stability of sHBD-3

Recombinant porcine β-defensins have been reported to be stable after different heat treatments (Li et al., 2013). In order to investigate the thermal stability of sHBD-3, a peptide solution (in 0.1% acetic acid) was heated at 100 °C for 14 min (baking time used in this study). After cooling, the activity of sHBD-3 (final concentration = 15 μg·ml^{-1}) was measured against *F. culmorum* FST 4.05 in a 96-well microtiter assay as described above.

2.7. Yeast strains

Two *Saccharomyces pastorianus* strains were used in this study. The parental strain *S. pastorianus* CMBS-33 is a bottom-fermenting lager yeast strain. CMBS-33 was used to create a novel strain, by integrating the gene coding for HBD-3 into its genome. The novel strain, called CM-INT-51, secreted recombinant HBD3 (rHBD-3) into its growth medium (James et al., 2014). *S. pastorianus* CMBS-33 and CM-INT-51 were from the Moyne Institute culture collection, Trinity College Dublin.

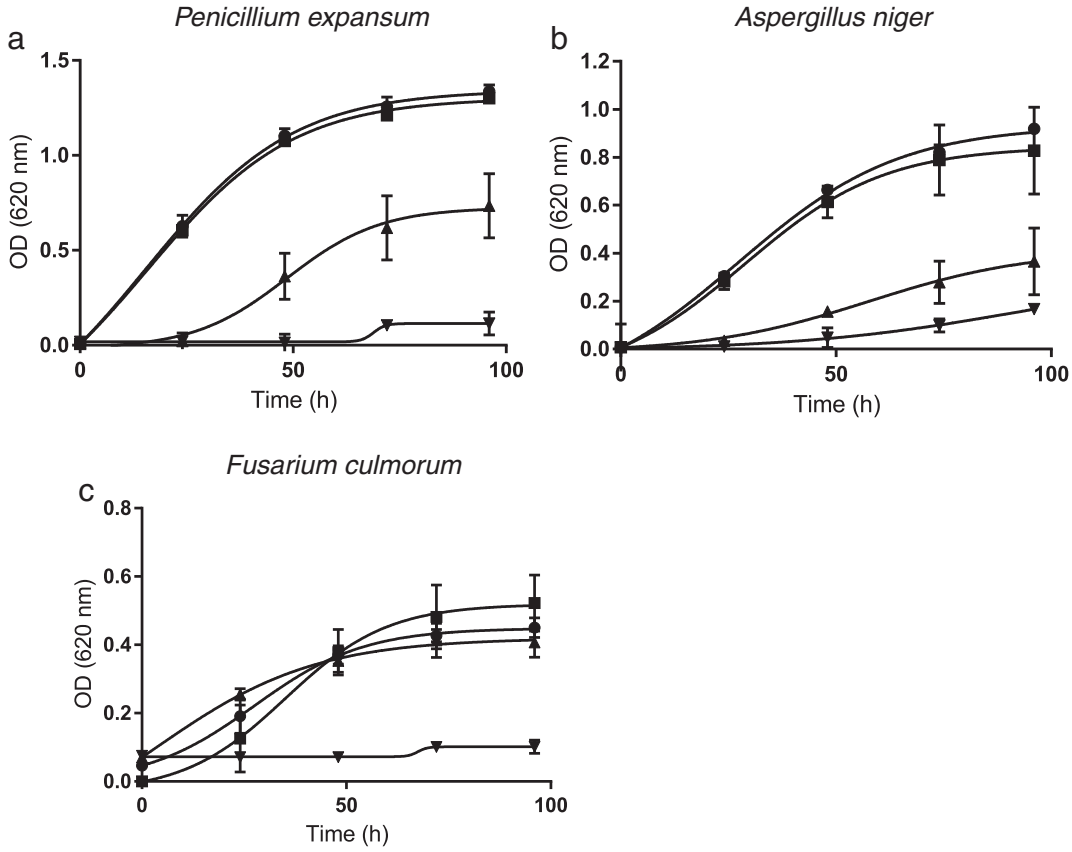


Fig. 1. Fungal growth inhibition of sHBD-3. (a) *Penicillium expansum* FST4.21, (b) *Aspergillus niger* FST 4.22, (c) *Fusarium culmorum* FST 4.05. Panels a and b: circles: 0 $\mu g \cdot ml^{-1}$, squares: 5 $\mu g \cdot ml^{-1}$; triangles: 50 $\mu g \cdot ml^{-1}$; reversed triangles: 150 $\mu g \cdot ml^{-1}$. Panel c: circles: 0 $\mu g \cdot ml^{-1}$; squares: 5 $\mu g \cdot ml^{-1}$; triangles: 10 $\mu g \cdot ml^{-1}$; reversed triangles: 15 $\mu g \cdot ml^{-1}$.

A commercial *Saccharomyces cerevisiae* strain (Pante instant dry yeast, Puratos, Belgium) was used as a control for the baking process.

2.8. Preparation and growth of cells

The *S. pastorianus* strains from a glycerol stock were first grown on yeast peptone sucrose (YPS) agar plates (0.3% (*w*/*v*) yeast extract, 1% peptone, 1% sucrose, and 2% agar) at 30 °C. For the selection of the recombinant CM-INT-51, the yeasts were grown on YPS agar containing geneticin (200 $\mu g \cdot ml^{-1}$) (Sigma Aldrich, St. Louis, MO) as described by James et al. (2014). After 72 h, the yeasts colonies were harvested and inoculated in YPS medium at 30 °C and the growth was followed by measuring the optical density (OD 620 nm) and counting the cells using a Neubauer chamber haemocytometer every 6 h. After 48 h of growth (between the diauxic shift and post-diauxic shift phase) which corresponds to the best phase to obtain superior bread characteristics according to Rezaei et al. (2014), the yeasts were harvested as described by Rezaei et al. (2014); the yeast culture was centrifuged at 870*g* for 3 min in an Eppendorf centrifuge 5417D (Eppendorf AG, Hamburg, Germany). Cultures were stored at 4 °C to be used in baking trials. Since a part of rHBD-3 has been shown to be expressed in the growth medium of CM-INT-51 (3–10 $ng \cdot ml^{-1}$) (James et al., 2014), the supernatant was kept at −20 °C for analysis or freeze-dried for further use in bread preparation.

2.9. Quantification of metabolites in the fermentation medium

The concentrations of different sugars (sucrose, glucose and fructose) and organic acids (citric, succinic and acetic acids) in the supernatant were determined as described by Rezaei et al. (2014). Briefly, the supernatant collected previously was centrifuged at 11,000*g* for 3 min and filter-sterilized with 0.20 μm filters (Macherey-Nagel, GmbH & Co., Germany). 20 μl of each sample were injected into a HPLC system (Agilent technologies, 1260 Affinity) equipped with a refractive index detector (RID) and an ultraviolet-diode array detector (UV/DAD) to quantify carbohydrates and organic acids. The analyses were performed twice on two different fermented growth media The standard calibrations curves were determined at the beginning of the set measurement with four different concentrations of acids (10–100 mM) and sugars (0.5–5 mM). The separation of sugars was carried out using Hi-Plex H column (300 × 7.7 mm, 8 μm, Agilent, Cork, Ireland), equipped with a guard column (50 × 7.7 mm, 8 μm, Agilent, Cork, Ireland), using water at a flow rate of 0.6 ml/min at 25 °C. The organic acids were quantified using the same column, after elution with 5 mM H_2SO_4.

2.10. Quantification of rHBD-3

rHBD-3 levels expressed by CM-INT-51 were quantified by capture enzyme-linked immunosorbent assay (capture ELISA) kit (Peprotech Co., Rocky Hill, NJ, USA) as described by James et al. (2014) with some modifications. The levels of rHBD-3 produced by CM-INT-51 in the supernatant or in the cell lysate were determined. Briefly, after 48 h of fermentation, the yeasts cells and the supernatant were separated by centrifugation at 870*g* for 3 min. The supernatant was filtered, purified on Sep.-Pak C18 cartridge (Merck Millipore, Cork, Ireland) and freeze-dried. Yeast cell pellets (2.5-ml packed cell volume) of CM-INT-51 were washed twice in 20 volumes of phosphate-buffered saline (PBS). Total cell lysates were prepared as using CelLytic-Y (Yeast Cell Lysis/Extraction Reagent; Sigma-Aldrich) as described by James et al. (2014).

Wells of a Nunc Maxisorb (96-well) plate (Thermofisher, Loughborough, UK) were coated overnight at RT with 100 μl of polyclonal anti-HBD3 capture antibody in PBS (1 $\mu g \cdot ml^{-1}$). The wells were washed four times with 0.05% Tween 20 (PBS-Tween). The wells were then blocked for 2 h with 1% bovine serum albumin (BSA) in PBS, washed

and incubated with 100 μl of different concentrations of sHBD-3 (as standard), previously diluted in 0.05% Tween 20, 0.1% BSA, PBS or samples.

Following incubation overnight at 4 °C, the wells were washed and incubated for 2 h with biotinylated polyclonal anti-HBD3 antibody (0.1 $\mu g \cdot ml^{-1}$) and washed as before. The wells were incubated for 30 min with 100 μl of streptavidin-HRP (1:2000 in 0.05% Tween 20, 0.1% BSA, PBS) and then washed with PBS-Tween. Antibody-binding was detected with 3,3′,5,5′-tetramethylbenzidine (TMB, T8665; Sigma-Aldrich) and the colour development was followed for 20 min by reading OD405 with a wavelength correction at 650 nm. Following incubation, sulphuric acid (to 0.1 M) was added and the absorbance measured at 450 nm.

2.11. Bread preparation

The dough was prepared according to a UCC standard recipe, based on flour weight (baker's flour, Odlums, Ireland) with some modifications: 3% (w/w) of commercial fat, 2% (w/w) of sugar from Siucra (Ireland), 0.3% (w/w) of sodium chloride from Glacia British Salt Limited (UK), 0.5% of sodium stearoyl lactate (SSL) (Danisco, Copenhagen, Denmark) , 0.1% of ascorbic acid (AllinAll Ingredients, Dublin, Ireland). The yeast strains were *S.pastorianus* CMBS-33, *S. pastorianus* CM-INT-51 and commercial *S. cerevisiae* dry yeast (Pante instant yeast, Puratos, Belgium). Sixty-three % (*v/w*) of tap water (for the rehydration of Puratos strain) or fermentation medium (YPS) for the *S.pastorianus* strains was used. The yeast concentration was $6 * 10^{10}$ cells/100 g of flour. Each dough of 50 g was fermented in a proofer (KOMA Sunriser, Roermond, Netherlands) for 1 h at 30 °C and 85% RH, and then baked for 14 min at 210 °C in a pre-steamed deck oven (MIWE, Arnstein, Germany). Finally, the breads were left for cooling for 2 h at room temperature before analysis. Three batches were prepared for each yeast strain.

Another baking trial was conducted with the Pante yeast strain, in which the tap water used in the recipe was supplemented with sHBD-3 (concentration = 80 $\mu g \cdot ml^{-1}$). A trial was performed with 0.3% (w/w, based on flour weight) calcium propionate (Sigma-Aldrich) as preservative agent commonly used in industry.

2.12. Bread characteristics

The breads were characterized as previously described by Hager et al. (2012). Specific volume was measured using a Volscan profiler (Stable Micro Systems, UK). After cutting the breads into 10-mm slices, three slices from the centre of each loaf were used for further analysis. Firstly, the crumb colour was analyzed, using a chroma meter CR- 400 (Konica Minolta Inc., Tokyo, Japan) with CIE standard illuminant D65. The L*-value, as calculated by the software, was used to evaluate the crumb lightness. C-cell Bread Imaging System (Calibre Control International Ltd., UK) was used to characterize the crumb structure. The wall thickness and the average diameter of the cells were evaluated. The crumb texture was determined by texture profile analysis (TPA), using a TA- XT2i texture analyser (Stable Micro Systems, Surrey, UK), equipped with a 25-kg load cell and a 10-mm aluminum cylindrical probe. A speed of 5 mm/s and a force of 0.98 N were applied to compress the middle of the crumb to 50% of its original height. Three slices per batch were analyzed on the baking day.

2.13. Analysis of metabolites in breads

The concentrations of sugars and acids in the different breads were measured by HPLC as described by Rezaei et al. (2014). Briefly, slices of 10 mm of loaf were blended (Kenwood chef classic) for 30 s in two volumes of MilliQ water. After centrifugation at 11,000g for 3 min, the supernatant was filter-sterilized with 0.20 μm filters and analyzed as described for the fermentation medium.

2.14. Quantification of rHBD-3 in the bread

To determine the concentration of rHBD-3 in the bread, 10 g of bread were soaked into 100 ml of water overnight at room temperature. After centrifugation, the supernatant of bread/water mix was filtered and purified on Sep.-Pak C18 cartridge and freeze-dried. The lyophilisate was resuspensed in 0.5–1.0 ml of PBS and the concentration of rHBD-3 was determined as described above.

2.15. Shelf-life

The study of the effect of the yeast strain CMBS-33 and the novel strain CM-INT-51 on the shelf life of bread was made as described by Dal Bello et al. (2007) with some modifications. Loaves were cut transversely in a sterile manner to obtain uniform slices of 10-mm thickness and left 5 min on each side exposed to the ambient air. Then, the slices were packed in heat sealed plastics bags. Filter pipette tips were inserted in small slots on each side of the bag and the bags were incubated at room temperature. The slices were examined for mould growth over 13 days and the shelf life was determined by the day of appearance of the first fungal colony. The slices were sorted into five categories based on the percentage of contamination as described by Axel et al. (2015).

2.16. Statistical analysis

The results in the tables are presented as means ± SDs. A Kruskal–Wallis Test was performed with XLSTAT Version 2015.5.01. (Adinsoft Inc., New York, USA) to compare the metabolite concentrations in the growth media of the *S.pastorianus* strains, and to analyse the textural properties of breads and metabolites concentrations in the breads. Values of $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1. Antifungal assays

The growth curves of the three studied fungi (*P. expansum*, *A. niger* and *F. culmorum*) are presented in Fig. 1. sHBD-3 showed the highest antifungal activity against *F. culmorum* with an IC_{50} value of 11 $\mu g \cdot ml^{-1}$ and a MIC of 15 $\mu g \cdot ml^{-1}$. Higher concentrations of sHBD-3 were required to inhibit *A. niger* and *P. expansum* (respectively IC_{50} of 36.4 $\mu g \cdot ml^{-1}$ and 44.3 $\mu g \cdot ml^{-1}$) (Table 1). The inhibition of the fungal strains *F. culmorum* FST 4.05, *A. niger* FST4.21, *P. expansum* FST 4.22 was confirmed on PDA plates after 96 h (Fig. 2).

Apart its typical CSαβ motif, the configuration of human defensins is characterized by the presence of three disulfide bonds between the cysteine residues. Unlike native HBD-3, a synthetic HBD-3 does not have disulfide bonds. Despite this, sHBD-3 is able to inhibit fungal growth, which supports the idea that disulfide bonds are not essential for the antimicrobial activity (Hoover et al., 2003). However, the presence of disulfide bonds in the peptide has been shown to improve the antibacterial activity and has been proven to be involved in the chemotactic action of HBD-3 (Wu et al., 2003).

The mechanism behind the inhibition of *F. culmorum* by sHBD-3 was analyzed by using phase contrast microscopy. As seen in Fig. 3, after 24 h of incubation, the spores in the control medium have

Table 1
Inhibition of fungal growth by a synthetic Human β-defensin 3.

Fungal species	IC_{50} ($\mu g \cdot ml^{-1}$)	MIC ($\mu g \cdot ml^{-1}$)
Fusarium culmorum FST 4.05	11.0	15.0
Penicillium expansum FST4.21	44.3	>150
Aspergillus niger FST 4.22	36.4	>150

>150: no total inhibition in the range of concentration and time used in this study.

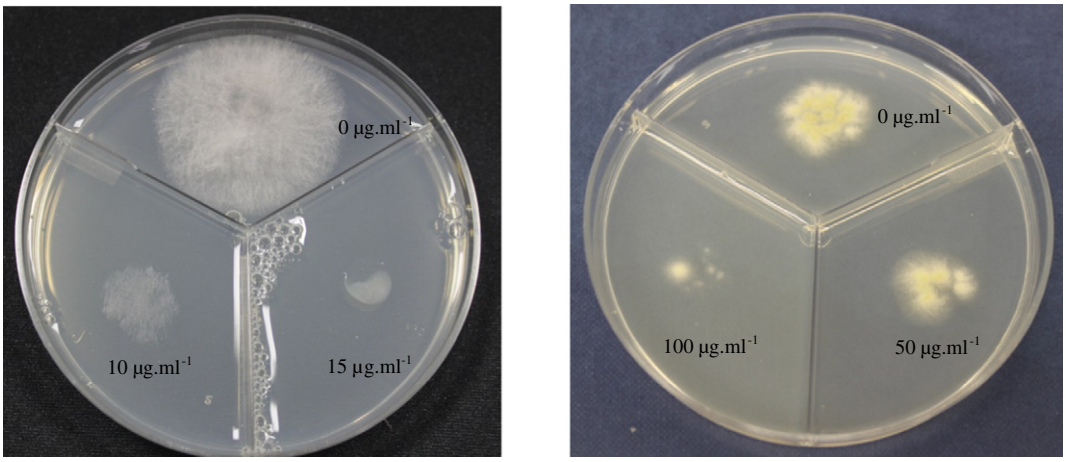


Fig. 2. Examination of fungal inhibition on agar plates after 96 h of incubation in 1/2PDB with different concentrations of sHBD-3. Left: *F. culmorum*; Right: *A. niger*. The concentrations of sHBD-3 in the broth are indicated in the different compartments.

germinated. With 10 µg · ml^{-1} of sHBD-3, the conidia remained in the same state. Moreover, the intracellular content of the *Fusarium* spores appears to be released in the medium after disruption of the membrane.

Two major models are used to explain the mechanism of action of the defensins: the carpet and the pore-forming model. In both models, it is proposed that defensins interact with the negatively charged molecules of the microbial cell membrane, leading to an increase of

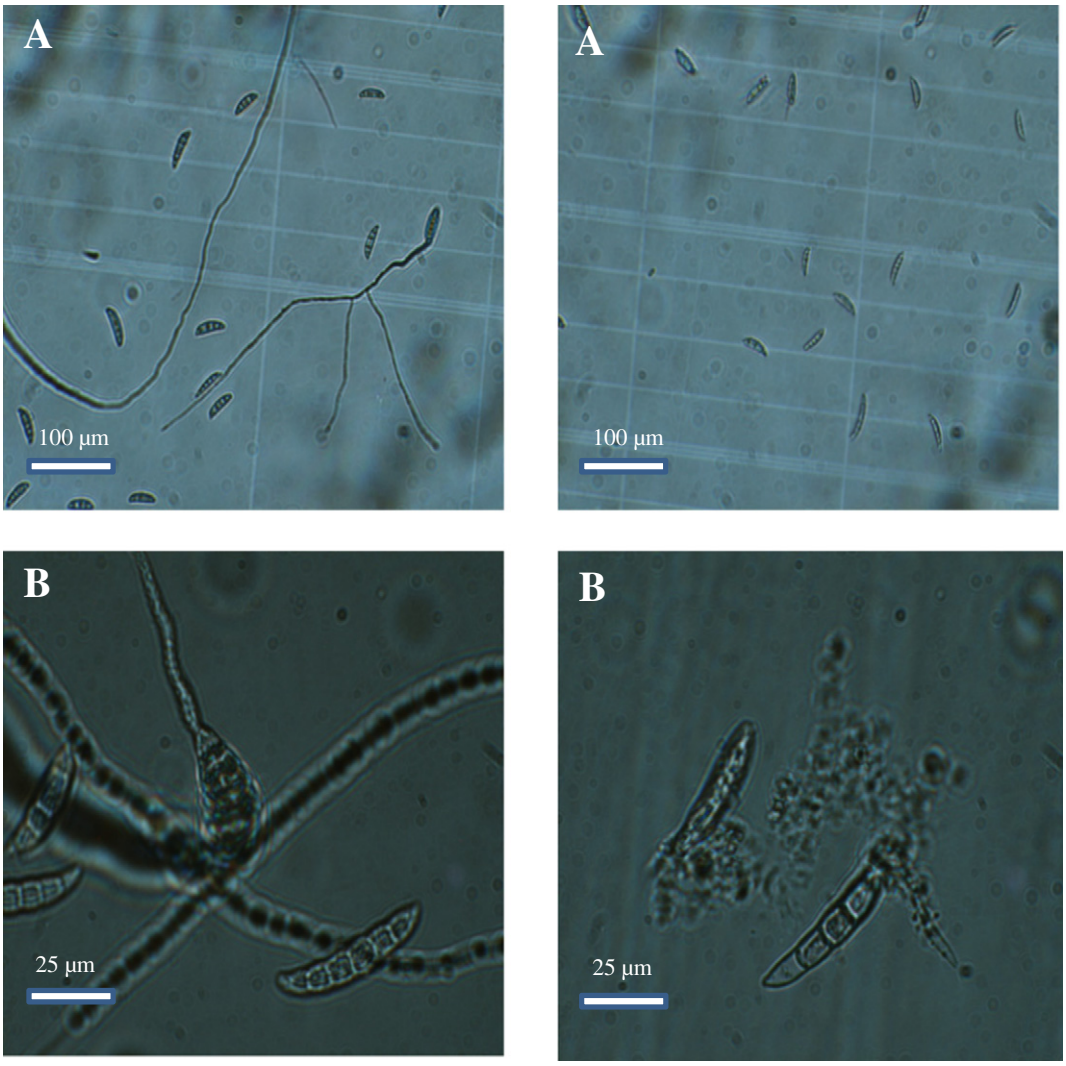


Fig. 3. Phase contrast microscopy of *Fusarium culmorum* (10^3–10^4 spores · ml^{-1}) after 24 h of fungi growth in the presence of control medium (left) or medium supplemented with synthetic HBD-3 (10 µg · ml^{-1}) (right). The content of the magnification bars = 100 µm (A), 25 µm (B).

permeabilization and cell leakage. However, for a given peptide and microbial genera, the mechanism may depend on the species. It has also been suggested that the native peptides and their linear analogues can use different mechanisms of action (Wu et al., 2003).

3.2. NaCl effect on HBD-3 inactivation of F. culmorum

Sodium chloride is an important ingredient of baking process and its effect on antimicrobial peptides has already been studied. It has been reported by Wu et al. (2003) that the presence of salt tends to decrease the antimicrobial activity of this type of peptides. This effect is due to the weakening of electrostatic interactions between the cationic peptides and the negatively charged membrane of microbial cells (Wu et al., 2003). Here, the effect of salt on the activity of sHBD-3 against *F. culmorum* was studied.

The activity of sHBD-3 (15 $\mu g \cdot ml^{-1}$) against *F. culmorum* was tested in solutions with 50 mM NaCl and 150 mM NaCl. The percentage of inhibition of *F. culmorum* is presented in Fig. 4a. In the salt solutions containing up to 50 mM NaCl, sHBD-3 revealed antifungal activity (~90% of the control activity after 96 h) against *F. culmorum*. However, under physiological salt concentrations (150 mM NaCl), the activity of sHBD-3 was reduced by ~90% after 96 h. These results reveal that the activity of sHBD-3 against *F. culmorum* was reduced by the addition of salt.

Native HBD-3 has previously been reported to be salt-insensitive (up to a level of 100 mM NaCl) in its antibacterial activity (Hoover et al., 2003). Its ability to form oligomers has been proposed to explain its antibacterial activity against Gram-positive bacteria at physiological salt concentrations (Dhople et al., 2006). However, only linear HBD-3 holds its activity against *E. coli* at concentrations up to a level of 150 mM NaCl (Hoover et al., 2003). The high cationicity of the peptide (Sahl et al., 2005) is essential for its antibacterial activity and the upholding of this latter in salt solutions. It has been shown that the antifungal activity of HBD-3 is much more salt sensitive than the antibacterial activity (Vylkova et al., 2007). The mechanisms of action of HBD-3 against fungi and bacteria may be behind the difference in sensitivity. Indeed, the net charge and the secondary structure of the peptide do not appear to be critical for the inactivation of *C. albicans* (Dhople et al., 2006; Vylkova et al., 2007).

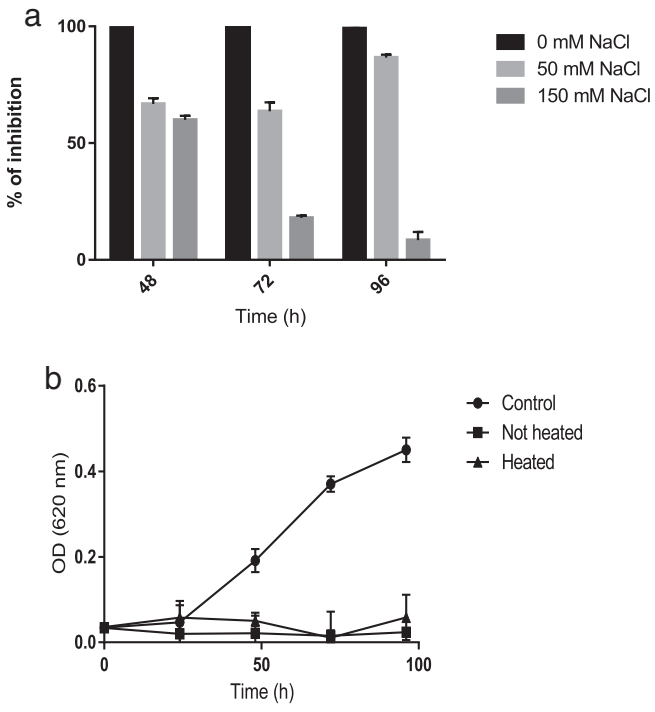


Fig. 4. Antifungal activity stability of a synthetic HBD3. The synthetic peptide (final concentration = 15 $\mu g \cdot ml^{-1}$) was tested against *F. culmorum* (10^3–10^4 spores$\cdot ml^{-1}$). Panel a: Effect of NaCl concentration on sHBD-3 antifungal activity. Panel b: Effect of heat treatment (100 °C/14 min) on sHBD-3. Circles: control (sHBD-3 = 0 $\mu g \cdot ml^{-1}$); squares: sHBD-3 = 15 $\mu g \cdot ml^{-1}$ without heating treatment; triangles: sHBD-3 = 15 $\mu g \cdot ml^{-1}$ with heating treatment.

3.3. Thermal stability

A solution of synthetic HBD-3 (15 $\mu g \cdot ml^{-1}$) in 0.1% acetic acid was treated at 100 °C for 14 min. After cooling at RT, the antifungal activity was determined against *F. culmorum*. As seen in Fig. 4b, the antifungal activity of sHBD-3 was maintained after heat treatment.

The heat stability of different recombinant or native defensins has been studied by several authors (Lacerda, Vasconcelos, Pelegrini, & Grossi de Sa, 2014; Liu et al., 2008) and explained by the resistance of the secondary structure to heat denaturation. In the case of a synthetic HBD-3, the disulfide bonds are absent and the secondary structure of the peptide may be modified. However, the high flexibility of the linear forms of defensins (Hoover et al., 2003) could confer different configurations to the peptide. Many linear AMPs, including LL-37 and CP-29, are unstructured in an aqueous environment, and adopt an alpha helical structure when they come into contact with microbial membrane (Wu & Hancock, 1999). This α-helical structure is usually amphipathic, allowing the peptide to interact with phospholipid bilayers, key to membrane disruption and translocation into membranes.

In addition to this, it has been proven that the secondary structure of HBD-3 has a little role in the antifungal activity of the peptide (Vylkova et al., 2007). It is generally accepted that heat treatment can enhance surface hydrophobicity of protein. Now, the hydrophobicity has been proven to play a role in the antimicrobial activity of cationic antimicrobial peptides (Laverty & Gilmore, 2014). Specifically, the hydrophobicity of HBD-3 is important for its activity against Gram-positive bacteria and *C. albicans* (Hoover et al., 2003; Lee, Raman, Gellman, Lynn, & Palecek, 2014). Thus, the maintaining of the antifungal activity of HBD-3 after heat treatment could be attributed to the flexibility and the hydrophobicity of the linear peptide.

3.4. Quantification of rHBD-3

The concentration of rHBD-3 expressed by the yeasts was determined by ELISA assay. After 48 h of fermentation, the level of rHBD-3 in the supernatant was 8.5 ± 1.2 ng/10^8 cells. It appears that an important part of rHBD-3 remained bound to the yeast cell or is present into the cell, since the concentration in the total lysate is 13 ± 3 ng/10^8 cells. The presence of rHBD-3 was not detected in the bread. The expression of rHBD-3 extracellularly and intracellularly has already been reported by James et al. (2014).

Although the heterologous expression of peptide in yeast is more complex than in *E. coli*, the recombinant peptide expressed in yeast has been proven to be more efficient (Kant, Liu, & Pauls, 2009). The use of yeast to produce a recombinant defensin did not affect the peptide conformational features and allows post-translational modifications, such as the creation of disulfide bonds (Lacerda et al., 2014). Besides, recombinant human defensins have been proven to maintain antimicrobial activities (Pazgier et al., 2006).

3.5. Evaluation of the effect of defensins on bread characteristics

Three different yeast strains were used for the fermentation of the dough, namely *S. pastorianus* CMBS-33, *S. pastorianus* CM-INT-51 and a commercial *S. cerevisiae* strain (Pante dry yeast) from Puratos. Doughs of 50 g were fermented for 1 h and baked at 210 °C for 14 min. The pictures of the bread are presented in Fig. 5.

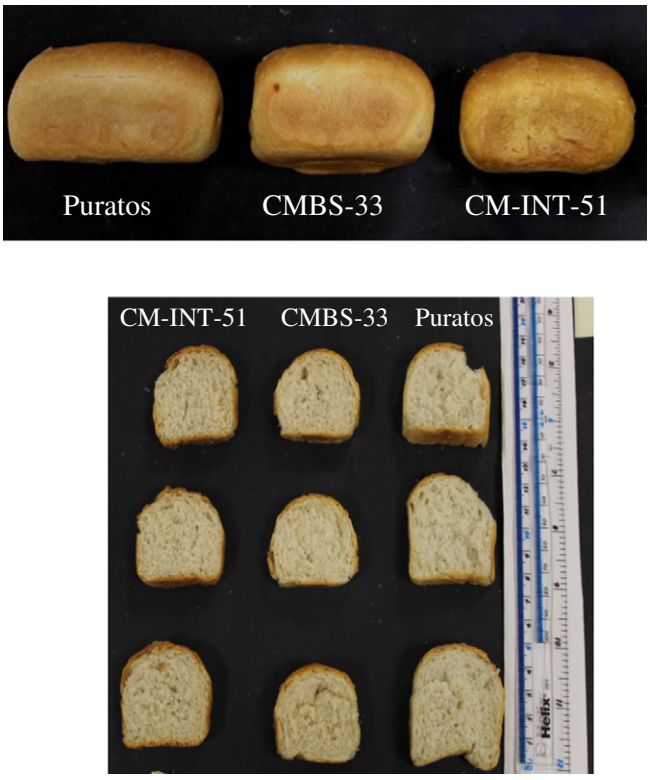


Fig. 5. Pictures of breads made with the three different yeast strains. The doughs fermented with the fresh *S. pastorianus* strains lead to smaller breads, which can be explained by the difference of ability of fermentation between *S. cerevisiae* and *S. pastorianus* species.

3.6. Characterization of the breads

The effect of the different yeast strains on different rheological characteristics of the bread was evaluated and is presented in Table 2. The different types of bread presented overall similar characteristics. The differences in loaf specific volume and hardness were not significant ($p = 0.65$ and 0.25 respectively) between the breads made the three yeast strains. The breads made with the commercial yeast strain appeared to have a higher specific volume (~4.9 ml/g) and this can be attributed to the specific adaptation of this strain to the bread environment.

Another batch was made from the commercial yeast strain and the dough was supplemented with sHBD-3. The fermentation of the dough by the commercial yeast strain was not affected by the presence of sHBD-3, the supplemented bread showing similar characteristics to the control (Table 2).

Table 3
Acids and sugars concentrations of fermentation medium (YPS) with the yeast strains CMBS-33 and CM-INT-51[a].

	Yeast strains		
	Pante (*S.cerevisiae*)	*S.pastorianus* CMBS-33	*S.pastorianus* CM-INT-51
Sugars (mM)			
Sucrose	<0.01[a]	1.30 ± 0.94[a]	2.36 ± 1.38[a]
Glucose	<0.01[a]	<0.01[a]	<0.01[a]
Fructose	<0.01[a]	0.05 ± 0.02[a]	0.04 ± 0.03[a]
Acids (mM)			
Citric	0.9 ± 1.5[a]	1.7 ± 1.47[a]	0.35 ± 0.6[a]
Succinic	<0.01[a]	<0.01[a]	1.72 ± 2.9[a]
Acetic	4.13 ± 0.48[b]	2.52 ± 0.08[a]	1.31 ± 1.10[a]

Values with different letters in a row differ statistically (Kruskal-Wallis test, $p < 0.05$).
[a] Mean ± SDs calculated with duplicates of two growth media.

3.7. Analysis of metabolites

The concentrations of the different sugars (sucrose, glucose and fructose) and organic acids (citric, succinic, acetic) in the supernatant of the fermentation media of the three yeast strains is presented in Table 3. As nutrients, the concentration of sugars in bread influences the development of fungal colonies. Organic acids, particularly succinic acid, are usual metabolites of the baking process (Rezaei et al., 2014; Yoshida & Yokoyama, 2012). The presence of organic acids produced during the fermentation plays a role as well in fungal growth. Indeed, these acids have been shown to have antifungal activity (Shokri, 2011).

After 48 h of growth, the concentration of the different metabolites was not significantly different between the media fermented with the strains CMBS-33 and CM-INT-51. The Pante strain appeared to ferment more efficiently sucrose (<0.01 mM) and to produce more acetic acid (4.13 mM) than CMBS-33 and CM-INT-51 (2.52 and 1.31 mM respectively). The differences can be attributed to the ability of fermentation at 30 °C between the two species. The concentrations of metabolites in the breads highlight the better ability of the commercial yeast strain (Pante) to ferment dough at 30 °C (Table 4).

The concentration of sugars and acids were slightly different between the breads made with CMBS-33 and CM-INT-51. The insertion of a gene expressing HBD-3 in the yeast genome could slightly affect the yeast performance (Nevoigt, 2008).

3.8. Shelf-life

The breads produced with the different yeast strains were sliced and the appearance of moulds was monitored over a 13-day period. Each slice was classified among one of the five categories (based on the percentage of contamination) presented in Fig. 6.

The use of the fresh *S. pastorianus* yeast strains delayed the appearance of the fungal colonies, in comparison with the commercial Pante

Table 2
Structural characteristics of breads[a].

Characteristic	Yeast strains				
	Pante (*S.cerevisiae*)	Pante (*S.cerevisiae*) + sHBD-3	*S.pastorianus* CMBS-33	*S.pastorianus* CM-INT-51	*p* *p*-Value
Loaf specific volume (mL/g)	4.9 ± 1.8[a]	4.22 ± 0.14[a]	4.31 ± 1.3[a]	3.84 ± 0.5[a]	0.65
Crust colour	52 ± 2.7[b]	50.5 ± 1.6[b]	52.6 ± 2.0[b]	46.5 ± 1.9[a]	0.003
Hardness (g)	327 ± 60[a]	257 ± 50[a]	362 ± 62[a]	273 ± 39[a]	0.25
Springiness (%)	0.97 ± 0.01[b]	0.94 ± 0.07[ab]	0.94 ± 0.02[ab]	0.91 ± 0.02[a]	0.04
Cohesiveness	0.58 ± 0.05[a]	0.69 ± 0.06[ab]	0.68 ± 0.01[b]	0.68 ± 0.02[b]	0.06
Chewiness (g)	187 ± 44[a]	165 ± 29[a]	230 ± 39[a]	170 ± 27[a]	0.2
Cell diameter (mm)	2.13 ± 0.24[a]	1.74 ± 0.24[a]	2.7 ± 1.1[a]	3.9 ± 1.7[a]	0.33
Wall thickness (mm)	0.44 ± 0.01[a]	0.42 ± 0.01[a]	0.49 ± 0.04[a]	0.49 ± 0.04[a]	0.77

Values with different letters in a row differ statistically (Kruskal-Wallis test, $p < 0.05$).
[a] Mean ± SDs calculated with at least three slices of two bread batches.

Table 4
Sugars and acids concentrations in bread with the three yeast strains[a].

Yeast strains	Pante (*S.cerevisiae*)	*S. pastorianus* CMBS-33	*S. pastorianus* CM-INT-51
Sugars (mM)			
Sucrose	7.35 ± 0.00[a]	7.91 ± 0.01[b]	9.61 ± 0.11[c]
Glucose	0.24 ± 0.00[a]	2.85 ± 0.02[c]	1.53 ± 0.37[b]
Fructose	3.93 ± 0.03[a]	7.79 ± 0.04[c]	5.88 ± 0.04[b]
Acids (mM)			
Citric	<0.01[a]	1.29 ± 1.32[a]	1.71 ± 1.63[a]
Succinic	10.7 ± 1.98[a]	13.24 ± 0.11[a]	7.12 ± 2.30[a]
Acetic	0.56 ± 0.01[c]	0.17 ± 0.01[a]	0.3 ± 0.02[b]

Values with different letters in a row differ statistically (Kruskal-Wallis test, $p < 0.05$).
[a] Mean ± SDs calculated with duplicates of three batches.

strain. Some differences in the composition of the fermentation medium could explain this delay.

In addition to this, it appeared that the appearance of fungal colonies on the bread made with the recombinant yeast strain CM-INT-51 is delayed. Indeed, compared to the breads made with the strain CMBS-33, the first fungal colonies appeared 3 days later. It has been shown by James et al. (2014) that the yeast strain CM-INT-51 expresses rHBD-3 in its growth medium. We can assume that the presence of rHBD-3 plays a major role in the decreasing of the fungal growth. This is supported by the fact that the breads prepared with a supplement of sHBD-3 (80 $\mu g.ml^{-1}$) did not present any fungal contamination over 13 days (data not shown). Furthermore, the breads made with CM-INT-51 presented the same extension of shelf-life as "industrial" breads formulated with 0.3% calcium propionate (*w*/w) (Fig. 6D).

Besides, during baking, yeast cells are susceptible to release their intracellular content (Boekhout & Robert, 2003), including rHBD-3. Surprisingly, rHBD-3 was not detected in the bread made with the yeast strain CM-INT-51. To detect rHBD-3, the method of extraction and potential links between HBD-3 and the bread matrix will be further studied.

4. Conclusion

In conclusion, it is shown that a synthetic HBD-3 has a deleterious effect on the growth of fungal contaminants. This reinforces the potential of defensins to fight microbial spoilage of cereal-based products. Since the antifungal activity of sHBD-3 solution is both relatively heat- and salt- resistant, this peptide is a good model candidate for baking applications. A *S.pastorianus* strain expressing the defensin gene was used in baking process. Despite slight differences in the breads made with this strain and the bread made with a commercial yeast strain, the "modified" bread has an extended shelf life with a decrease of the fungal growth. Due to the lack of knowledge on the safety of sHBD-3 and the yeast strain CM-INT-51, no sensory analysis was performed. The use of HBD-3, and more generally of defensins, in synthetic form or produced by modified yeasts, may be a novel alternative to common preservatives in bread protection.

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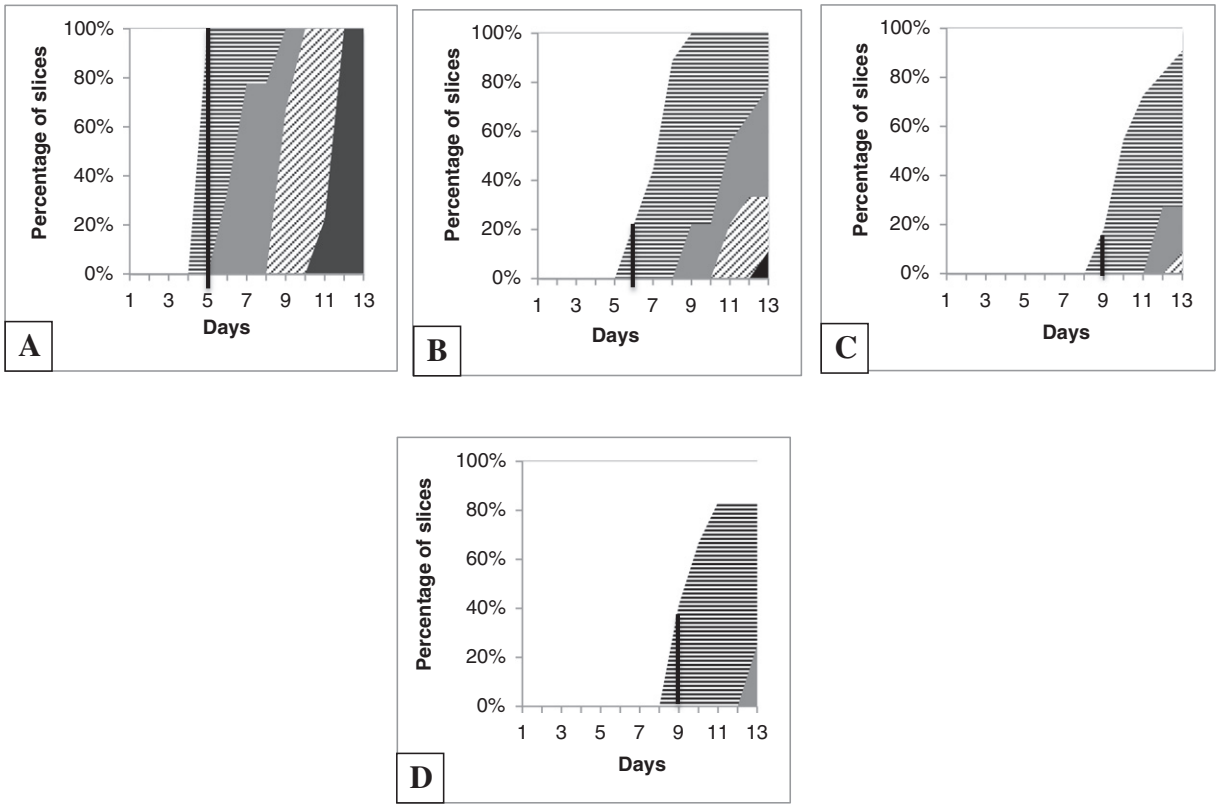


Fig. 6. Shelf life of the four types of breads (A: Puratos strain; B: CMBS-33; C: CM-INT-51; D: Puratos strain with 0.3% calcium propionate). The slices are categorized depending on the percentage of contamination: ▭Mould free ▤<10% ▩10–24% ▨25–49% ■>50% The black line indicates the first day of appearance of fungal colony.

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Antifungal activity of synthetic cowpea defensin Cp-thionin II and its application in dough

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ABSTRACT

Plant defensins are small, cysteine-rich antimicrobial peptides of the immune system found in several organs during plant development. A synthetic peptide, KT43C, a linear analogue of the native Cp-thionin II found in cowpea seeds, was evaluated for its antifungal potential. It was found that KT43C displayed antifungal activity against *Fusarium culmorum*, *Penicillium expansum* and *Aspergillus niger*. Like native plant defensins, KT43C showed thermostability up to 100 °C and cation sensitivity. The synthetic peptide decreased the fungal growth without inducing morphogenic changes in the fungal hyphae. Non-inhibitory concentrations of the peptide induced permeabilization of the fungal membrane. In addition, high concentrations of KT43C induced the production of reactive oxygen species in the granulated cytoplasm. To investigate potential applications, the peptide was used as an additive in the preparation of dough which did not contain yeast. This peptide delayed the development of fungal growth in the dough by 2 days. Furthermore, KT43C did not induce red blood cell lysis up to a concentration of 200 $\mu g.ml^{-1}$. These results highlight the potential for the use of synthetic antimicrobial defensins for shelf-life extension of food products.

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1. Introduction

Plants have developed an ancient and complex defense strategy through their immune system to combat pathogens and abiotic stresses (de Beer and Vivier, 2011; Lacerda et al., 2014). Among their many defense systems, the production of cationic antimicrobial peptides (cAMPs) is a major contributor to plant resistance to phytopathogens, thanks to their broad spectrum of activity (Stotz et al., 2009). Plant AMPs have been divided into several categories based on their amino acid structure, sequence identity or tertiary structure (Nawrot et al., 2014). Among these AMPs, plant defensins were first discovered in the seeds of wheat and barley (Colilla et al., 1990; Mendez et al., 1990). Plant defensins can be divided into three groups: defensins leading to morphogenic changes in the fungal hyphae, defensins causing reduction of hyphae without morphogenic changes, and defensins without antifungal activity (Broekaert et al., 1995). The expression of plant defensin genes has been reported to be increased in response to pathogens, which supports the idea that these peptides constitute a major defence mechanism (Garcia-Olmedo et al., 1998). In addition, the localization of the plant defensins in different plant organs and tissues, with a preferential cell-wall location in epidermal cells (Lacerda et al., 2014), is highly consistent with a defensive role. The defensins also play a role in the protection against insects, abiotic stress and metal tolerance (Carvalho and Gomes, 2009).

Plant defensins are cysteine-rich cationic peptides of 5–8 kDa whose mature domain comprises 45 to 54 amino acid residues, with an isoelectric point around 9. The global fold of plant defensins consists of a cysteine-stabilized motif (CSα/β) made up of an α-helix and a triple-stranded β-sheet stabilized by four disulfide bridges (C1-C8/C2-C5/C3-C6/C4-C7) (Lay et al., 2003). In addition to this motif, Yount et al. (2004) reported the importance of an additional conserved motif, named γ-core, for the antimicrobial activity of the peptide.

The mode of action of several plant defensins has been extensively studied and different mechanistic steps have been identified but are yet not completely understood (Thevissen et al., 2004; Thomma et al., 2002). In many cases, it involves the recognition and binding to a cell wall (e.g., glucosylceramide) or membrane (e.g., phosphatidic acid) receptor. Then, plant defensins are either

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internalized into the fungal cell or stay at the surface of the membrane and induce several mechanisms: activation of enzyme pathways, modification of ion fluxes, inhibition of conidial germination or induction of apoptosis. In contrast to mammalian and insect defensins, the insertion of plant defensins in the fungal membrane via pore-formation is uncommon and the mechanism of translocation of the peptides has not yet been identified. Plant defensins are generally non-cytotoxic to mammalian and plant cells (Thevissen et al., 2004). However, some plant defensins have been reported to display Ca^{2+} channel inhibition, anticancer activity and mitogenic activity towards mammalian cell lines (Guzman-Rodriguez et al., 2015). The toxicity of defensins and cAMPs in general has been attributed to the presence of disulfide bonds, their high hydrophobicity and amphipathicity (Hollmann et al., 2016; Jenssen et al., 2006). Consequently, the design of linear analogues of AMPs and defensins showing antimicrobial properties has gained strong interest. For example, a linear variant of human β-defensin 3 has been shown to partially retain its antimicrobial activity, without any toxicity towards human erythrocytes and conjunctiva epithelial cells (Liu et al., 2008).

Fungal spoilage is a major issue in cereal-based foods and in the wider food industry. In addition to economic losses due to unpleasant flavours and appearance, mould has the potential to cause health issues due to the production of mycotoxins (Pitt and Hocking, 2012). Among the different cereal-based products, chilled dough is recognized as a convenient solution to the consumer's desire for oven-fresh baked products. Despite the chilled conditions, bacterial and fungal growth can appear during the storage of this dough. In order to prevent spoilage, a range of preservatives are used, but the overuse of these artificial preservatives has a negative image with the consumer. Alternative solutions to such preservatives, including natural antimicrobial peptides such as nisin, are already in use but their application is still challenging, mainly due to their narrow spectrum of activity (Dielbandhoesing et al., 1998). In contrast, due to their natural role in plant protection, defensins present promising properties, such as inhibition of a broad range of microorganisms and this can be exploited in food applications.

The defensin Cp-thionin II has been extracted from cowpea seeds and displayed antimicrobial activity against Gram-positive and Gram-negative bacteria (Franco et al., 2006). The objective of this study was to characterize the antifungal activity of a linear analogue of Cp-thionin II, KT43C (see Table 1). The application of this synthetic peptide for the prevention of fungal growth in dough was also studied. In addition, the effect of the peptide on red blood cell integrity was examined to determine its toxicity.

2. Material and methods

2.1. Defensin

A synthetic peptide (purity >70%), named KT43C (5.2 kDa), a linear analogue of the plant defensin Cp-thionin II, was synthesized by and purchased from GLBiochem, Shanghai, China. Synthesis was made by solid-phase method using Fmoc chemistry, as described by the manufacturer. Fmoc group was removed by treating the resin with a mix of piperidine/DMF (20/80). A MALDI-TOF mass spectrometry (SHIMADZU AXIMA Assurance) was performed by GLBiochem to confirm the stability of the peptide after synthesis (see Supplementary information). The lyophilized peptide was reconstituted in 0.1% acetic acid (pH 3.5) and stored in vials at different concentrations at −20 °C.

2.2. Fungal strains

The filamentous fungi used in this study represent some of the main contaminants of cereal-based products (Beuchat, 1987) including *Fusarium culmorum*, *Aspergillus niger* and *Penicillium expansum*. The fungal strains *F. culmorum* FST 4.05, *A. niger* FST4.21 and *P. expansum* FST 4.22 originated from the culture collection of the School of Food and Nutritional Sciences, University College Cork (Cork, Ireland).

2.3. Fungal growth inhibition assay

To determine the antifungal activity of the synthetic peptide, the fungal growth was followed in a microtiter plate assay as described previously by Van Der Weerden et al. (2008) with some modifications. Briefly, fungal spores were collected from mature colonies grown for 72 h on potato dextrose agar (PDA) (Sigma-Aldrich) plates at 25 °C, counted on a haematocytometer and inoculated in synthetic nutrient-poor broth. Fungal spores were then diluted in half-strength potato broth (1/2 PDB) (Sigma-Aldrich) to obtain a final concentration of 1×10^4 spores.ml^{-1}. Spore suspensions (80 µl) were added to the wells of a 96-well microtiter plate.

Filter-sterilized synthetic peptides (20 µl) at concentrations ranging from 5 µg.ml^{-1} to 200 µg.ml^{-1} (final concentration) were then added to the fungal cells. Fungal growth was assessed by measuring the absorbance at 595 nm for 96 h at 25 °C (Multiskan TM, Thermo Scientific). Cell suspensions in 0.1% acetic acid (in ½ PDB, pH = 5.0) were used as negative controls. Each test was performed in triplicate.

The inhibition of fungal growth by KT43C was then examined on PDA plates. The same solutions were prepared as described above

Table 1
Characteristics of the linear peptide KT43C and comparison with plant defensins.

Name	Source	Sequence	Identity	Activity	Function	Reference
Linear peptide KT43C	Synthetic peptide	KTCMTKKEGWGRCLIDTTCAHSCRKYGYMGGKCQGITRRCYCLLNC	–	*F. culmorum* *A. niger* *P. expansum*		This study
Cp-thionin II	*Vigna unguiculata*	KTCMTKKEGWGRCLIDTTCAHSCRKYGYMGGKCQGITRRCYCLLNC	100%	Gram-positive *S. aureus* Gram-negative *E. coli* *P. syringae*	γ-thionin	(Franco et al., 2006)
Cp-thionin	*Vigna unguiculata*	RVCESQSHGFKGACTGDHNCALVCRNEGFSGGNCRGFRRRCFCTLKC	42%	Unknown	Trypsin inhibitor	(Melo et al., 2002)
VrD1	*Vigna angularis*	RTCMIKKEGWGKCLIDTTCAHSCKNRGYIGGNCKGMTRTCYCLVNC	73%	Insecticidal	α-amylase inhibitor	(Liu et al., 2006)

Underlined cysteine residues are involved in disulfide bonds (C1-C8/C2-C7/C3-5/C4-C6).

and after 24 h incubation at 25 °C, 50 µl were spot-inoculated on PDA plates. Pictures of the plates were taken at 24 h intervals for a period of three days.

2.4. MIC/IC_{50} determination

After 96 h of incubation at 25 °C, the MIC was read as the lowest concentration of KT43C resulting in complete inhibition of fungal growth. The required concentration to inhibit 50% of the growth (IC_{50}) was determined by non-linear regression, using the software Graph PRISM (GraphPad Software, Inc., La Jolla, CA) with the microplate reader data.

2.5. Thermal stability of KT43C

In order to investigate the thermal stability of KT43C, a peptide solution was heated at 100 °C for 15 min. After cooling at RT, the activity of KT43C (MIC = 20 µg.ml^{-1}) was measured against *F. culmorum* in a 96-well microtiter assay as described above.

2.6. Effect of cations on the antifungal activity of KT43C

To determine the effect of cations on KT43C activity, an antifungal assay was performed in different salt solutions. Fungal spores (1×10^4 spores.ml^{-1}) were inoculated in medium containing either 100 mM NaCl, 50 mM KCl, 5 mM $CaCl_2$ or 5 mM $MgCl_2$, as described previously (Terras et al., 1992). After the addition of the peptide, the inhibitory activity of KT43C (concentration = 20 µg.ml^{-1}) was measured against *F. culmorum* in a 96-well microtiter assay as described above.

2.7. Characterization of the antifungal activity

To visualize the action of KT43C against *F. culmorum* spores, the peptide, at concentrations of 5 and 20 µg.ml^{-1}, was added to ½ PDB inoculated with fungal spores. After 24 h, 500 µl of each solution was freeze-dried for further analysis. Five hundred µl of 70% glycerol was added to the solutions to avoid damage due to freeze drying. The lyophilized samples were then analysed using a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan), as described by Oliveira et al. (2012), with some modifications. Briefly, fungal mycelium was mounted onto plain aluminium stubs using carbon double surface adhesive and coated with a 7 nm gold layer using a Gold Sputter Coater (BIO-RAD Polaron Division, SEM coating system, England) and observed under a constant accelerating voltage of 5 kV.

2.8. Membrane permeabilization assay

To characterize the mode of action of the synthetic peptides against *F. culmorum*, a SYTOX® Green (Molecular Probes) uptake assay was performed as described by Van Der Weerden et al. (2008), with some modifications.

F. culmorum hyphae were grown in ½ PDB from a suspension of 1×10^4 spores.ml^{-1} for 18 h at 25 °C. The hyphae were then incubated with 10, 20 and 50 µg. ml^{-1} of KT43C at room temperature with gentle agitation. SYTOX® Green was added (final concentration of 0.5 µM) and the hyphae were allowed to stand for 10 min. Fluorescence of hyphae was measured using a fluospectrophotometer (Varioskan® LUX reader) for 2 h with excitation and emission wavelengths of 488 and 538 nm, respectively, or visualized using a fluorescence microscope (Olympus Fluoview) (excitation wavelength 460–490 nm). Solutions of hyphae without peptides or with 1% Triton X-100 (Sigma-Aldrich) were used as negative and positive control, respectively. For each concentration of KT43C, the experiment was performed in triplicate.

2.9. Induction of reactive oxygen species (ROS) assay

The detection of ROS was performed as described by Van Der Weerden et al. (2008) with some modifications. *F. culmorum* hyphae were grown as described above and then treated with KT43C or water for 12 h. Following the incubation, the hyphae was incubated with 10 µg.ml^{-1} dihydrorhodamine 123 (Sigma-Aldrich) for 2 h followed by extensive washing with 0.6 M KCl. Fluorescence of hyphae was measured using a fluospectrophotometer with excitation and emission wavelengths of 488 and 538 nm respectively, or visualized using a fluorescence microscope (Olympus) (excitation wavelength 460–490 nm). Solutions of hyphae without peptides or with 1% Triton X-100 (Sigma-Aldrich) were used as negative and positive control, respectively. For each concentration of KT43C, the experiment was performed in triplicate.

2.10. Circular dichroism

The analysis of secondary conformers of KT43C in different solutions was observed by using circular dichroism (CD) spectroscopy as described by Liu et al. (2008). Peptide (1 mg.ml^{-1}) was diluted into deionized water or 20 mM sodium dodecyl sulfate (SDS). CD measurements were performed by using a Chirascan™ CD Spectrometer (Applied Photophysics), at 27 °C within a wavelength range of 180–260 nm. Measurements were made in triplicate and the solvent CD were subtracted from the sample CD.

2.11. Chilled dough shelf life

Dough of 10 g was prepared according to a standard recipe (without yeast) consisting of 63% (w/w) flour, 1.5% (w/w) sugar, 3% (w/w) fat and 0.3% (w/w) salt. A solution of 20 µg.ml^{-1} KT43C in sterile distilled water was inoculated with 1×10^2 spores.ml^{-1} (final concentration) of *F. culmorum* and added to the mix. Water, without peptide, was used as control. After preparation, the dough was packed into sealed plastic bags and filter pipette tips were inserted into the bag to allow gas exchange. Dough was stored at 4 °C. The shelf life of the dough was monitored over 14 days, as described by Dal Bello et al. (2007).

2.12. Fungal contamination in the dough

Fungal contamination of dough was estimated by measuring the ergosterol concentration as described by Jedlickova et al. (2008) with some modifications. Briefly, 10 g of ground dough was treated with 50 mL of methanol in a closed vessel and was shaken for 30 min. Then, 25 mL of cleaned extract was placed into a test tube and 3 g of KOH was added and stirred until dissolution of KOH. Ten mL of *n*-hexane was then added and the solution was incubated at 65 °C in a water bath for 30 min. After cooling at RT, 5 ml of distilled water were added and the hexane layer was placed into a beaker. The extraction process using hexane was repeated three times using 10 mL of the solvent. The extracts were then pooled and evaporated until dry in a vacuum dryer (Scanvac, Labogene) and redissolved in 5 mL of methanol. The extracted samples were analysed using HPLC with a DAD. The mobile phase was methanol, and 1.0 mL/min was the optimal flow rate. The column temperature was held at 25 °C. A control was made with the addition of standard ergosterol (Sigma-Aldrich) into fresh dough. A 95% recovery was obtained with the standard ergosterol.

2.13. Haemolysis assay

KT43C was assayed for its ability to induce haemoglobin release from fresh defibrinated sheep erythrocytes as described by Laverty et al. (2010). Fresh sheep red blood cells (Thermo Fisher Scientific) were washed three times with equal volumes of PBS. After centrifugation for 15 min at 900*g*, erythrocytes were resuspended to 4% (v/v) in PBS. Eythrocyte suspension (80 μL) was added to the wells of a 96-well microtiter plate, followed by the addition of 20 μl of KT43C in PBS at different concentrations. After incubation at 37 °C for 1 h, the suspension was clarified by centrifugation at 1,000*g* for 10 min. Supernatant was transferred to a fresh 96-well microtiter plate and haemoglobin release was measured spectrophotometrically at 405 nm. Erythrocytes were treated with 0.1% Triton X-100 (in PBS) and PBS alone as positive and negative control, respectively. The percentage haemolysis was calculated as described by Laverty et al. (2010):

$$\%\ \text{Haemolysis} = \frac{(Abs405\ peptide\ treatment) - (Abs405\ PBS)}{(Abs405\ 0.1\%\ Triton\ X - 100) - (Abs405\ PBS)}$$

The release of haemoglobin was determined for six replicates.

2.14. Statistical analysis

Statistical analysis was carried out using Microsoft XLSTAT Version 2015.5.01. (Adinsoft Inc, New York, USA). Standard deviations were calculated for absorbance values at each concentration of KT43C based on triplicates, except for the haemolysis assay for which six replicates were used.

The effect of the different salts on the antifungal activity of KT43C was analysed with a one-way ANOVA followed by a Tukey-Kramer HSD test to identify differences relative to the positive control (KT43C in 0.1% acetic acid). In all cases, a probability of $p < 0.05$ denoted significance. The same statistical analysis was carried out to determine individual differences in haemolysis activity for each concentration of KT43C in comparison to the negative control.

3. Results

3.1. Antifungal activity of KT43C

The growth of spores of *F. culmorum*, *P. expansum* and *A. niger* was followed in presence of different concentrations of the peptide KT43C, in a microtiter plate assay. After 96 h, KT43C showed the highest antifungal activity against *F. culmorum* with an IC_{50} of 12.5 μg.ml^{-1} and a MIC of 20 μg.ml^{-1} (Fig. 1A). Growth of *A. niger* was delayed but the MIC, after 96 h, was not apparent within the concentration range of the peptide used in the study (up to 200 μg.ml^{-1}) (Fig. 1B). The growth of *P. expansum* was only inhibited with a peptide concentration of 200 μg.ml^{-1} (Fig. 1C). The inhibition of fungal growth was controlled on PDA plates (Fig. 2). KT43C inhibited fungal growth without induction of morphogenic changes in *F. culmorum* hyphae, e.g. branching, as observed with scanning electron microscopy (Fig. 3).

3.2. Effect of heat and salt on the antifungal activity of KT43C

A solution of KT43C was treated at 100 °C for 15 min and the antifungal activity was determined against *F. culmorum*. The antifungal activity of KT43C (MIC = 20 μg.ml^{-1}) was completely maintained after heat treatment (99.6 ± 0.004% of fungal growth inhibition), demonstrating the thermostability of the synthetic peptide.

The effect of cations on the activity of KT43C against *F. culmorum* is presented in Fig. 4. The presence of cations, especially divalent cations such as Ca^{2+} and Mg^{2+}, partially or totally inhibited the antifungal activity of KT43C.

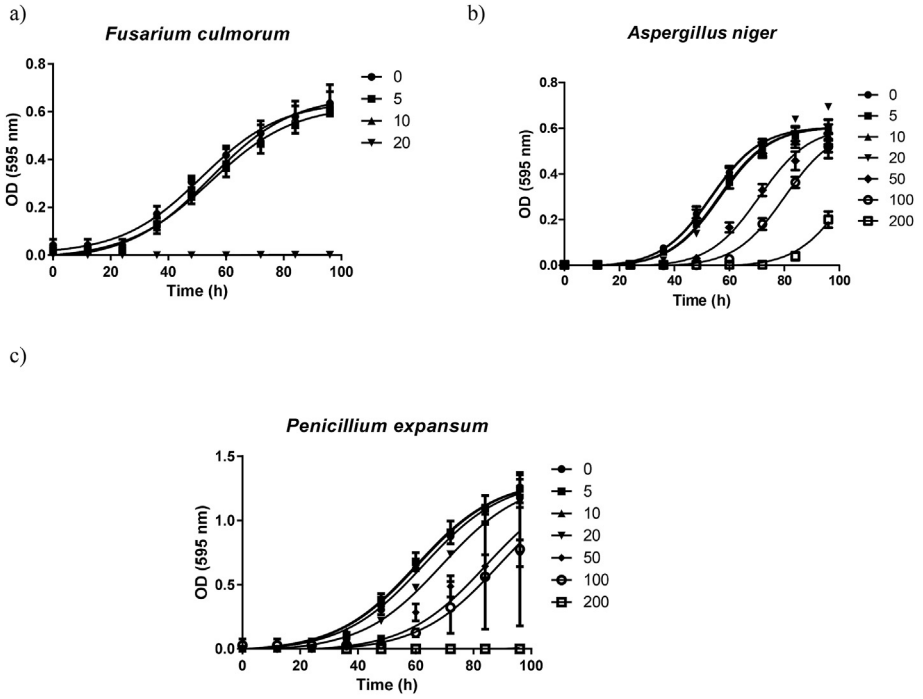


Fig. 1. Fungal growth inhibition by KT43C. Concentrations of KT43C are indicated in μg.ml^{-1}. (a) *Fusarium culmorum* FST 4.05, (b) *Aspergillus niger* FST 4.22, (c). *Penicillium expansum* FST4.21. All absorbance values are the mean of three replicates.

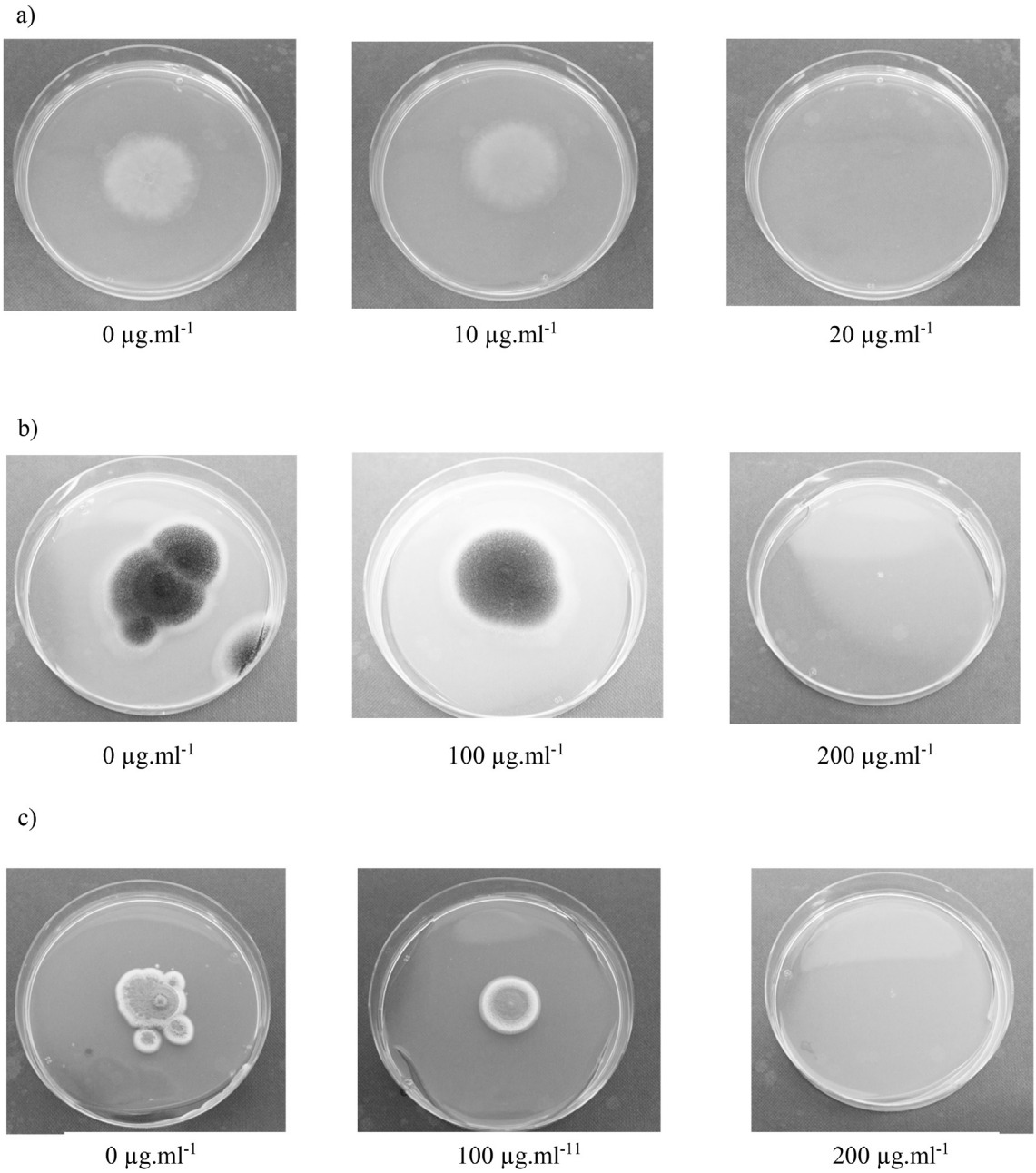


Fig. 2. Examination of fungal inhibition after 24 h of incubation in 1/2PDB with different concentrations of KT43C followed by 72 h of incubation on agar plates. (a) *F. culmorum* FST 4.05, (b) *A. niger* FST 4.22, (c). *P. expansum* FST4.21. The concentrations of KT43C are indicated below the pictures.

3.3. Mode of action of KT43C

To identify some characteristics of the mode of action of KT43C, a SYTOX® Green uptake assay was performed on *F. culmorum* with different concentrations of KT43C. The peptide induced permeabilization of *F. culmorum* hyphae at a concentration of 10 µg.ml^{-1} and higher (Fig. 5). Permeabilized hyphae had significant cytoplasmic granulation at higher concentrations. However, KT43C-induced permeabilization appeared to be required for inhibition but was not sufficient to cause cell death.

Another inhibitory mechanism is the increased generation of free radicals, mostly from a mitochondrial source, which can lead to an excessive level of reactive oxygen species (ROS), commonly known as oxidative stress. Significant production of ROS appeared only at concentrations of KT43C well above those required to inhibit fungal growth (Fig. 6). These results suggest that the overproduction of ROS was not a primary mechanism of antifungal action of KT43C against *F. culmorum*, but may, at high concentrations, enhance its activity.

3.4. Circular dichroism

Native defensins possess a characteristic CSαβ motif, in which tertiary structures, such as an alpha helix and antiparallel β-sheet are stabilized by disulfide bonds. In aqueous solutions, in the absence of disulfide bonds, the linear analogues of defensins present random conformations and therefore are thought to be more flexible. In contrast, in trifluoroethanol or SDS micelles (a

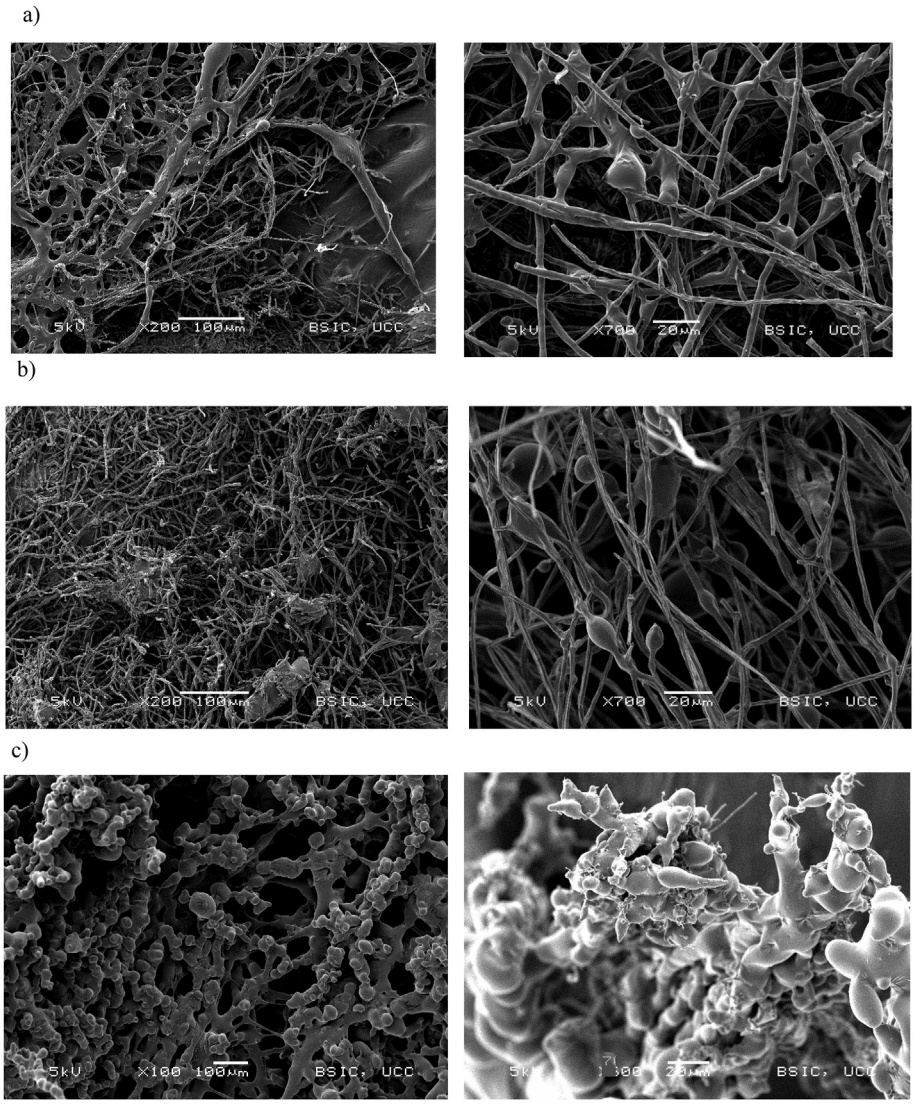


Fig. 3. Inhibition of *F. culmorum* germination in presence of KT43C observed with a scanning electron microscopy: a: Control; b: 5 μg.ml^{-1}; c: 20 μg.ml^{-1}. The magnifications are indicated on the pictures. The absence of conidial germination and mycelium are clearly visible with a concentration of 20 μg.ml^{-1} of KT43C.

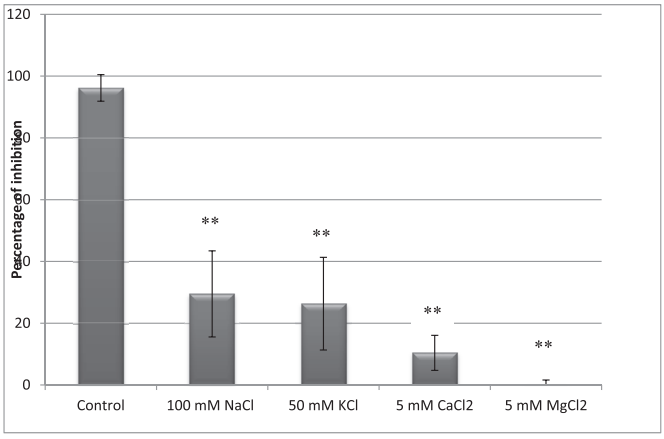


Fig. 4. Cation sensitivity of the synthetic cowpea defensin, KT43C (20 μg.ml^{-1}). Percentage of inhibition of *F. culmorum* growth after 96 h in growth medium in presence of different salts: **p < .01, significant difference of the percentage of inhibition between the control and the tested media.

membrane-mimicking model), the analogues could adopt structured conformations.

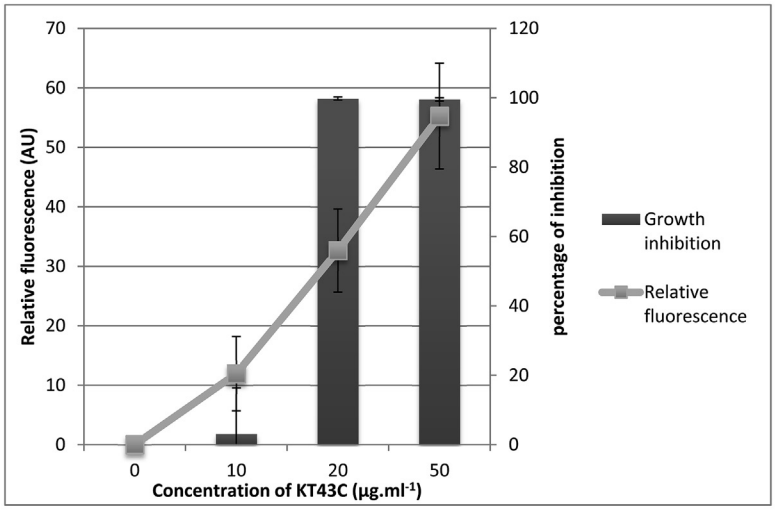
The secondary structures of KT43C were studied by CD spectroscopy (Fig. 7). In deionized water, the peptide appeared unstructured with a minima at ~200 nm, which is consistent with the presence of transient β-hairpin or turn-like conformations. A transition is observed in presence of SDS with a crossover at ~195 nm that correlates with the presence of more structured conformers. The slightly positive peak at ~190 nm and the double minima at ~205 nm and ~223 nm indicate a propensity for helical conformations.

3.5. Application of KT43C in dough

Spoilage fungi, such as *Fusarium* spp. in wheat or barley, are ubiquitous in cereal environments. Although the baking step essentially kills spoilage fungi, the presence of heat-stable mycotoxins remains a potential health issue. The protection of raw material or a microbiologically unstable cereal-based product against fungal growth is therefore an absolute necessity.

The synthetic peptide, KT43C, was used as an ingredient in the

a)



b)

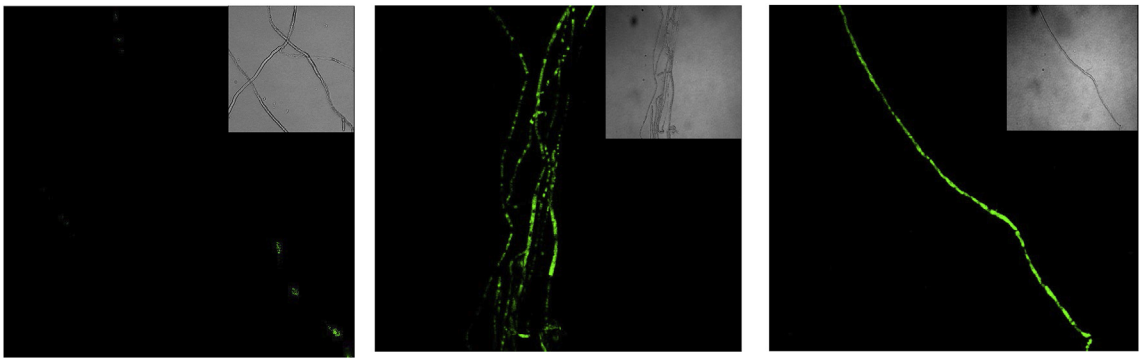


Fig. 5. SYTOX® Green uptake assay. a) After 18 h of growth, *F. culmorum* hyphae were treated for 24 h with KT43C (0, 10, 20 and 50 µg.ml^{-1}). Permeabilization of the fungal membrane was determined by fluorescence with SYTOX® Green (excitation 488 nm, emission 538 nm) and correlated to the percentage of inhibition. Values are the mean of three different experiments. b) *F. culmorum* hyphae were observed with a confocal laser scanning microscope, with an excitation wavelength 460–490 nm. Left: no KT43C; middle: 20 µg.ml^{-1} KT43C; right: 50 µg.ml^{-1}. High concentrations of KT43C induced permeabilization of fungal membrane, highlighted by fluorescence of the dye, and granulation of the fungal cytoplasm.

preparation of bread dough (20 µg.ml^{-1}), based on total flour weight. The monitoring of the shelf-life of chilled dough, previously inoculated with *F. culmorum* spores is presented in Fig. 8. For 14 days, dough was monitored each day and classified into different categories based on the percentage of fungal contamination present on the surface. The development of fungal colonies on the treated dough was delayed by 2 days compared to the dough without peptide. To quantify the fungal contamination, the concentration of ergosterol in the dough was determined. After 14 days of storage, the level of ergosterol in the control dough was twice as high as in the treated dough (17.5 ± 3.5 and 7.6 ± 0.9 µg.ml^{-1}, respectively). The synthetic defensin was thus shown to be an efficient tool against fungal spoilage of dough. In addition, the thermostability of KT43C could allow its exploitation as a novel preservative in baked goods.

3.6. Haemolysis assay

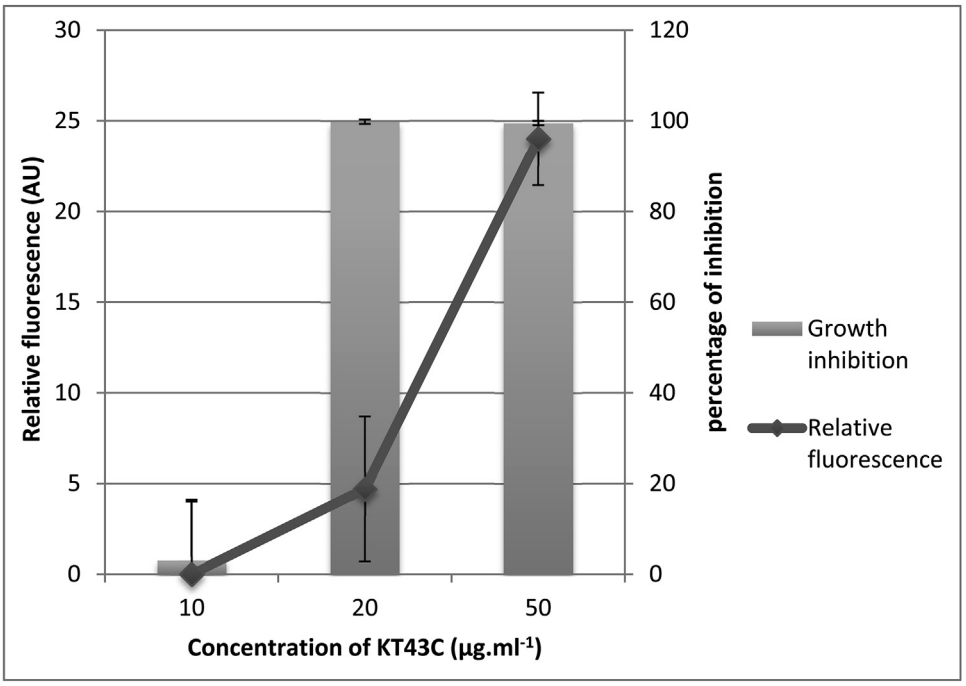
In order to determine the safety of KT43C against mammalian cells, the activity of the peptide against red blood cells was measured. The release of haemoglobin from fresh sheep erythrocytes after treatment with KT43C was measured in a microtiter plate assay. At concentrations up to 200 µg.ml^{-1}, KT43C did not lyse the red blood cells (data not shown). The safety of KT43C against mammalian blood cells is an essential parameter for potential applications in food products.

4. Discussion

Previous studies have highlighted the antifungal (Carvalho et al., 2001) and antibacterial (Franco et al., 2006) activity of antimicrobial peptides isolated from cowpea seeds, such as the defensin, Cp-thionin II as described by Franco et al. (2006). In this study, a synthetic linear analogue of Cp-thionin II, namely KT43C, exhibited antifungal activity against *F. culmorum*, *P. expansum* and *A. niger* (Fig. 1); while KT43C has been previously shown to inhibit Gram-positive bacteria (Kraszewska et al., 2016), to our knowledge, the antifungal activity of cowpea antimicrobial peptides or derivatives has never been described for the fungi studied here. A synthetic linear analogue of Cp-thionin II, namely KT43C, exhibited antifungal activity against *F. culmorum*, *P. expansum* and *A. niger* (Fig. 1).

Despite the absence of disulfide bonds in its linear structure, KT43C displays antifungal and antibacterial activity. Disulfide bonds are determinants of defensins' integrity and have been

a)



b)

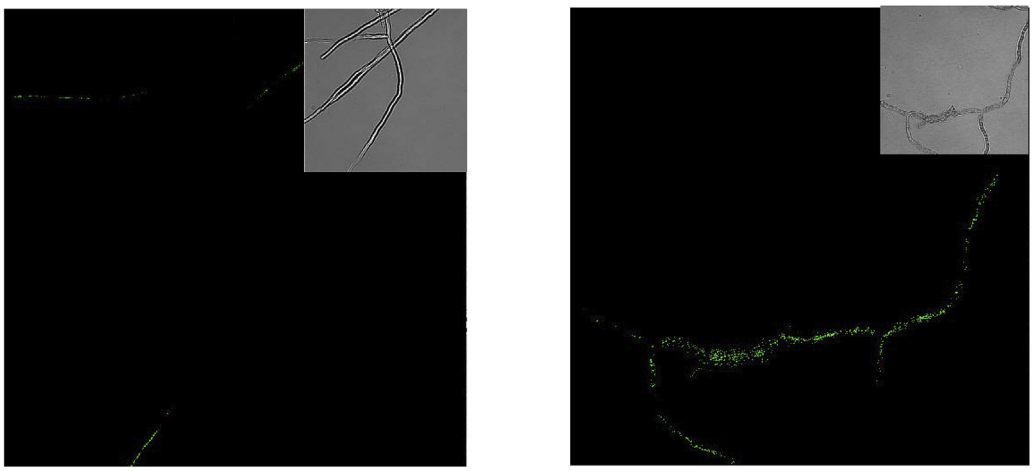


Fig. 6. Detection of reactive oxygen species (ROS) production. a) After 24 h of growth, *F. culmorum* hyphae were treated for 12 h with KT43C (0, 10, 20 and 50 µg.ml^{-1}). Production of ROS was determined by fluorescence of DHR 123 (excitation 488 nm, emission 538 nm), and correlated to the percentage of inhibition. Each value is the mean of triplicates b) *F. culmorum* hyphae were observed with a confocal laser scanning microscope, with an excitation wavelength 460–490 nm. Left: no KT43C; right: 50 µg.ml^{-1}.

reported to increase the antimicrobial activity (Jenssen et al., 2006). However, some linear derivatives of AMPs (Wu and Hancock, 1999) and defensins (Liu et al., 2008) are able to retain partial or complete antimicrobial activity after removal of the disulfide bonds. Other parameters, such as net charge, hydrophobicity, amphipathicity and flexibility, are essential for the antimicrobial activity of AMPs. Although disulfide bridges are not essential for the antimicrobial and antifungal activity of Cp-thionin II, the presence of free cysteine residues may modify the hydrophobicity of the peptide and then change its activity. However, this effect seems unclear for linear analogues of defensins (Liu et al., 2008; Nagano et al., 1999).

Dimerization of plant defensins is also a highly significant criterion for their antifungal activity (Song et al., 2011). Plant defensins that form dimers become highly efficient molecules against pathogenic fungi due to a stronger interaction with the negatively charged proteins of the fungal cell wall and membrane (Lay and Anderson, 2005). However, the oligomerization of defensins does not appear to be crucial, as shown for the antibacterial activity of Cp-thionin II (Franco et al., 2006).

KT43C displayed antifungal activity against *F. culmorum, A. niger* and *P. expansum*. These three fungal species belong to the same subdivision, *Pezizomycotina*, but *F. culmorum* belongs the class of *Sordariomycetes* while *P. expansum* and *A. niger* both belong to the class of *Eurotiomycetes*. Although the mechanistic action of KT43C on these fungi may have similarities, differences in cell/wall composition or fungal physiology between these species may be

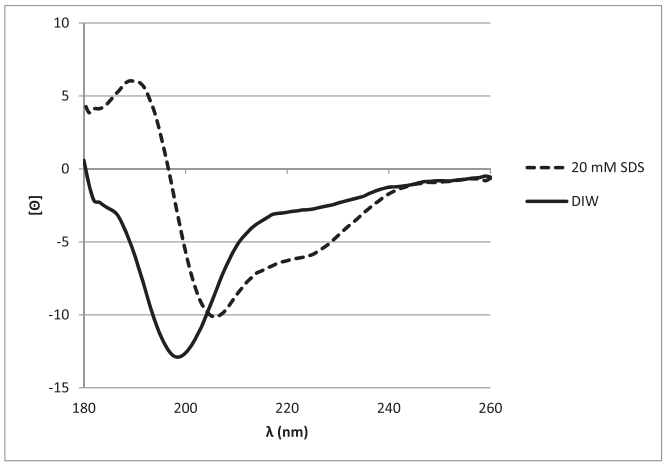


Fig. 7. CD spectra of KT43C in presence of DIW (solid line) or 20 mM SDS/DIW (dashed line). In DIW, the absence of crossover and the minimum at ~200 nm indicate a mostly random coiled configuration with possible transient β-hairpins. In SDS, KT43C adopts a more structured conformation with the presence of helical features.

pertinent to the mode of action of KT43C and its antifungal potency. Differences in the mode of action of the plant defensin MtDef4 against *Neurospora crassa* and *F. graminearum* have been described by El-Mounadi et al. (2016).

KT43C inhibited growth of *F. culmorum* without inducing morphogenic changes in the hyphae (Fig. 3). This finding is in agreement with the ability of KT43C to inhibit Gram-positive bacteria (Kraszewska et al., 2016) and the antibacterial activity of native peptide (Franco et al., 2006). Indeed, only non-morphogenic defensins appear to have an effect on bacteria (Carvalho and Gomes, 2009).

After heat treatment, KT43C retained its antifungal activity against *F. culmorum*. The heat stability of KT43C has also previously been shown regarding its antibacterial potency (Kraszewska et al., 2016). Terras et al. (1992) and Broekaert et al. (1995) have reported the thermal stability of defensins from radish and other plant species. The role of disulfide bonds in defensins stabilization was highlighted by Terras et al. (1992). KT43C appeared unstructured in aqueous solutions but adopts an α-helical conformation in a membrane environment (Fig. 7). The random conformations and flexibility of the peptide could protect from thermal denaturation. The adoption of a structured conformation in presence of SDS may indicate possible interactions between KT43C and a membrane-mimic environment. This new conformation could be related to its antifungal activity (Domingues et al., 2008; Liu et al., 2008).

The presence of ions, especially divalent cations, has been proven to decrease the antifungal activity of native plant defensins (Vriens et al., 2014). The antifungal activity of this linear analogue of Cp-thionin II was demonstrated to be also affected by the presence of cations (Fig. 4). Kraszewska et al. (2016) reported that the peptide keeps its antibacterial activity in the presence of NaCl, up to 50 mM, but loses it at 100 mM. The loss of activity in presence of cations is a common feature for plant defensins and AMPs linear derivatives in general (Adem Bahar and Ren, 2013; Vriens et al., 2014). This effect is due to the weakening of electrostatic interactions between the cationic peptides and the negatively charged membrane of microbial cells (Wu et al., 2003). Other potential effects may include structural changes in the peptide (Oard and Karki, 2006), or stabilization of the microbial membrane by cations (Thevissen et al., 1999).

Plant defensins (Vriens et al., 2014) and linear AMPs (Domingues et al., 2015; Liu et al., 2008) have been shown to present several modes of action. Among these different mechanisms, permeabilization of the fungal membrane has been described as a secondary effect of plant defensin action (Vriens et al., 2014), but is primarily involved in the antimicrobial activity of linear peptides (Bechinger and Lohner, 2006). Like native plant defensins, such as NaD1 (Van Der Weerden et al., 2010) or linear antimicrobial peptides (Van Der Weerden et al., 2010), KT43C causes fungal membrane permeabilization of *F. culmorum* at the MIC (Fig. 5), but at a slower rate of action than the native peptide (data not shown). The time difference can be explained by the formation of oligomers of the synthetic peptide or the necessity to reach a sufficient concentration of peptide at the surface of fungal membrane (Thevissen et al., 2004). Another hypothesis would be a limited mobility of aggregates of KT43C that tend to form into the bilayer environment because of changes in hydrophobicity, as described for tachyplesin (Han and Lee, 2015).

At high concentrations, KT43C induces a high production of ROS in *Fusarium* hyphae (Fig. 6). The generation of ROS is involved in mechanisms related to oxidative stress and damage, leading generally to cell-death. The interaction with intracellular targets and the overproduction of ROS in the fungal cytoplasm has been highlighted for several defensins (Vriens et al., 2014) and linear AMPs (Huang et al., 2010). In addition, a model, involving pore-formation and intracellular target strategy, has already been considered for several linear AMPs (Mason et al., 2007). Like KT43C, ROS production with NaD1 was only observed at concentrations greater than the MIC, even when membrane permeabilization was observed, suggesting a partial role for oxidative stress in fungal inhibition (Hayes et al., 2013).

A major issue with the use of cAMPs in pharmaceuticals or food applications is their potential toxicity towards mammalian cells. The reduction of hydrophobicity and the absence of disulfide bridges in linear derivatives have been pointed out as key elements in reducing their cytotoxicity (Liu et al., 2008). KT43C did not induce red blood cells lysis in the range of concentration used for the antifungal assays. Due to the presence of cholesterol, mammalian cell membranes have been shown to be less sensitive to destabilization by linear cationic AMPs than fungal membranes (containing mostly ergosterol) (Mason et al., 2007). Another study from our group has shown that the synthetic cationic peptide OOWW-NH2 is inactive against gut Caco-2 cell lines (Thery et al., 2018). The cytotoxicity of plant defensins has already been proven to be low, even negligible (Thevissen et al., 2004). In addition, Liu et al. (2008) showed that a linear analogue of hBD-3 displayed lower cytotoxicity compared to the native form of HBD-3. The decreased cytotoxicity towards mammalian cells of linear derivatives of AMPs and defensins has been attributed to the removal of the disulfide bridges, decreasing the overall hydrophobicity (Liu et al., 2008).

KT43C (20 $\mu g.ml^{-1}$) was used as an ingredient in the preparation of chilled dough and delayed the growth of *F. culmorum* by 2 days in a challenge test. The use of natural (Lucera et al., 2012; Rai et al., 2016; Rydlo et al., 2006) and synthetic (Appendini and Hotchkiss, 2000; Thery et al., 2018) AMPs to prevent spoilage of food products has been reported. Thus, the synthetic analogue of the human β-defensin 3 protects bread against environmental contaminants, with a shelf-life extension of 3 days (Thery et al., 2016). Although the concentration of KT43C used in this test was the MIC against *F. culmorum*, the conidial germination was not completely inhibited. The presence of other dough ingredients and proteases resulting from the preparation process may affect the antifungal action of the peptide. The sensitivity of AMPs to proteolytic digestion is a major concern for a potential use as food additive to avoid further action once in the intestinal system.

In this study, a synthetic linear analogue of the Cp-thionin II,

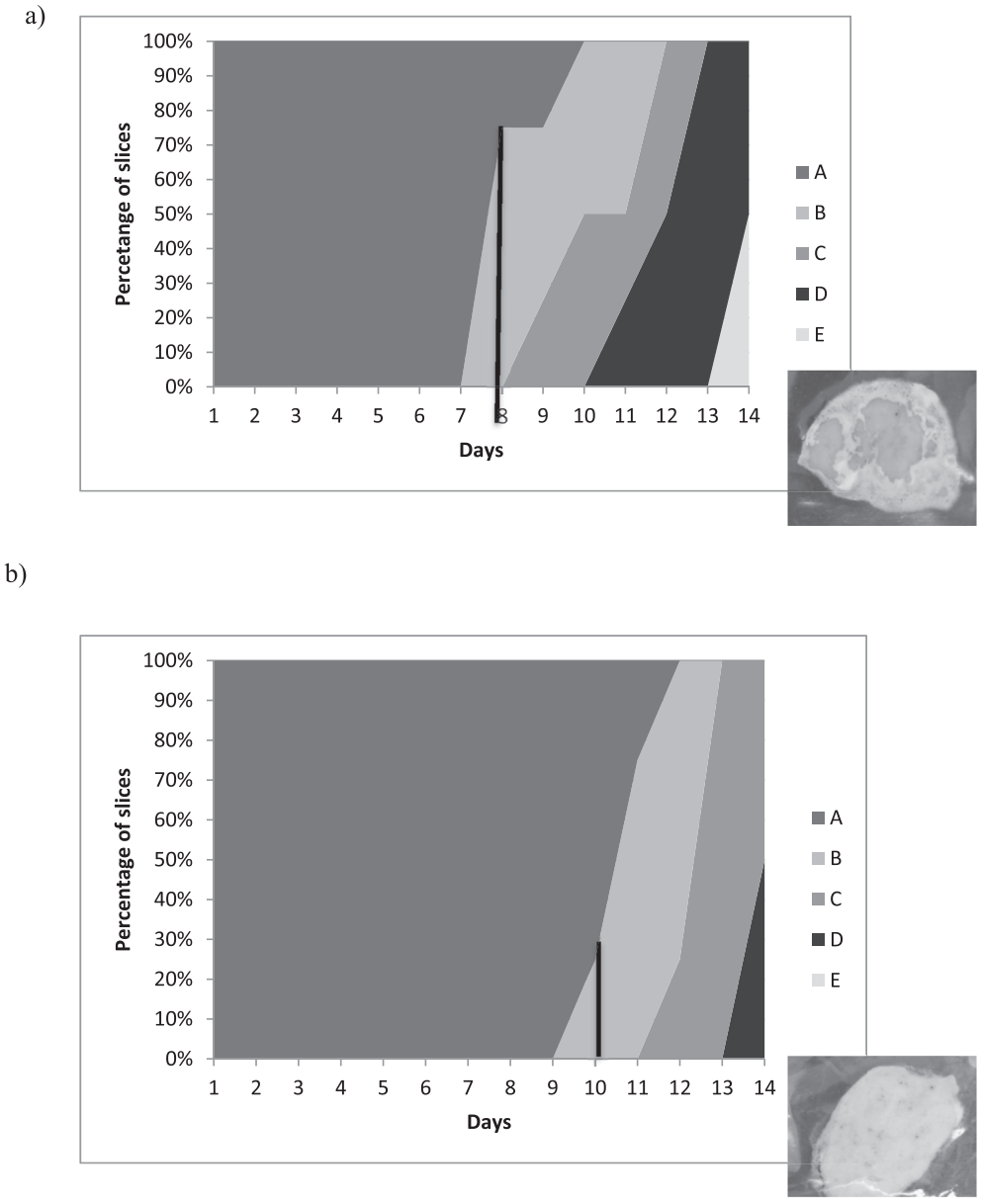


Fig. 8. Shelf-life of chilled dough inoculated with *F. culmorum* (1×10^2 spores.ml^{-1}). The development of fungal mycelium was monitored over 14 days and the dough was classified according to the percentage of contamination (from A (0% contamination) to E (50% contamination)). a) Control dough; b) Treated dough with KT43C (20 μg.ml^{-1}). Three doughs were used in each experiment.
Pictures of fungal contamination on chilled dough after 14 days of storage are presented with the charts.

showed antifungal activity against common fungal contaminants of cereal and cereal-based products. The analogue defensin, KT43C, presented similar characteristics to native plant defensins, such as heat stability and cation sensitivity. The synthetic peptide appears to be an efficient tool against fungal contamination of dough. Given its harmlessness towards erythrocytes, KT43C may represent a novel alternative to commonly used chemical preservatives in the baking industry.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2018.01.006.

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Optimisation of the antifungal potency of the amidated peptide H-Orn-Orn-Trp-Trp-NH2 against food contaminants

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ABSTRACT

The design of novel efficient antimicrobial peptides (AMPs) faces several issues, such as cost of synthesis, proteolytic stability or cytotoxicity. The identification of key determinants involved in the activity of AMPs, such as cationicity and amphipathicity, allowed the synthesis of short peptides with optimized properties. An ultrashort peptide made of the sequence H-Orn-Orn-Trp-Trp-NH2 (O3TR) showed antifungal activity against several contaminants from food products. This peptide inhibited the growth of the filamentous fungi *Fusarium culmorum*, *Penicillium expansum* and *Aspergillus niger* within a range of concentration of 12.5–50 μg/ml. In addition, O3TR inhibited the growth of the yeast *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Debaryomyces hansenii* and *Kluyveromyces lactis* within the range 12.5–50 μg/ml. A derivative peptide, called C12O3TR, made by the addition of lauric acid at the N-terminus of O3TR was 2- to 8-fold more active than O3TR against every species. In addition to the inhibition of conidial germination, O3TR and C12O3TR killed *F. culmorum* hyphae at 100 and 50 μg/ml respectively.

The MIC of the two peptides against *F. culmorum* and *Z. bailii* after heat treatment at 100°C for 60 min and within the pH range 3–10, were not changed. However, the activity of O3TR against *F.culmorum* and *Z. bailii* was strongly reduced in salt solutions, whereas the lauric acid peptide kept its antifungal activity and resistance to proteolytic digestion.

The conjugation with lauric acid reduced the random coiled structure and increased the α-helical content of O3TR. After conjugation with the dye tetramethylrhodamine (TMR), both peptides entered *F. culmorum* spores. They also both induced permeabilization of *F. culmorum* hyphae but only C12O3TR permeabilized *Z. bailii* membrane.

In contrast to the lipopeptide, O3TR did not show haemolytic or cytotoxic activity when applied at the concentrations that exhibited antifungal potency. The two peptides were challenged against a yeast cocktail of *S. cerevisiae* and *Z. bailii*, and *A. niger* in different commercial beverages. After 7 days, O3TR was able to inhibit the yeast cocktail in a commercial lager and carbonated drink. Due to its antifungal potency, high stability and low cytotoxicity, the tetrapeptide could represent a promising starting point of a novel food preservative.

1. Introduction

In order to provide sustainable and safe food, protection from microbiological contamination is essential. Food supply is facing two major issues, due to microbial safety: the risk to human health and economic losses due to food loss. In 2015, foodborne diseases have been estimated by the WHO to affect 1 in 10 people every year and 420,000 cases resulted in death (WHO [World Health Organization], 2015). Beverage industry is not spared by microbial spoilage. Although the low pH of soft drinks prevents most of microbial spoilage, fungal growth still occurrs. Yeasts are the primary contaminants in carbonated products, due to their ability to grow in highly carbonated and with low pH beverages (Tribst et al., 2009; Tserennadmid et al., 2011). In case of poor hygiene, mould can grow thanks to high water availability and acidity (Kregiel, 2015). In addition to health risk, fungal spoilage induces undesirable modifications of flavours and tastes. Chemical preservatives are commonly used to inhibit food spoilage but the intensive overuse of these preservatives has led to a rise of resistant microorganisms. The common preservatives used in soft drinks are weak acid preservatives such as sorbic or benzoic acids (Battey et al., 2002). However, yeast species including *S. cerevisiae* and mostly *Z. bailii* have shown resistance to these acids (Tribst et al., 2009). In addition, the increasing interest of the consumer for products of natural origin generated an increasing development of novel natural antimicrobials.

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Among these natural compounds, antimicrobial peptides (AMPs) are short (12–50 amino acid residues) peptides of the innate immune system found in every kingdom. They contain 2 to 9 basic residues and approximately 50% hydrophobic residues and a diversity of structures. The main advantage of these peptides is their mode of action which, for most of them, does not involve specific targets on bacteria or fungi thereby avoiding the development of resistance (Bechinger and Lohner, 2006; Huang et al., 2010). *In vivo*, apart from their direct action, some cationic antimicrobial peptides (CAPs) are involved in immunomodulatory function reinforcing their antimicrobial potential (Hancock et al., 2016). Several AMPs and AMP-derived lipopeptides derived from AMPs have already been approved by the FDA. However, only the bacteriocin nisin is an antibacterial used as food preservative (Domalaon et al., 2014). Nisin present some limitations, such as its limited spectrum of activity, in the absence of additional treatments (Rydlo et al., 2006).

Positive net charge, hydrophobicity and amphipathicity are the key parameters of the antimicrobial activity of these peptides and many potent peptides have been synthesized based on these properties. However, the high cost of these peptides is a hurdle which led to research on shorter peptides. The shortest peptides are called ultrashort peptides (USPs) whose length is below seven amino acid residues. Among the different USPs, the 4 amino acid H-Orn-Orn-Trp-Trp-NH2 has been characterized (Bisht et al., 2007; Laverty et al., 2010). This peptide displayed antimicrobial activity against several human pathogens (Laverty et al., 2015). The use of conjugated fatty acids has been revealed by different groups to increase the antimicrobial potential and act synergistically with USPs (Laverty et al., 2010; Yadav et al., 2013).

In this study, the *in vitro* activity of the USP H-Orn-Orn-Trp-Trp-NH2 (O3TR) was characterized against some representatives of fungal contaminants found in cereal-based products, such as beer, or soft drinks. In addition to potency and safety, promising antimicrobials in food must present an interesting “cost in use”. Thus, the efficiency of this low-cost 4-residue peptide and a derived lipopeptide to reduce the contamination of pre-inoculated drinks was also determined.

2. Material and methods

2.1. Peptides

The ultrashort amidated tetrapeptide H-Orn-Orn-Trp-Trp-NH2 (O3TR) and its derivatives, (C2H3O)- Orn-Orn-Trp-Trp-NH2 (Ac-O3TR) and a lauric acid ester of O3TR, the lipopeptide (C12H23O)-Orn-Orn-Trp-Trp-NH2(C12O3TR), were synthesized by and purchased from GLBiochem, Shanghai, China. Synthesis was performed by solid-phase peptide synthesis technique on Fmoc MBHA Rink amide resin, followed by removal of the Fmoc group. The purity of all peptides exceeded 90% according to the manufacturer. The lyophilized peptide was reconstituted in deionized water and stored in vials of different concentrations at − 20 °C.

2.2. Fungal strains and growth conditions

The following fungal strains were used in this study: *Fusarium culmorum* FST 4.05, *Aspergillus niger* FST 4.21, *Penicillium expansum* FST 4.22. These fungi were chosen because they represent some of the main contaminants of beverages (Kregiel, 2015). The three strains originated from the culture collection of School of Food and Nutritional Sciences, University College Cork (Cork, Ireland). Spore suspensions were prepared according to Oliveira et al. (2012). Briefly, fungal mycelium was cultured on Potato Dextrose Agar (PDA, Sigma-Aldrich) plates at 28 °C for 7 days. Following incubation, hyphae was inoculated in nutrient-poor broth until sporulation. Fungal spores were collected by filtration (30 μm), counted on a haematocytometer and inoculated in fresh nutrient-poor broth.

The yeast strains were purchased from the Leibniz Institute DSMZ (Braunschweig, Germany) and Puratos (Belgium). *Zygosacharromyces bailii* DSM 70492, *Zygosaccharomyces rouxii* DSM 2531, *Kluyveromyces lactis* DSM 4394 and *Debaryomyces hansenii* DSM 3428, brewing *Saccharomyces cerevisiae* (Fermentis) were chosen since they represent some of the main contaminants of food and beverages products (Wareing and Davenport, 2004). The yeasts were cultured on Yeast Potato Dextrose Agar (YEPD, Sigma-Aldrich) at 25 °C for 2–5 days, depending on the strain.

2.3. Antifungal activity assays

Antifungal *in vitro* assays were conducted as described by Munoz et al. (Muñoz et al., 2014) with some modifications. Briefly, fungal conidia (1×10^4 conidia/ml) were inoculated in synthetic fungi media (SFM) without calcium (50 μM MgSO4/7H2O, 5 μM FeSO4/7H2O, 0.1 μM CoCl2, 0.1 μM CuSO4/5H2O, 2 μM Na2MoO4/2H2O, 0.5 μM H3BO3, 0.1 μM KI, 0.5 μM ZnSO4/7H2O, 0.1 μM MnSO4/1H2O, glucose, asparagine, 20 mg of methionine, 2 mg of myo-inositol, 0.2 mg of biotin, 1 mg of thiamine-HCl, 0.2 mg of pyridoxine-HCl, 0.5 mM K2HPO4/3H2O), as described previously (Thevissen et al., 1999).

Fungal suspension (90 μl) was added to 96-well microplate wells followed by the addition of 10 μl of peptides (0–200 μg/ml). Deionized water was used as negative control. Fungal growth was followed at 595 nm over 96 h at 28 °C on a microplate reader (Multiskan TM, Thermo Scientific). The MIC was defined as the lowest concentration of peptide that showed no growth at the end of the experiment (4 days of incubation) in all the experiments. The IC50 was defined as the concentration required inhibiting 50% of fungal growth and calculated *via* PRISM software based on the microplate data.Each peptide concentration was tested in 3 different wells.

A similar procedure was used for yeasts cells with slight differences. The initial yeast concentration was 1×10^5 cfu/ml in yeast minimal media (YMM), as described previously (Thevissen et al., 1999) and the yeast growth was followed at 25 °C. YMM was made of 0.8 g of complete supplement mixture – URA [CSM-URA] per liter, 6.5 g of yeast nitrogen base (YNB) without amino acids per liter (Formedium, Norfolk, UK) and 10 g of glucose (Sigma-Aldrich) per liter.

2.4. Fungal cell viability assays

Cell viability assays were performed as described by Van Der Weerden et al. (2010) with some modifications. *F. culmorum* hyphae were grown in SFM from a concentration of 5×10^4 spores/ml for 18 h at 28 °C with some modifications. Hyphae were collected by centrifugation (10,000 g for 5 min) and resuspended in fresh medium in an equal volume. Hyphal suspension (90 μl) was transferred in wells of a 96-well microtiter plate with 10 μl of peptides (0–200 μg/ml). Deionized water was used as negative control as described above. After 2 h of incubation at 28 °C, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/ml; Sigma) was added to the wells. After 16 h of incubation at room temperature, medium was removed and 100 μl of MTT solvent (0.1 N HCl in anhydrous isopropyl alcohol) were added to the wells and left for 15 min until cell lysis. The presence of MTT/formazan was monitored by measuring absorbance at 570 nm and subtracting background at 690 nm.

2.5. Modification of fungal cell-wall

In order to identify a potential receptor for the peptides on the fungal cell-wall, the latter was modified as described by Van Der Weerden et al. (2010). *F. culmorum* hyphae were grown from a starting solution of 5×10^4 spores/ml for 18 h at 28 °C. The hyphae were collected by centrifugation (10,000 g for 10 min) and treated with β-glucanase (1 ml of 2 mg/ml) for 1 h at 30 °C. After 3 washings (10 min) in SFM, hyphae were treated with the two peptides as described above. Cell viability assay was monitored using MTT assay after 2 h of

incubation.

2.6. Heat and pH resistance

To investigate their stability, the peptides were treated at a range of temperatures from 25 to 100 °C for 15, 30 or 60 min, in a heating block (Grant Instruments, Cambridge). After cooling at room temperature for 30 min, the antifungal activity of the peptides was determined against *F. culmorum* and *Z. bailii*, as described above.

The inhibitory effect of the non-heated peptides was determined in the respective media at pH of 3–10.

2.7. Effect of cations on the antifungal activity

To determine the effect of cations on the activity of O3TR and C12O3TR, an antifungal assay against *F. culmorum* and *Z. bailii* was performed in different salt solutions, as described in previous work (López-Garcia et al., 2000). Fungal spores (1×10^4 spores/ml) and yeast (1×10^5 cfu/ml) were inoculated in SFM and YMM respectively, supplemented with 50 mM/150 mM NaCl or 1 mM/5 mM $CaCl_2$. After the addition of the peptide, the inhibitory activity of the peptide/lipopeptide was determined as described above.

2.8. Resistance to proteolytic digestion

Resistance to chymotrypsin was conducted as described by Kim et al. (2014) in a growth inhibition assay. Peptides were incubated with α-chymotrypsin (Sigma, St Louis, MO, USA) at different molar ratios peptide:enzyme (60:1, 250:1, 2500:1), in a digestion buffer (50 mM Tris–HCl, pH 7.4, 5 mM $CaCl_2$) at 37 °C for 4 h. To stop enzymatic reaction, the solution was treated at 80 °C for 10 min and 10 µl of this solution (0–50 µg/ml) were added to *F. culmorum* spores solution (90 µl). Fungal growth was followed as described above.

2.9. Circular dichroism spectroscopy

The analysis of the peptides secondary structure was conducted as described by Liu et al. (2008). Circular dichroism spectroscopy was performed by using a Chirascan™ spectropolarimeter (Applied Photophysics) with the peptides dissolved in deionized water (DIW), in 50% trifluoroethanol (TFE) in DIW or 20 mM sodium dodecyl sulphate (SDS). The two peptides were analysed at a concentration of 1 mg/ml. CD measurements were performed at 27 °C within a wavelength range of 180–260 nm. Every sample and solvent was scanned three times and solvent CD was subtracted from the sample CD.

2.10. Membrane permeabilization

To characterize the mode of action of the synthetic peptides against *F. culmorum*, a SYTOX® Green (SG) (Molecular Probes) uptake assay was performed as described by Van Der Weerden et al. (2010) with some modifications. *F. culmorum* hyphae were grown in SFM from a starting concentration of 5×10^4 spores/ml for 18 h at 28 °C. Hyphal suspension (90 µl) was then transferred to 96-well microtiter plates and exposed at O3TR and C12O3TR. Water alone and Triton X-100 (0.1%) were used as negative and positive controls respectively. After 1 or 2 h of incubation, SG (final concentration 0.5 µM) was added to the wells and incubated for 10 min before visualization. SG uptake was visualized by fluorescence microscopy (Olympus FluoView™ FV1000) (excitation wavelength 460–490 nm).

A kinetic procedure was used to follow SG uptake in yeast cells (10^5 cfu/ml in YMM). Peptides and SG (final concentration of 20 µM) were added simultaneously and the SG uptake was measured every 10 min for 2 h, using a fluorospectrophotometer (VarioSkan) with excitation and emission wavelength of 488 and 538 nm respectively.

2.11. Internalization assay

In order to detect internalization of labelled O3TR and C12O3TR, tetramethylrhodamine (TMR) was added at the N-terminal of the original peptides. The labelled peptides were provided by GLBiochem (Shanghai, China). The antifungal activity of the labelled peptides was determined as described above.

Labelled peptides (10 µl) were added to *F. culmorum* spores solutions (1×10^5 conidia/ml) in a 96-well microplate. After 10, 30 and 60 min of incubation, spores solutions were visualized with laser-scanning confocal microscopy (Olympus FluoView™ FV1000). TMR-O3TR and TMR-C12O3TR were excited at 543 nm and fluorescence detected at 570–700 nm.

2.12. Haemolysis assay

O3TR and C12O3TR were assayed for their ability to induce haemoglobin release from fresh sheep erythrocytes (Oxoid) according to the method of Papanastasiou et al. (2009). Fresh blood cells (1 ml) were prepared by centrifugation at 1620 g and the pellet was then resuspended in an equal volume of PBS. This process was repeated three more times. After a final centrifugation at 900 g for 15 min, the pellet was resuspended at 4% (*v*/v) in PBS. Erythrocytes (100 µl) were then combined in the wells of 96-well microtiter plate with peptides at different concentrations, previously diluted in PBS. The solutions were incubated for 1 h at 37 °C followed by centrifugation at 1000 g for 2 min. Aliquots of the supernatant were transferred to a fresh 96-well microtiter plate and the absorbance (haemoglobin release) was measured spectrophotometrically at 405 nm. PBS and 0.1% Triton X-100 were used as negative and positive control respectively. All peptide concentrations are reported as the mean of six replicates. The percentage of haemolysis was calculated as followed:

$$\%\text{Haemolysis} = \frac{(Abs405\ peptide\ treatment) - (Abs405\ PBS)}{(Abs405\ 0.1\%Triton\ X - 100) - (Abs\ 405\ PBS)} \times 100$$

2.13. Cytotoxicity assays

The cytotoxicity of O3TR and C12O3TR was performed as described by Laverty et al. (2010) by measuring cell viability using a MTT cell viability kit (Roche, Switzerland). Human colonic cells, Caco-2, (ECACC) were cultured in a 96-well microtiter plate in Dulbecco's modified Eagle's medium (DMEM), supplemented with non-essential amino acids (1%) and FBS (10%), at 37 °C in 5% CO_2. Cells were allowed to adhere overnight, media was removed and 200 µl of fresh medium supplemented with peptides (0–200 µg/ml) was added to the wells and incubated for 24 h at 37 °C. After removal of the medium, fresh medium (100 µl) and 3-[4,5- dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (10 µl) (Cell viability kit, Roche) was added to each well and incubated for 4 h at 37 °C. The solubilisation solution (100 µl) was added to each well and the plate was incubated overnight at 37 °C. Cell viability was measured spectrophotometrically at 570 nm with a background reading at 690 nm. The cytotoxicity of each peptide was determined in 4 wells for 5 replicates.

2.14. Applications in beverages

Cultures of *S. cerevisiae* and *Z. bailii* were grown in malt extract broth solutions (for 2 days at 25 °C at 160 rpm). Cell counting was performed with decimal dilutions on malt extract agar (Oxoid Ltd.), incubated at 25 °C for 5 days. The individual solutions were blended together mixed thoroughly to form the yeast cocktail at a final concentration of 5×10^4 cfu/ml, as described by (Battey et al., 2002). The cocktail was stored at 5 °C until further use.

Four beverages were used: commercial lager [Heineken (g/per 100 g): protein: 0.4; carbohydrates: 3.4; alcohol: 4; pH = 4.7];

Table 1
MIC (μg/ml) of O3TR and derivatives against fungal and yeast contaminants.

	O3TR	C12O3TR	Ac-O3TR
F. culmorum	12.5	6.25	> 200
P. expansum	50	12.5	> 200
A. niger	50	25	> 200
Z. rouxii	50	6.25	N.D.
K. lactis	25	6.25	N.D.
Z. bailii	12.5	3.12	N.D.
D. hansenii	25	6.25	N.D.
S. cerevisiae	12.5	3.12	N.D.

All peptides possess a NH2 group at the C-terminus; Fmoc-protection was removed at the N-terminus.
C12 = C12H23O, is lauric acid tail; Ac is an acetyl group (CH3CO).
N.D.: not determined.

carbonated orange soft dirnk [Fanta: carbohydrates (= sugars) 12.2 g/100 ml; pH = 2.73]; orange juice (Tesco)[10 g sugars/100 ml; pH = 3.7]; apple juice (Tesco) [9.6 sugars / 100 ml, pH = 3.4]. The filter-sterilized beverages (were inoculated with the yeast cocktail (10^2 cfu/ml) or *A. niger* spores (10^2 conidia/ml). Beverage solutions (90 μl) were transferred to the wells of a 96-well microtiter plate and 10 μl of O3TR or C12O3TR were added to the wells. Fungal growth was followed spectrophotometrically at 595 nm for 7 days at RT.

3. Results & discussion

3.1. Antifungal activity

The growth of some representatives of fungal food and beverage contaminants was conducted in the presence of the ultrashort peptide O3TR and two derivatives: a lipopeptide C12O3TR and an acylated Ac-O3TR. O3TR and C12O3TR displayed antifungal activity against all the species at different levels (Table 1). The presence of the NH2 group removes the negative charge at the C-terminus, increasing the net charge of the peptide. Amidation can be used to increase antimicrobial activity of AMPs, such as lactoferrin (Pan et al., 2007), or restore the activity of an inactive peptide (Wu and Hancock, 1999). The acylated peptide (C2H3O)-OOWW-NH2 did not show any antifungal activity in the range of concentrations (0–200 μg/ml) used in this study. The addition of the acetyl group removes the positive charge at the N-terminus of O3TR. This removal has been shown to abrogate the antimicrobial activity of several peptides, such as dermaseptin (Radzishevsky et al., 2005) or lactoferrin (Lu et al., 2016), mainly due to a reduced electrostatic interaction. On the contrary, Bisht et al. (2007) showed that the *N*-acylation with acetic anhydride improves the antimicrobial activity of the amidated peptide H-OOWW-NH2.

To verify if the peptides only affect conidial germination, a MTT cell viability assay was performed on *F. culmorum* hyphae. Both peptides induced hyphal death but at higher concentrations than the ones required for inhibition of conidial germination (Fig. 1). In order to identify a peptide receptor on fungal cell-wall, a pre-treatment of *F. culmorum* hyphae with β-glucanase was performed to degrade the β-glucans of fungal cell wall. This treatment did not modify the percentage of hyphal survival, after challenging with both peptides (data not shown), which indicated that both peptides did not require specific receptor on fungal cell-wall.

The net charge and the hydrophobicity of O3TR are carried by the ornithine and the tryptophan residues respectively. The use of the unnatural amino acid residue ornithine reduces its degradation by proteases (Bisht et al., 2007). N-terminal modification of the peptide increases synergistically the antifungal potential of the peptide and of lauric acid. The fatty acid chain tail of lauric acid was shown to be the optimal chain tail for O3TR regarding its antimicrobial activity (Laverty et al., 2010). With longer fatty acid chains, high hydrophobicity may induce an important oligomerization of the peptide which prevents it entering the microbial membrane (Matsuzaki et al., 1997). The improvement of the activity has been attributed to an increase of the overall hydrophobicity of the lipopeptide.

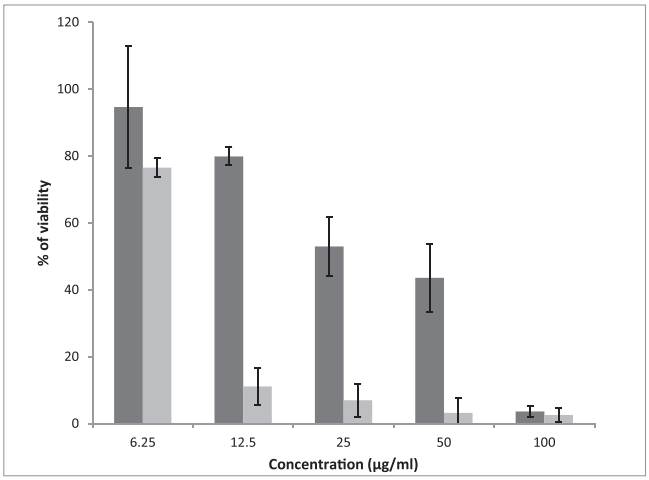


Fig. 1. Survival of *F. culmorum* hyphae challenged with O3TR(dark grey) or C12O3TR (light grey). Errors bars represent the standard deviation between three replicates.

3.2. Resistance of the antifungal peptides

The resistance of the two antifungal peptides was tested after different heat treatments, at different pH and in presence of salt solutions. Both peptides showed heat resistance up to 100 °C for 1 h and pH resistance in the range 3–10. They presented the same MIC against *F. culmorum* and *Z. bailii* as showed in Table 1. The ultrashort peptide O3TR has been reported to keep its antibacterial activity after heat treatment (Kraszewska et al., 2016). The small size of the peptides and their flexible structures make them less sensitive to thermal degradation. The two peptides presented different conformations in aqueous solutions. While O3TR is mostly unstructured in aqueous solutions, C12O3TR adopted a more structured conformation. The absence of structure in aqueous solutions has been highlighted both for natural and synthetic AMPs (Liu et al., 2008). In addition to the absence of structural elements (*e.g.* disulphide bonds), the limited size of the peptides may contribute to the difficulty of creating defined structure. However, small derivative proteins (Nedwidek and Hecht, 1997) or linear hexapeptide (Guo et al., 2013) have been observed to form ordered structures. In addition, self-assembly has been reported for several AMPs (Bechinger and Lohner, 2006), including short peptides (Han and Lee, 2015).

In salt solutions, O3TR lost most of its antifungal potency (Table 2). O3TR was only able to inhibit *F. culmorum* growth in presence of 50 mM NaCl and 1 mM $CaCl_2$ at concentrations of 50 and 100 μg/ml respectively, well above the concentration required in non-supplemented SFM. C12O3TR kept its antifungal potency against *F. culmorum* and *Z. bailii,* regardless of the salt concentrations. The reduction of the antibacterial and antifungal activity with an increase of ionic strength is a common feature for antimicrobial peptides, linear or non-linear (Jenssen et al., 2006). Several hypothesis have been suggested: the increase in the ionic strength increases the net charge of the membrane, reducing electrostatic interactions; the changing in the global zeta-potential of the membrane; binding of some ions to the membrane, reducing the ability of the peptides to find receptors; changes in the configuration of the peptide. Thus, AMPs with a high positive net charge, such as HBD-3, are thought to be less sensitive to the presence of cations. The high hydrophobicity of C12O3TR is suggested to maintain its antifungal activity despite the loss of electrostatic affinity induced by the presence of cations.

Table 2
MIC (μg/ml) of O3TR and C12O3TR in presence of different salt concentrations.

	F. culmorum				*Z. bailii*			
	50 mM NaCl	150 mM NaCl	1 mM $CaCl_2$	5 mM $CaCl_2$	50 mM NaCl	150 mM NaCl	1 mM $CaCl_2$	5 mM $CaCl_2$
O3TR	50	> 100	100	> 100	> 100	> 100	> 100	> 100
C12O3TR	6.25	6.25	6.25	6.25	3.12	3.12	3.12	3.12

Table 3
MIC (μg/ml) against *F.culmorum* after treatment with α-chymotrypsin.

Ratio peptide/chymotrypsin	O3TR	C12O3TR
0 = digestion buffer	> 50	6.25
60/1	> 50	> 50
250/1	> 50	12.5
2500/1	> 50	6.25

The resistance of the peptides to proteolytic digestion was performed by measuring antifungal activity after treatment with α-chymotrypsin in a digestion buffer (Table 3). O3TR lost its antifungal activity in a digestion buffer alone, but the presence of Ca^{2+} in the digestion buffer is a limiting factor for the study of the resistance of the peptide to proteolytic digestion. C12O3TR showed total resistance to chymotrypsin digestion at a ratio peptide:enzyme of 2500:1, and partial resistance with a ratio at 250:1. The antifungal activity of C12O3TR was largely inhibited using a peptide:enzyme ration of 60:1, since the MIC against *F. culmorum* was over 50 μg/ml. The degradation by proteolytic enzymes of peptides used as food preservatives is desirable to avoid undesirable effect on gut microbiota.

3.3. Secondary configurations of O3TR-NH2 and C12-O3TR-NH2

The secondary structures of O3TR-NH2 and C12-O3TR-NH2 were analysed by CD spectroscopy in DIW, 50%TFE and 20 mM SDS micelles (Fig. 2). In aqueous solution, O3TR profile showed a negative peak at ~200 nm, which indicates a mostly unstructured configuration or transient B-turn or polyproline II (PPII) motif (Rozek et al., 2003). In presence of TFE, known to stabilize configurations, the reduction of the peak intensity suggested a reduction of unordered conformation. In addition, a second negative peak appeared at ~222 nm, possibly due to the tryptophan residues (Rozek et al., 2003). In presence of SDS, used to mimick a membrane-environment, the conformation was more ordered with a potential increase of α-helix content, as suggested by the second negative peak and the presence of positive peak. A small peak at ~230 nm could correlate with PPII structural features.

The presence of the positive peak below 200 nm and a reduced negative peak at ~220 nm in CD spectra of C12O3TR in DIW and TFE suggested a more ordered conformation than O3TR. In SDS, the negative peak at ~200 nm moved to ~205 nm, and the second negative peak moved to ~225 nm separated by a maximum at ~218 nm. This change suggested a more structured conformation. In addition, a strong positive peak appeared at ~190 nm with a crossover at 200 nm, indicating a mix of α-helical structures and random coil. The presence of Trp residues could explain the positive peak at ~235 nm in both peptides (Crabtree effect).

C12O3TR conformation comprised α-helix and β-turn structural elements. The fatty acid conjugation has been reported to increase the α-helicity of peptides which then display higher antimicrobial activity (Chu-Kung et al., 2004). Indeed, this conformation enhances the amphipathicity of the peptide improving its antimicrobial action, enabling a better interaction and penetration into the membrane (Chu-Kung et al., 2004). However, Yadav et al. showed that a linoleic acid derivative of OOWW-NH2, did not show α-helical or β-sheet configuration (Yadav et al., 2013).

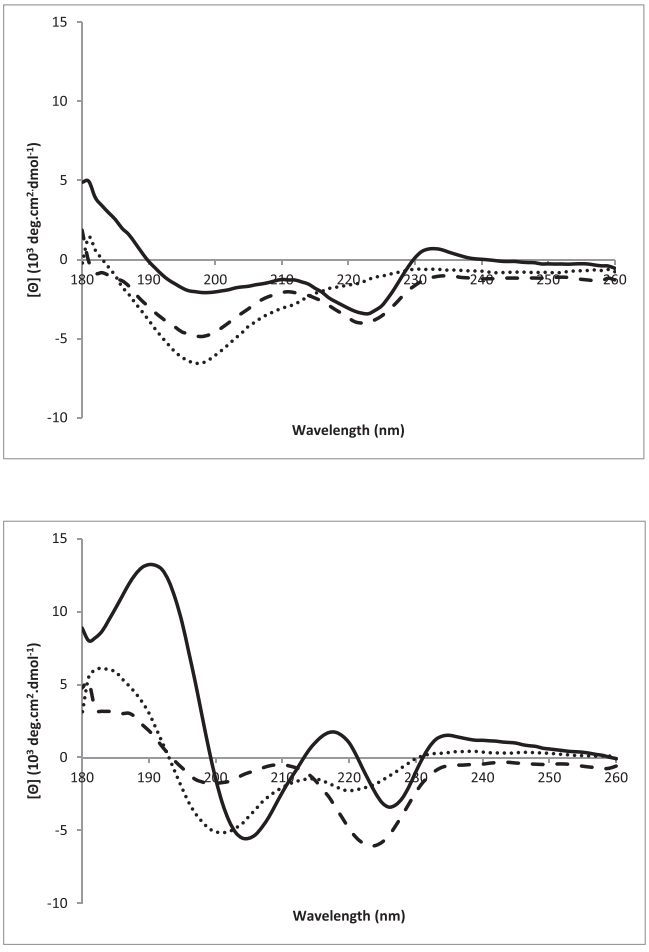


Fig. 2. CD spectra of O3TR (A) and C12O3TR (B) in DIW (▪▪▪▪), 50% TFE (▬ ▬) or 20 mM SDS (▬▬).

3.4. Permeabilization of fungal membrane

In order to characterize the mode of action of the peptides O3TR and C12O3TR, a SG assay was performed on *F. culmorum* hyphae and *Z. bailii*.

After 1 h of incubation, C12O3TR started to induce membrane permeabilization at 6.25 μg/ml. At higher concentration, the permeabilization is more important O3TR only induced permeabilization of fungal membrane at high concentrations (100 μg/ml) after 1 h of incubation After 2 h of incubation, both peptides induced membrane permeabilization at concentrations that induce hyphal death.

The kinetics of membrane permeabilization of *Z. bailii* was followed by fluorospectrophotometry for 4 h (Fig. 3). The yeast solutions treated with O3TR did not show fluorescence for 4 h even at peptide concentration of 100 μg/ml, much higher than its MIC against *Z. bailii.* On the contrary, fluorescence was rapidly detected in solutions treated with C12O3TR indicating a rapid permeabilization of yeast membrane. The permeabilization appeared to be correlated to the peptide concentration. In addition, a plateau was reached after almost 3 h of

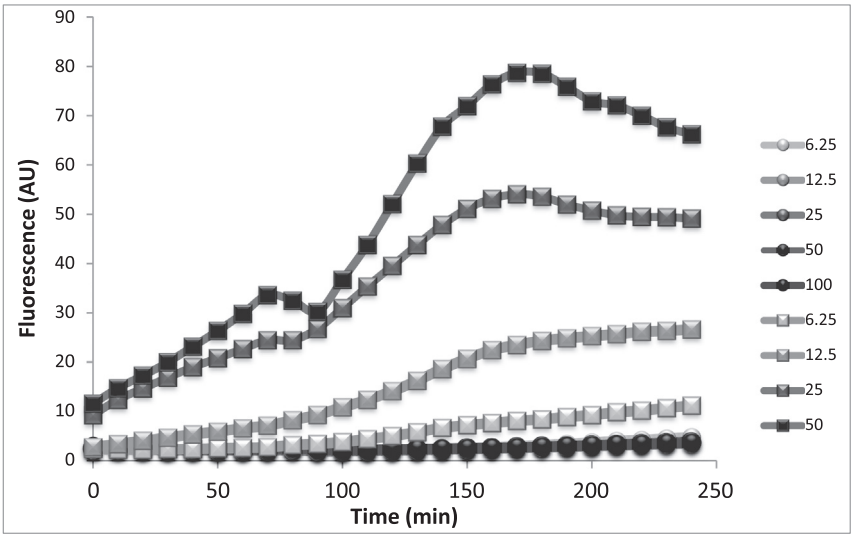


Fig. 3. Kinetics of membrane permeabilization of *Z. bailii*, in presence of C12O3TR (squares) or O3TR (circles). Peptide concentrations (μg/ml) are indicated in the legend.

incubation, which suggests a potential saturation in membrane permeabilization.

In addition to the inhibition of the conidial germination, O3TR and C12O3TR kill fungal hyphae of *F. culmorum*, involving permeabilization of the fungal membrane. Membrane permeabilization has been identified for many short synthetic peptides (Matsuzaki et al., 1997). Interestingly, despite its small size (compared to the 7–10 nm of cell membrane), O3TR permeabilized the membrane so is thought to create at least transient pores. Self-assembly has been observed for antimicrobial peptides, increasing the size of the peptide and facilitating the pore creation (Bechinger and Lohner, 2006).

However, no fluorescence was detected in SG assay performed on yeast cells with O3TR. Yeast resistance to the antifungal protein AFP has been attributed to the activation of several signalling pathways (Ouedraogo et al., 2011).On the contrary, C12O3TR induced a rapid permeabilization of the yeast membrane. The two peptides appeared to use different mechanisms to inhibit yeast and this may explain the higher activity of C12O3TR. The addition of fatty acid chain tail increases the ability of the peptide to induce membrane permeability, generally correlated to antimicrobial activity of the peptide. However, an excessive hydrophobicity can drastically reduce the activity if the peptide cannot penetrate the outer membrane of the microbial target (Makovitzki et al., 2006). The formation of aggregates at the surface of the membrane may induce a peptide too large or change the mode of action of the peptide (Han and Lee, 2015).

3.5. Internalization assay

Apart from the modification of fungal membrane permeability, the presence of intracellular targets has been highlighted for several AMPs, as well as the mechanisms of entry (El-Mounadi et al., 2016). To highlight their internalization into *F. culmorum* conidia, the peptides were labelled with TMR at the N-terminus. The labelled peptides inhibited *F. culmorum* germination with a MIC of 12.5 μg/ml for both peptides.

Within 10 min of treatment, the labelled peptides, highlighted by the red dye TMR, had already bound to the conidial surface and slowly entered the fungal cells After 30 min of incubation, TMR-C12O3TR completely entered some of the fungus cell whereas TMR-O3TR remained mostly at the conidial surface After 1 h of treatment, with a concentration of 6.25 μg/ml, the internalization of TMR-O3TR was limited compared to TMR-C12O3TR. At a concentration of 12.5 μg/ml, TMR-O3TR and TMR-C12-O3TR were internalized in all the *F. culmorum* conidia.

It is noteworthy that the entry of the labelled peptide appeared at concentrations below the ones required to inhibit germination. This suggests that a minimum concentration is required to inhibit specific pathways causing germination. TMR-C12O3TR entered into the fungus cell faster than TMR-O3TR, which can also explain its higher activity.

3.6. Haemolytic and cytotoxic activities

In addition to important costs of synthesis, the limited use of AMPs as novel pharmaceuticals is due to their resistance and toxicity to human cells. O3TR and C12O3TR were tested against fresh sheep RBCs and Caco-2 human cell line. In presence of O3TR, RBCs and Caco-2 cells remained alive at peptide concentrations up to 200 μg/ml (Figs. 4 and 5).

On the contrary, a C12O3TR concentration of 50 μg/ml induced total RBCs lysis and significant cytotoxic activity towards Caco-2 cells (Fig. 5).

C12O3TR lysed sheep RBC and induced Caco-2 cell death at low concentrations in contrast to O3TR. In agreement with these results, Laverty et al. (2010) showed that the acyl chain addition increases the cytotoxicity of the peptides towards keratinocytes and horse RBC. The

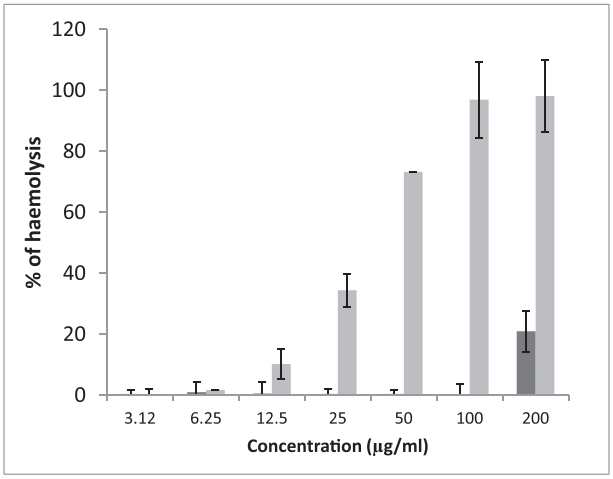


Fig. 4. Haemolytic activity of O3TR (dark grey) and C12O3TR (light grey) towards sheep RBCs. Error bars represent the standard deviation between six replicates.

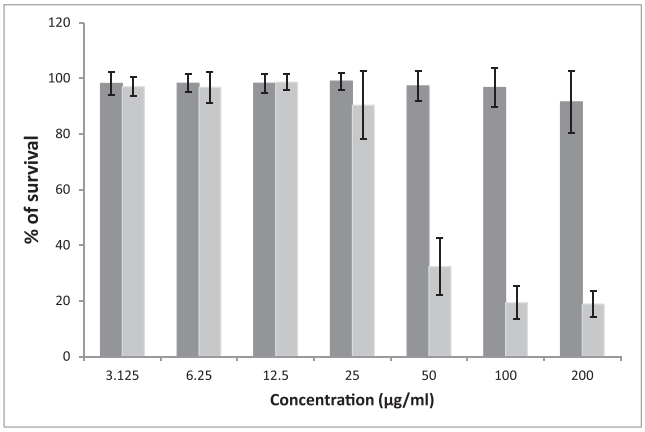


Fig. 5. Percentage of survival of Caco-2 cells in presence of O3TR (dark grey) or C12O3TR (light grey). The experiment was performed in 4 wells in 5 independent experiments.

presence of cholesterol or ergosterol in mammalian or fungal membranes respectively has been shown to reduce the potency of these peptides (Mason et al., 2007) in comparison to antimicrobial activity. Indeed, sterols stabilize the interaction between the phospholipids of the bilayer and prevent degradation. Structural differences (*e.g.* fused rings) in yeast ergosterol, fungal ergosterol or cholesterol, are involved in the lipid acyl chain order and consequently in their sensitivity to AMPs (Shrivastava and Chattopadhyay, 2007). Despite their cholesterol-rich zwitterionic membranes mammalian cells were sensitive to C12O3TR. Lipopeptides are non-selective so an increase of their hydrophobicity and helical structural features increase their cytotoxicity towards human cells (Makovitzki et al., 2006). It is thought that peptides with suitable level of hydrophobicity can penetrate deeper in the bilayer and are able to induce membrane lysis (El-Mounadi et al., 2016).

Although desired in certain treatments, such as cancer, this toxicity is usually a limiting factor in the use of CAPs (Haug et al., 2016). To reduce the toxicity of C12-OOWW-NH2, modifications can increase the specificity of the peptide. Thus, the addition of specific receptor (Yang et al., 2015) or a pH-dependent conjugate (Burns et al., 2016) have been proven to be efficient.

3.7. Application as beverage preservatives

Despite hostile environment for most microorganisms, beverages can be suitable for some yeast and mould growth, leading to undesirable flavours and taste (Wareing and Davenport, 2004). In addition, yeast species such as *Z. bailii* shows resistance to common weak-acid preservatives used for soft drinks. To avoid this resistance, the multiple mode of action of AMPs is a promising way. To date, nisin is the only antimicrobial peptide allowed for food applications, but its use is limited, due to its narrow spectrum (Rydlo et al., 2006).

To foresee potential applications, the two peptides were added to different commercial beverages in a challenge test against yeast and fungi contaminants. After 7 days of incubation, O3TR at concentrations of 100 and 25 µg/ml inhibited yeast growth in the commercial lager and the carbonated soft drink, respectively. The peptide was not efficient to prevent yeast growth in apple or orange juice and slightly retarded *A. niger* growth (Tables 4 and 5).

After 7 days, C12O3TR inhibited the growth of yeast and *A. niger* in all the drinks, except in orange juice, where *A. niger* was not affected (Table 5). The inhibition of yeast growth in orange juice required higher concentration of C12-O3TR (100 µg/ml) (Table 4).

O3TR was able to inhibit yeast growth in beer and carbonated drink. However, high concentrations were required. Interactions between antimicrobials and food components, including sugars, have been highlighted, resulting in a limited activity. To avoid these interactions, novel formulations such as encapsulation are currently used for nisin (Carmona-Ribeiro and Carrasco, 2014). C12O3TR was efficient in preventing or delaying fungal growth in all the beverages, even at concentrations that did not induce cytotoxicity. Other rationally designed peptides have been proven to delay fungal growth on food products (López-Garcia et al., 2000).

4. Conclusion

Antimicrobial peptides represent promising alternatives to common antibiotic and food preservatives. The ease and the reduction of costs for the synthesis of ultrashort peptides make them ideal candidates. The addition of a C12 fatty-acid chain tail at the N-terminus of the tetrapeptide amine H-Orn-Orn-Trp-Trp-NH2 improved its antifungal activity. Structural differences between the two peptides and in their mode of action were correlated to their antifungal potency. The high hydrophobicity of the lipopeptide was suggested to play a role in these differences as well as in its cytotoxicity and haemolytic activity. By optimizing their properties, these peptides could represent a novel

Table 4
Yeast growth in commercial beverages in presence of O3TR and C12O3TR.

	Day 2				Day 7			
	Commercial lager	Carbonated soft drink	Apple juice	Orange juice	Commercial lager	Carbonated soft drink	Apple juice	Orange juice
Control	+	−	+ +	+ +	+ +	+	+ +	+ +
O3TR (µg/ml)								
25	+	−	+ +	+ +	+ +	−	+ +	+ +
50	−	−	+ +	+ +	+ +	−	+ +	+ +
100	−	−	+ +	+ +	−	−	+ +	+ +
200	−	−	+	+ +	−	−	+ +	+ +
C12O3TR (µg/ml)								
25	−	−	−	+	−	−	−	+ +
50	−	−	−	+	−	−	−	+ +
100	−	−	−	−	−	−	−	−
200	−	−	−	−	−	−	−	−

−: no growth (OD_{600} < 0.2; +: moderate growth (OD_{600}: 0.2–0.6); + +: strong growth (OD_{600} > 0.6).

Table 5
A. niger growth in commercial beverages in presence of O3TR and C12O3TR.

	Day 2			Day 7		
	Commercial lager	Apple juice	Orange juice	Commercial lager	Apple juice	Orange juice
Control	+	++	++	++	++	++
O3TR (μg/ml)						
25	+	+	++	++	++	++
50	+	+	++	++	++	++
100	+	+	+	++	++	++
200	+	+	+	++	++	++
C12O3TR (μg/ml)						
25	−	−	−	−	++	++
50	−	−	−	−	−	++
100	−	−	−	−	−	++
200	−	−	−	−	−	++

−: no growth (OD_{600} < 0.2; +: moderate growth (OD_{600}: 0.2–0.6); ++: strong growth (OD_{600} > 0.6).

preservative against beverage contaminations. Although the use of novel additives requires the food regulatory approval, this study shows the feasibility of using USP to protect beverages and more generally food products from fungal spoilage, especially against preservative-resistant species.

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RESEARCH ARTICLE

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Antifungal activity of a de novo synthetic peptide and derivatives against fungal food contaminants

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The development of novel solutions to fight microbial food contaminants rests upon two pillars, which are the development of resistant strains and consumers' desire for a reduced consumption of synthetic drugs. Natural antimicrobial peptides possess the qualities to overcome these issues. De novo synthesis of novel antifungal compounds is a major progress that has been facilitated by the identification of parameters involved in the antimicrobial activity. A 14-residue peptide named KK14, with the sequence KKFFRAWWAPRFLK-NH_2, was designed and inhibited conidial germination and fungal growth of food contaminants within the range 6.25 to 50 μg/ml and 6.25 to 100 μg/ml, respectively. The study of three analogues of the peptide highlighted the role of some residues in the structural conformation of the peptide and its antifungal activity. The substitution of a Pro residue with Arg increased the helical content of the peptide not only its antifungal activity but also its cytotoxicity. The insertion of an unnatural bulky residue β-diphenylalanine or a full D-enantiomerization overall increased the antifungal potency. The four peptides showed similar behaviour towards salt increase, heat treatment, and pH decrease. Interestingly, the Denantiomer remained the most active at high pH and after proteolytic digestion. The four peptides did not present haemolytic activity up to 200 μg/ml but had different behaviours of cytotoxicity. These differences could be crucial for potential application as pharmaceutical or food preservatives.

KEYWORDS

antimicrobial peptide, cytotoxicity, D-enantiomer, food spoilage, substitutions

1 | INTRODUCTION

Although less commonly associated with grave symptoms than bacteria or viruses, fungi are frequently involved in severe infections.[1-3] These lethal infections generally involve *Candida*, *Aspergillus*, and *Cryptococcus* species.[1,4] Food-related fungi are not lethal, but detrimental effects have been observed in immuno-suppressed people. Fungal infections of the eye are also classified as mucosal fungal infections but are caused more frequently by *Fusarium* or *Aspergillus* species rather than *Candida* species.[3] In addition, a prolonged exposure to mycotoxin-producing fungi, such as *Aspergillus niger*, may lead to the development of several diseases, such as gastrointestinal disorders, to neurological issues or cancers.[5] When submitted to inefficient or inappropriate treatments, the fungal threat remains and represents a health hazard and may cause major food spoilage. Either in a therapeutic setting or as a food preservative, there is a need for novel antifungal compounds.

Cationic antimicrobial peptides (CAPs) are members of the innate immune system and act as primary agents against infective pathogens. These peptides play an important role in novel treatments, and many are currently under clinical trial[6-8] or used in food products.[9,10] However, few of the latter target filamentous fungi. Plants and insects are particularly powerful producers of antifungal peptides (AFPs) effective against filamentous fungi.[11,12] However, the use of natural AFPs faces

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several issues, such as poor stability, potential toxicity, and a time-consuming extraction. To overcome these problems, synthetic peptides with a broad antimicrobial activity have been developed. However, the high cost of production of long peptides reduces the potential applications. Thus, de novo peptides have been produced by Hodges and coworkers[13-15] based on the key effectors of peptide efficacy, such as cationicity and hydrophobicity.[13-15] To a lesser extent, other parameters such as flexibility, with a nondefined but inducible structure in hydrophobic environment, or amphipathicity play a role in the antimicrobial and cytotoxic activities.[16]

Peptide production is achieved via recombinant and chemical synthesis, both methods presenting advantages and limitations. Recombinant production peptides are usually cheaper with a useful material for large peptides and are expected to play a major role in novel drug development. In addition, recombinant methods are more demanding in terms of process and personnel[17,18] than chemical synthesis. In spite of high prices, the cost of peptide synthesis via Fmoc chemistry has decreased significantly, and chemical synthesis appears easier to apply at large scales. It also facilitates the insertion of unnatural peptides, commonly recognised as necessary for the design of drugs.

Based on these parameters and on the comparison of several peptides sequences, we designed novel peptides sequences. Among the numerous sequences used as frameworks, indolicidin is a potent 13-mer Trp-rich peptide with an hydrophobic core, ILPWKWPWWPWRR[19,20]; LTX-315 (KKWWKKDipK) is an anticancer 9-mer cationic peptide comprising an unnatural β-β diphenylalanine residue[21]; V13KL[13] is 26-residue amphipathic α-helical peptide, studied to highlight the role of hydrophobic residues in the activity of helical peptides. The insertion of Lys and Arg residues was meant to increase the cationicity while the choice between Lys and Arg was due to a will to improve reduced haemolytic activity. A similar rationale was used to select the hydrophobic residues Trp and Phe. Because of its helix propensity, Ala was inserted in the sequence whereas the presence of Pro and its associated helix-breaking properties aimed at reducing the toxicity. Ultimately, after comparison of predicted antimicrobial activity (accessible online http://www.camp.bicnirrh.res.in/prediction.php), we decided to work with a sequence of a 14-residue novel peptide, KKFFRAWWAPRFLK-NH_2, named KK14. In the present study, the influence of specific residues and conformation on the antifungal activity of the peptide was studied with the generation of three analogues. The peptide presented the following characteristics: presence of cationic residues with a hydrophobic centre; a medium size to avoid dimerization but to give a defined structure and limit the cost of synthesis. The peptide was amidated at the C-terminus to increase the net charge of the peptide. The choice between Lys/Arg and Trp/Phe was made to limit haemolytic activity while maintaining antimicrobial potency.[22,23] The positions of the polar and apolar residues were also chosen to limit the haemolysis of the peptides. Indeed, Jiang et al showed that disruption of a perfect α-helix by the substitution of Val to Lys on the non-polar face reduced toxicity while maintaining the antimicrobial potency.[16] The modifications of the initial peptide KK14 included: a substitution with the unnatural residue β, β-diphenylalanine (Dip) (KK14(Dip)), in order to improve salt stability and activity; D-enantiomerization of KK14 (kk14), known to give protease resistance; the substitution of Pro_{10} with Arg_{10} (KK14(R)), to increase the net charge of the peptide. The effect of sequence modifications on the antifungal potency, mechanisms of antimicrobial action, the structure, and the toxicity of the peptides were studied. The rational design and the correlation with predictors are effective ways to create powerful antifungal agents and to improve the understanding of the mechanisms involved in antifungal activity. The generation of this novel antifungal peptide and derivatives could aid in the cost-effective development of novel, efficient, and safe antifungal agents.

2 | MATERIAL AND METHODS

2.1 | Peptides

Four newly designed peptides (purity >90%, as specified by manufacturer) were synthesised by Fmoc solid-phase synthesis on resin (GLBiochem, Shanghai, China). The characteristics of the peptides are indicated in Table 1 and determined via the online tool (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py). The antimicrobial nature of the peptides was predicted with online tools available on the different databases. The analogues of the initial peptide KK14 (KKFFRAWWAPRFLK-NH_2) included a peptide where Trp in position 8 was replaced by an unnatural bulky residue β,β- diphenylalaline (Dip) (KK14(Dip)), a D-enantiomer of KK14 (kk14) and KK14(R) where Pro_{10} substituted with Arg_{10}. The peptides were resuspended in sterile deionized water at an initial concentration of 2 mg/ml.

2.2 | Antifungal assays

Fusarium culmorum, *A. niger*, and *Penicillium expansum* were chosen as representatives of filamentous fungal contaminants of ingredients and food. Fungal strains were originated from University College Cork (UCC, Cork, Ireland) collection. The inhibition of conidial germination was determined as described previously.[24] Fungal spores (final concentration, $1x10^4$ spores/ml) were inoculated in 90 µl of synthetic medium for fungi (SMF: 50 µM $MgSO_4$/7H2O, 5 µM $FeSO_4$/7 H_2O, 0.1 µM $CoCl_2$, 0.1 µM $CuSO_4/5H_2O$, 2 µM $Na_2MoO_4/2H_2O$, 0.5 µM H_3BO_3, 0.1 µM KI, 0.5 µM $ZnSO_4/7H_2O$, 0.1 µM $MnSO_4$/$1H_2O$, glucose, asparagine, 20 mg of methionine, 2 mg of myo-inositol, 0.2 mg of biotin, 1 mg of thiamine-HCl, 0.2 mg of pyridoxine-HCl, 0.5mM K_2HPO_4/3 H_2O), as described previously.[25] Peptide solution (10 µl, 0-100 µg/ml) was added to the wells and OD_{600} of the solution was monitored for 120 hours at 28°C using a Varioskan microplate reader (ThermoFischer Scientific).

The inhibition of fungal growth was performed on germinated conidia as described by Thevissen, Terras, and Broekaert.[25] Fungal spores were incubated for 24 hours in half-strength potato dextrose broth (½ PDB, Sigma-Aldrich) at 28°C. Following incubation, hyphae were washed twice with SMF. Aliquots of hyphal suspension (90 µl) were mixed with 10 µl peptides (0-100 µg/ml), and growth was monitored as described above. For each experiment, the minimum inhibitory concentration (MIC) was determined as the minimum concentration that inhibited completely conidial germination or mycelium growth in three replicates.

TABLE 1 Predicted characteristics of the synthetic peptides

Peptide	Sequence	Net Charge[a]	Hydrophobicity[a]	Amphipathicity[a]	Length	AMP activity[b]	pI[c]	MW (Da)[d]	Purity (%)
KK14	KKFFRAWWAPRFLK-NH_2	+6	0.566	0.175	14	0,999	14	1880.34	92.2
KK14(Dip)	KKFFRAW**Dip**APRFLK-NH_2	+6	ND	ND	14	ND	ND	1880.34	95.2
kk14	kkffrawwaprflk-NH_2	+6	ND	ND	14	ND	ND	1917.41	95.5
KK14(R)	KKFFRAWWA**R**RFLK-NH_2	+7	0.442	0.174	14	0,999	14	1868.33	94.5

Abbreviation: AMP, antimicrobial peptide; ND, not able to be determined with the online tools.

[a]The different parameters were determined with the online tool (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py) (using the non amidated peptide).

[b]The AMP potential of the peptides was predicted with the support vector machine (SVM) algorithm (available at http://www.camp.bicnirrh.res.in/predict_c/).

[c]The isoelectric point was calculated with the online tool (PepCal.com).

[d]Molecular weight (MW) was provided by the supplier GLBiochem.

2.3 | Peptides stability

The stability of KK14 and analogues was determined upon heat treatment, at low and high pH solutions and in different salt solutions. The four peptides (0-50 µg/ml) were heated at 100°C for 15 minutes using a heating block and left for cooling at room temperature for 30 minutes. Following cooling, the antifungal activity of the peptides was determined against *F. culmorum* conidia as described above. The pH stability of the antifungal activity of the peptides was tested in SMF at pH 3,5 and pH 9,5. The conidial germination in presence or absence of peptides (0-25 µg/ml) was monitored by absorbance as described above. Finally, the antifungal activity of the peptides was also determined in salt solutions with monovalent (50 mM and 150 mM of KCl) and divalent (1 mM and 5 mM $CaCl_2$) cations as described by Terras et al.[26]

2.4 | Effect of trypsin on antifungal activity

The protease effect on the antifungal activity was performed as described by Rozek, Powers, Friedrich, and Hancock.[27] Because of its presence in the gut tract, trypsin was chosen to mimic the fate of the peptides when used as food preservatives. KK14 and analogues were incubated at 37°C for 2 hours in the presence of trypsin (Sigma-Aldrich) at a peptide-to-trypsin ratio of 256:1 in 100-mM Tris buffer, pH 7.3. After treatment, trypsin was heat-inactivated at 80°C for 10 minutes. The remaining antifungal activity was performed against *F. culmorum* conidia (10^4 conidia/ml) as described above.

2.5 | Membrane permeabilisation

F. culmorum conidia (5x10^4 spores/ml) were cultured for 24 hours in ½ strength PDB at 28°C. Fresh *F. culmorum* mycelia was collected by centrifugation (8000 g/10 minutes), washed twice with SMF and resuspended in 100 µl of SMF. Mycelium was then treated with different concentrations of peptides (0, 6.25, 12.5, and 25 µg/ml) for 2 hours. Following incubation, mycelium was resuspended in 100 µl of SMF and incubated in 2 µM of propidium iodide (PI, Sigma-Aldrich) for 10 minutes and rinsed with distilled water three times. The mycelium was visualised by fluorescence microscopy (Olympus FluoView™ FV1000) (excitation 535 nm/emission 615 nm). The kinetics of PI uptake was determined as described previously.[28] Fresh mycelium was incubated with peptides and PI (2 µM), and the fluorescence was measured in a black 96-well plate for 2 hours at 28°C with a fluorospectrophotometer (VarioSkan, ThermoFischer Scientific).

2.6 | Circular dichroism spectroscopy

The secondary structures of KK14 and analogues were analysed by circular dichroism (CD) spectroscopy. The analysis of the peptides secondary structures was conducted as described by Liu et al.[29] CD spectroscopy was performed by using a Chirascan™ spectropolarimeter (Applied Photophysics) with the peptides dissolved in deionized water or 20-mM sodium dodecyl sulphate (SDS) micelles using a quartz cell of 1-mm path length. CD measurements were performed at 27°C within a wavelength range of 190 to 260 nm. Samples and solvents were scanned three times, and baseline was corrected by subtracting corresponding solvent spectrum. Ellipticities were converted to mean residue molar ellipticities (θ) in units of deg.cm^2/dmol and percentage of secondary structures obtained with deconvolution program CD spectroscopy software. A 3D model of KK14 and KK14(R) was generated via PEP-FOLD3 online tool.[30] In date of use, PEP-FOLD 3 did not process peptides with D-amino acids or unnatural L-amino acids, such as Dip. The structure of the four peptides was also predicted via in silico prediction using the online tool PEPstrMOD accessible online.[31,32]

2.7 | Haemolytic activity assay

Fresh sheep erythrocytes (Oxoid, ThermoScientific) were centrifuged at 1620 g and pellet was resuspended in an equal volume of 1xPBS. Washing steps were repeated three times, and the final pellet was resuspended at 4% (v/v) in PBS. Erythrocytes solution (180 µl) were added with 20 µl of peptides (0-200 µg/ml), diluted in PBS. The solutions were then incubated for 1 hour at 37°C. Supernatant (100 µl) was collected after centrifugation (1000 g/2 minutes) and absorbance was measured at 405 nm (Varioskan, ThermoFischer Scientific). PBS and 0.1% Triton X-100 were used to induce 0 and 100% haemolysis, respectively. The percentage of haemolysis was calculated as follows:

$$\%\text{Haemolysis} = \frac{(Abs405\ peptide\ treatment) - (Abs405\ )}{(Abs405\ 0.1\%Triton\ X - 100) - (Abs\ \ 405\ PBS)} \times 100.$$

2.8 | Cytotoxicity assays towards Caco-2 and mouse RAW 264.7 cell lines

The cytotoxicity of KK14 and analogs was performed against Caco-2 human colonic cell line ($1x10^5$ cells/ml) (ECACC), using the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously.[24] Briefly, Caco-2 cells were cultured in a 96-well microtiter plate in Dulbecco's modified Eagle's medium (DMEM), supplemented with non-essential amino acids (NEAA, 1%) and FBS (10%), at 37°C in 5% CO_2. A similar procedure was used to test the cytotoxicity of peptides towards mouse macrophages RAW 264.7 cell line ($1x10^5$ cells/ml). Cells were allowed to adhere and 200 µl of fresh medium (with 2.5% FBS) supplemented with peptides (0-200 µg/ml) was added to the wells and incubated for 24 hours at 37°C. The MTT reagent (Cell proliferation Kit I MTT; Sigma, Ireland) was added for 4 hours, followed by an overnight incubation with lysis solution. Cell viability was measured spectrophotometrically at 570 nm with a background reading at 690 nm.

2.9 | Applications in beverage systems

The potential application of the peptides was tested in several beverages upon challenges with fungal spores, as described previously.[24] Acid foods such as apple juice have been associated with foodborne illness outbreaks.[33] *A. niger* has been reported as a fungal contaminant of soft drinks, including apple juice, in poor hygiene conditions.[34] Filter-sterilized apple juice (for 100 mL, <0.5 g fat, 11 g carbohydrates, <0.5 g proteins, <0.01 g salt, pH = 3.7) was inoculated with spores of *A. niger* (10^5 spores/ml). Beverage solutions (90 µl) were transferred in the wells of a 96-well microtiter plate and peptides were added at a concentration range of 0-200 µg/ml (final concentration). After 24 hours of incubation at 28°C, beverage solution (50 µl) was drop-inoculated on a PDA plate and fungal growth monitored over 3 days at 28°C.

2.10 | Statistical analysis

Antifungal and stability experiments were conducted in three technical replicates. Haemolysis and cytotoxicity assays were conducted in three technical replicates and repeated at three occasions. Values are reported as means ± standard deviations of three technical replicates for antifungal and stability assays. A two-tailed *t* test was performed was conducted for each peptide to determine significant difference in cytotoxic assays. Significant difference was considered for *P* values of <0.05.

3 | RESULTS

3.1 | Antifungal activity

The reduction of food spoilage because of fungal contaminants is a health and economic necessity that requires novel approaches, such as the use of AMPs. Based on the key determinants of the antifungal activity of AMPs, a short AFP, named KK14, was constructed and tested against several food fungal contaminants. KK14 inhibited the germination of *F. culmorum*, *A. niger*, and *P. expansum* conidia with a MIC of 6.25-50 µg/ml (Table 2). The substitution of Trp in position 8 with the bulky unnatural residue Dip did not modify significantly the activity of KK14 (MIC: 12.5-25 µg/ml). A similar effect was obtained with the enantiomerization of KK14. The replacement of Pro (in position 10) by a polar residue Arg increased the MIC by 2- to 4-fold against *P. expansum* and *A. niger* (Table 2). The potency of the peptides to inhibit hyphal growth was only important against *F. culmorum* with a MIC of 6.25-25 µg/ml. *A. niger* and *P. expansum* hyphae appeared only sensitive to high concentrations of kk14 and KK14(R).

3.2 | Peptides resistance

The stability of the peptides was studied in salt solutions, low and high pH solutions, after heat treatment and following trypsin digestion. The antifungal potency of the peptides was determined as the ability to inhibit *F. culmorum* germination and growth. The antifungal activity of the peptides was altered in presence of 150 mM KCl, 1mM and 5 mM $CaCl_2$, with a MIC increase by 2- to 16-fold. However, in less concentrated solutions (50 mM KCl), the peptides showed the same activity against *Fusarium* conidia (Table 3) as in SMF.

At pH 3.5, *F. culmorum* had the same MIC to the four peptides as at pH 6.5. On the contrary, the activity of the peptides was reduced at pH 9.5, with an increase of MIC by 2- to 4-fold. This trend was even clearer for KK14(Dip), which did not inhibit *Fusarium* germination up to 25 µg/ml (Table 4). The peptides did not appear to be affected by heat treatment of aqueous peptide solutions (Table 5). Finally, following trypsin digestion, only the D-enantiomer kk14 kept its antifungal activity after 1 hour treatment. The other peptides did not inhibit conidial germination at a concentration up to 50 µg/ml. (Table 5).

3.3 | Secondary structure of the peptides

The secondary structure of KK14 and analogues was determined in aqueous and hydrophobic environment to evaluate the structural changes induced by sequence modifications (Figure 1). KK14 appeared unstructured in aqueous environment with a negative peak around

TABLE 2 Minimum inhibitory concentration (µg/ml) of KK14 and analogues against fungal food contaminants

	KK14	KK14(Dip)	kk14	KK14(R)
Non germinated conidia				
Fusarium culmorum	6.25	12.5	6.25	6.25
Penicillium expansum	25	25 (12.5)	25 (12.5)	12.5
Aspergillus niger	50 (25)	25	25	12.5
Germinated conidia				
Fusarium culmorum	25 (12.5)	25 (12.5)	6.25	25 (12.5)
Penicillium expansum	>100	>100	>100 (100)	>100 (50)
Aspergillus niger	>100	>100	100 (50)	100 (50)

The IC_{50} is indicated into brackets if reached.

TABLE 3 Minimum inhibitory concentration (IC_{50}) of KK14 and analogues for the inhibition of *Fusarium culmorum* conidial germination after 120 hours of incubation in different salt solutions

	Fusarium culmorum conidia			
	50 mM KCl	150 mM KCl	1 mM $CaCl_2$	5 mM $CaCl_2$
KK14	6.25	50 (25)	25 (12.5)	> 100 (25)
KK14(Dip)	12.5	25 (6.25)	25 (12.5)	100 (25)
kk14	6.25	25 (12.5)	12.5	100 (25)
KK14(R)	6.25	25 (6.25)	12.5	100 (25)

200 nm and positive peak at 220 nm. In presence of SDS micelles, a clear transition between coil-helix and helix was observed with two negative peaks at 205 and 220 nm and a slightly positive peak at 195 nm and a crossover at 200 nm. However, the low value and the undefined positive peak showed the remaining of a high proportion of random coil. The transition is clearer for KK14(R) with high values of ellipticities and clear peaks, which suggest a high α-helical content. The D-enantiomer of KK14 showed a mirror spectrum in aqueous environment. However, the D-enantiomer appeared even more

TABLE 4 Percentage of fungal growth inhibition at pH 3.5 and pH 9.5 in presence of the peptides

	pH 3.5			pH 9.5		
Concentration (μg/ml)	6.25	12.5	25	6.25	12.5	25
KK14	71.1 ± 14.3	63.1 ± 4.6	69.5 ± 10	20.6 ± 23.7	47.1 ± 20.8	86.3 ± 1.2
KK14(Dip)	84.2 ± 6.1	98.1 ± 0.3	97.3 ± 0.2	−18.9 ± 11.7	−6.81 ± 3.6	−23.6 ± 5.3
kk14	93.8 ± 4.6	95.7 ± 5.2	97.3 ± 0.4	62.7 ± 10.7	95.1 ± 1.7	97.6 ± 0.7
KK14(R)	81.6 ± 15.2	97.8 ± 0.6	98.7 ± 0.3	25.9 ± 30.0	63.4 ± 18.8	80.7 ± 6.4

Results are expressed as means of three technical replicates ± standard deviation.

TABLE 5 Antifungal activity of the peptides after heat treatment and trypsin treatment

	Heat Treatment				Trypsin Treatment			
Concentration (μg/ml)	6.25	12.5	25	50	6.25	12.5	25	50
KK14	15.7 ± 29.3	77.4 ± 41.0	96.8 ± 4.3	94.9 ± 2.8	8.3 ± 2.8	3.2 ± 9.5	−2.2 ± 5.6	12.7 ± 7.5
KK14(Dip)	51.0 ± 12.5	97.8 ± 3.5	93.8 ± 3.5	100.0 ± 5.5	3.0 ± 8.0	0.1 ± 3.4	−1.7 ± 7.5	8.0 ± 3.5
kk14	93.7 ± 1.7	88.7 ± 3.3	86.2 ± 7.7	86.1 ± 8.3	79.4 ± 5.6	84.5 ± 3.7	84.9 ± 5.6	93.6 ± 8.9
KK14(R)	93.5 ± 16.5	98.1 ± 4.4	93.9 ± 0.8	95.4 ± 8.3	1.8 ± 5.1	−2.3 ± 4.4	−10.4 ± 3.7	−10.8 ± 5.9

The remaining antifungal activity is expressed as the percentage of inhibition of *Fusarium culmorum* conidial germination, based on optical density (OD) monitoring. Results are expressed as means of three technical replicates ± standard deviation.

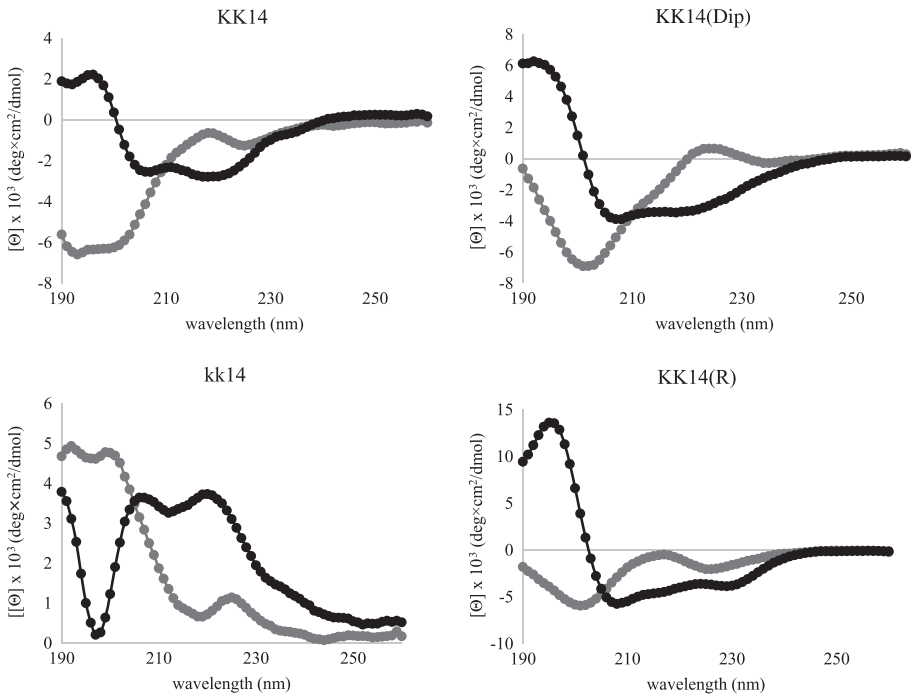


FIGURE 1 Circular dichroism spectra of the peptides dissolved in deionized water (grey) or 20 mM sodium dodecyl sulphate micelles solution (black) in the 190 to 260 nm region. Each curve is the average curve of three runs

structured than KK14 in hydrophobic environment with a more defined peak at 195 nm. Deconvolution analysis revealed a high proportion of random coil and antiparallel structure in aqueous and SDS solutions (Table 6). The values of structural features for KK14(R) showed a clear transition with an increase in helical content (17% to 29.8% of helical content in deionized water [DIW] and SDS, respectively). However, the very high-total sums of structural percentages make conclusions for the other peptides weak. An in silico prediction was also performed using the online tools PEP-FOLD3 and PEPstrMOD (Figure 2).

TABLE 6 Percentage of secondary structures of KK14 and analogues in deionized water and sodium dodecyl sulphate solution

	KK14		KK14(Dip)		kk14		KK14(R)	
	DIW	SDS	DIW	SDS	DIW	SDS	DIW	SDS
Helix	15.9	20	17.3	23.4	17.6	14.9	17	29.8
Antiparallel	57.4	32.5	54.3	22.7	32.2	47.2	50.1	10.6
Parallel	11.9	11.7	10.8	10.7	14.1	14.6	11.7	9.7
Beta-turn	22.3	20.1	22.2	19.1	19.8	21	21.6	17.5
Random. Coil	36.2	39.6	32.8	37.8	48.9	47.9	36.8	36.9
Total sum	143.6	124	137.4	113.8	132.7	145.6	137.3	104.4

Abbreviations: DIW, deionized water; SDS, sodium dodecyl sulphate. The total sum is indicated, highlighting the delicate interpretation of these results. Results are based on three runs of the same similar.

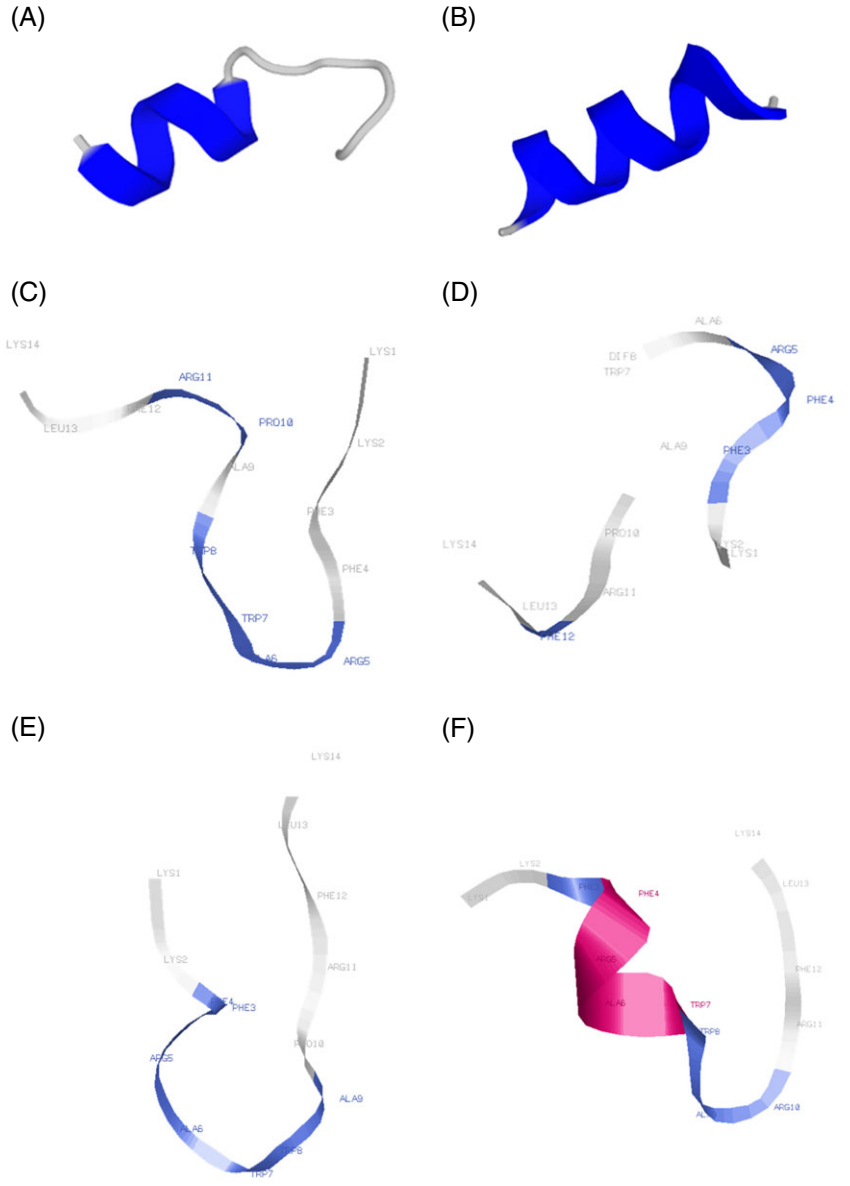


FIGURE 2 In silico prediction of secondary structure of the peptides using PEP-FOLD 3 tool (A and B) or PEPstrMOD (C-F). (A) KK14, (B) KK14(R), (C) KK14, (D) KK14(Dip), (E) kk14, (F) KK14(R)

The 3D model prediction of KK14 with PEP-FOLD3 online tool indicated the presence of random coil after the Pro residue in KK14. On the contrary, KK14(R) was represented as a perfect helix (Figure 2A,B) Using PEPstrMOD, only KK14(R) presented helical features in its secondary structure, whereas the other peptides presented a mostly random coiled configuration (Figure 2C-F).

3.4 | Membrane permeabilisation

A PI uptake assay was performed on *F. culmorum* hyphae in presence of the four peptides. After 2 hours of incubation, microscopic observation revealed the presence of PI into the mycelium treated with the peptides from a concentration of 6.25 µg/ml. The peptides kk14 and KK14(R) appeared as the most active peptides to permeate fungal membrane with a higher content of permeabilised hyphae at 12.5 µg/ml (Figure 3), as confirmed by the intensity of the fluorescence (Table 7).

3.5 | Haemolytic and cytotoxic activity

The haemolytic and cytotoxic activity of the four peptides was tested against fresh sheep erythrocytes, colonic Caco-2 cells, and mouse macrophages RAW 264.7. The peptides showed no or slight haemolytic activity (~10% at 200 µg/ml) with KK14 being the least haemolytic.

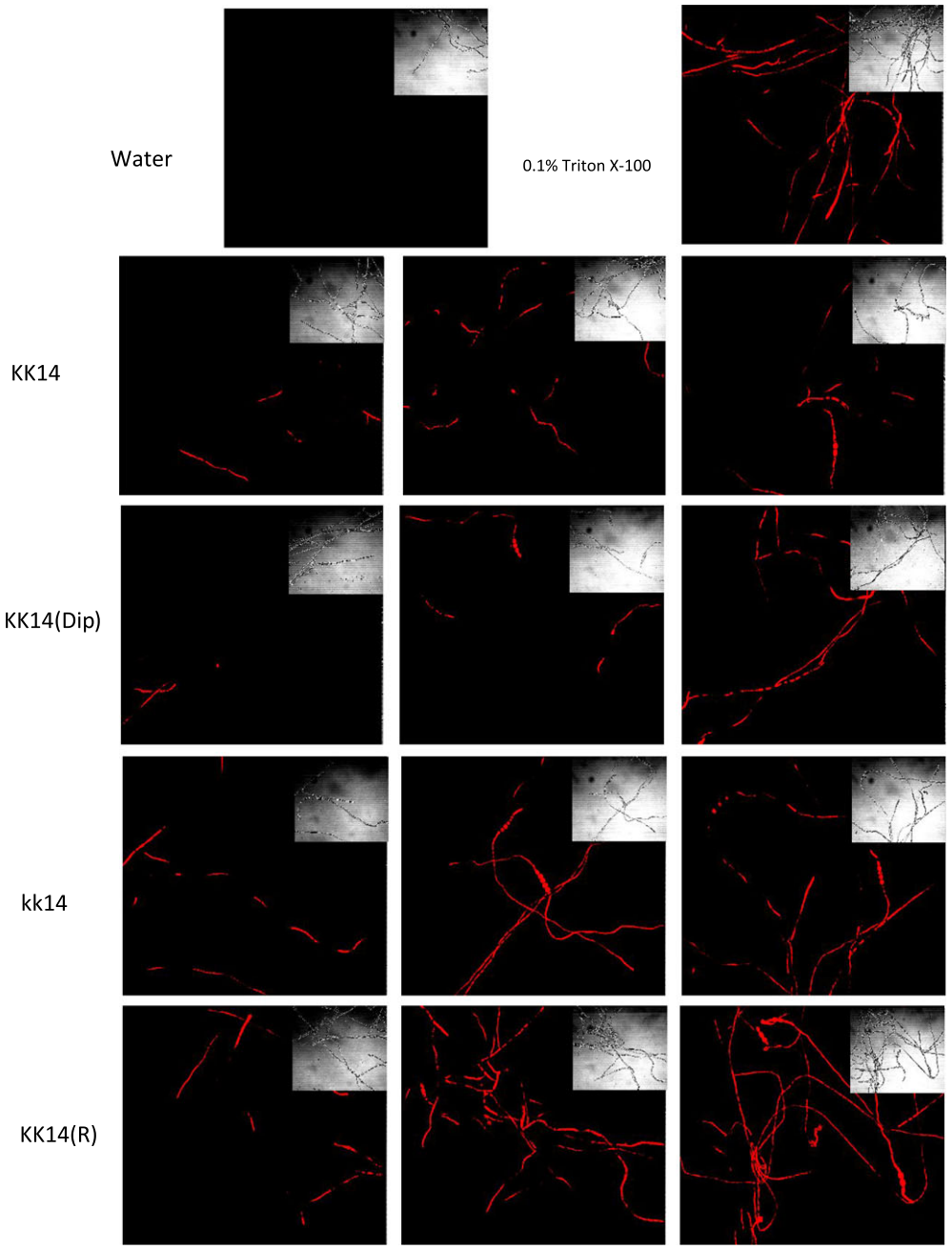


FIGURE 3 Propidium iodide uptake in *Fusarium culmorum* after treatment with KK14 and analogues: 6.25 µg/ml (left), 12.5 µg/ml (middle), 25 µg/ml (right). The mean intensity (mean gray value) was determined using the software ImageJ

TABLE 7 Mean intensity of propidium iodide fluorescence

Compound	Water	Triton X-100	KK14			KK14(Dip)			kk14			KK14(R)		
Concentration (μg/ml)			*6.25*	*12.5*	*25*	*6.25*	*12.5*	*25*	*6.25*	*12.5*	*25*	*6.25*	*12.5*	*25*
Mean intensity[a]	3.1E-04	10.4	1.1	3.1	2.7	1.1	1.1	5.7	1.8	5.6	4.4	2.4	7.1	10.9

[a]Results are based on mean gray intensity, calculated via ImageJ software.

Among the four peptides tested, only kk14 appeared toxic to Caco-2 cells at a concentration of 100 μg/ml, with a percentage of survival of cells of 55.4% ± 16.8% at 100 μg/ml and a 20.4% ± 10.4% viability at 200 μg/ml. Macrophages RAW 264.7 were also sensitive to KK14(Dip), kk14, and KK14(R) at a concentration 200 μg/ml, with a viability percentage of 29.8%, 13.5%, and 5.8%, respectively (Figure 4).

3.6 | Applications in beverages

The efficacy of the peptides to limit fungal spoilage of beverages was tested in an apple juice solution, contaminated with *A. niger* spores. After 3 days of incubation on PDA, the parental peptide KK14 and the D-enantiomer kk14 failed to reduce the growth of *A. niger*. A reduction of fungal growth was only observed after treatment with KK14(Dip) and KK14(R) at the highest concentration (200 μg/ml) (Figure 5). At lower concentrations of peptides, no inhibition of fungal growth was observed, which indicated an impairment of antifungal activity by 4- to 8-fold.

4 | DISCUSSION

A novel synthetic peptide KK14 (KKFFRAWWAPRFLK-NH_2) and analogues inhibited the germination and hyphal growth of *F. culmorum*, *A. niger*, and *P. expansum*. KK-14 possesses a C-terminal amidation,

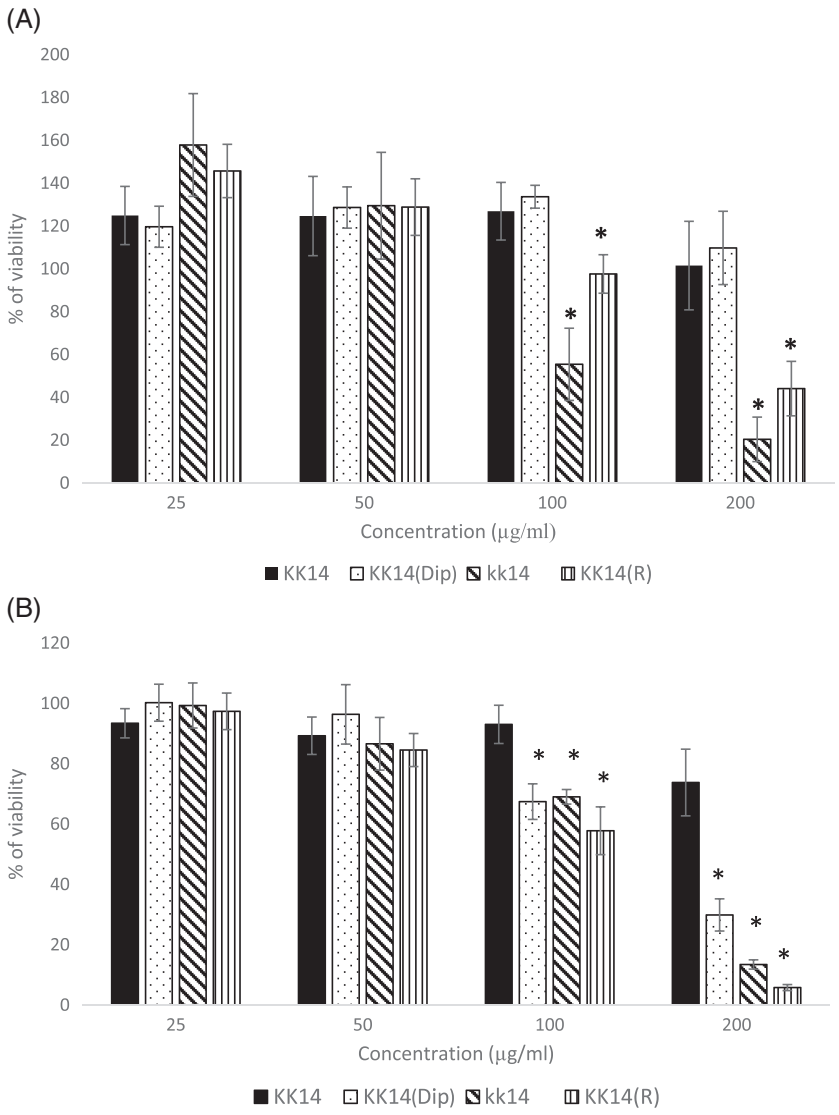


FIGURE 4 Viability of Caco-2 (A) and RAW 264.7 (B). cells Statistical analysis was calculated with a two-tailed *t* test. $P < 0.05$ was considered significant. An asterisk (*) indicates significant difference from negative control (100% viability). Results are expressed as means of three biological replicates ± standard deviation

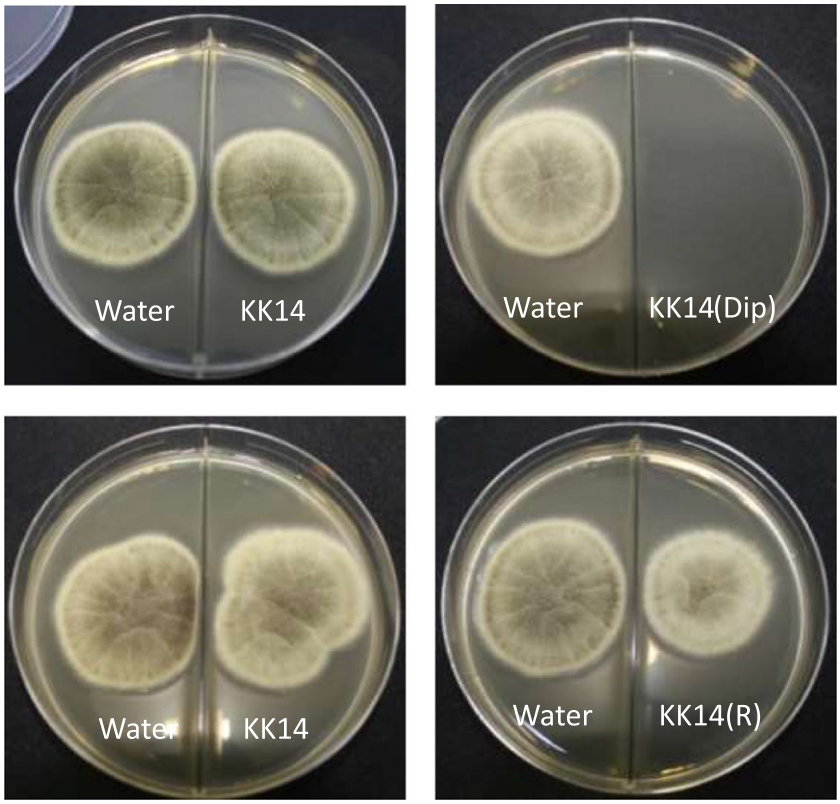


FIGURE 5 Growth of *Aspergillus niger* after incubation in apple juice in the absence (left compartment) or presence of peptides (200 μg/ml) (right compartment)

which is a common post-translational modification (PTM) used to increase the net charge and the stability of peptides. However, its role in serum stability and improvement of antifungal activity is relative (22, 23, 24). N-terminal acylation is another common PTM, and while it is used to improve the stability,[35,36] it decreases the net charge and can reduce antifungal activity.

The substitution of Trp_{10} by the unnatural residue Dip (peptide 2) induced a slightly stronger antifungal activity against *A. niger* and *P. expansum* and a change in the structure of the peptide. Dip increased the hydrophobicity of the peptide, probably resulting in higher antifungal activity. In addition, Dip possesses additional side chains involved in interactions such as hydrogen bonds (HBs) and salt bridges. Indeed, Dip interacts more efficiently than Trp with Arg to facilitate the insertion into the fungal membrane as revealed by the better membrane permeabilisation of *F. culmorum* hyphae. However, the presence of only one Dip residue did not change drastically the antifungal activity of the peptide, as observed for histatin P-113 derivatives.[37] Unnatural amino acids, such as 1-Bzl, Dab, and Nal, are also used to increase salt stability,[37,38] rigidity,[39] and antimicrobial[40] potency. However, these substitutions can lead to a higher membranolytic activity, resulting in cytotoxicity to normal and cancer cells and haemolysis.[21,38] Generally, the position and size of a bulky aromatic residue is critical. Its hydrophobic or charged side chains and their fit in position on the peptide affect the overall structure of the peptide, which regulates its activity. In addition, the additional side chains and the resulting HB and salt bridges influence the binding of peptides with membranes and other compounds.[41] These bridges also direct the lifetime of peptides/membrane interactions.[41] A common way to increase lifetime of peptides and serum stability is the blockage of terminal functional groups via cyclisation. Thus, cyclisation of the short peptide RRWWRF maintained its antibacterial activity in serum.[35] However, cyclisation of short peptides might restrict peptide flexibility inducing limited activity. The lack of secondary structure in aqueous environment allied to the presence of defined structures in hydrophobic environment was added to the list of determinants of antifungal activity of small peptides. The presence of a cationic residue on the non-polar face and the role of the self-association (to a certain extent) in membrane also regulate the activities of the peptides.[13,16]

The replacement of the Pro_{10} by Arg_{10} (KK14(R)) increased the antifungal activity by 2- to 4-fold. In addition to an increase in cationicity, KK14(R) acquired a more defined structure with an increase in α-helical content, as highlighted by CD spectroscopy and in silico predictions. Pro is recognised as an α-helix breaker because of its inability to establish HB, with the absence of side chains. The breaking ability of Pro is shown by the undefined spectrum at 190 to 200 nm. The higher cationicity and helical content are associated with the increase of antimicrobial activity and explains the higher toxicity of KK14(R).[42,43] With mirror structures, the L- and D-enantiomers showed an overall similar antifungal activity as demonstrated for other synthetic peptides.[44-46] This indicates that KK14 and its D-enantiomer do not use a specific receptor to inhibit fungal growth but rather use, at least partially, a membrane-permeabilising mechanism. Although D-enantiomerization of a single residue can reduce haemolytic activity, full enantiomerization is usually more advised to avoid the loss of activity and structural deficiencies of the peptide as shown with polybia-MPI.[47,48] Interestingly, L and D-enantiomers of thanatin display the same antifungal activity, although the D-enantiomer is much less active against Gram-negative bacteria.[49]

A major drawback of the use of CAPs is their sensitivity to high concentrations of salt. Mono- and divalent cations are thought to act

either on the structure of the peptide and/or decrease electrostatic interactions with microbial membrane. KK14 and its analogues were sensitive to salt increase, with a high reduction of their antifungal potency in KCl and $CaCl_2$ solutions. However, the peptides remained fully active in 50 mM KCl solution and partially active in 150 mM KCl and 1 mM CaCl2, which may indicate that their high cationicity (+6) and their hydrophobicity are sufficient to ensure antifungal potency.[50] Interestingly, KK14 appeared as the most sensitive peptide to the salt increase. The substitution of Trp residues with unnatural residues, such as β-naphthylalanine (Nal), has been successfully applied to increase salt stability of the histatin derivative P-113.[38] However, the number of Nal and their position appear critical to confer salt stability.[37] pH changes appeared to affect the activity of the peptides, with a stronger activity at low pH. Despite a high isoelectric point (pI), a basic solution (pH 9.5) partially altered the activity of the peptides, with a full abrogation of KK14(Dip) antifungal activity (up to 25 μg/ml). The better activity at a low pH enhances the single net charge of the residues, favouring electrostatic interactions. However, a low pH has been shown to be responsible for supplementary side chains interactions and thus changes in secondary structures, responsible for a decrease in activity. The peptides showed moderate stability after heat treatment in an aqueous environment because of the probable flexibility of the peptide and the absence of secondary structure, as highlighted by the CD spectra. Bigger AMPs are generally stable after heat treatment because of the presence of stabilising structural elements, such as disulfide bonds.

The four peptides modified the permeability to the plasma membrane of *F. culmorum* mycelium. Despite their short size, other peptides have been shown to retain their ability to disturb phospholipidic membrane[39] usually correlated with a high proportion of helical content and high amphipathicity. Indeed, a length of 22 residues is generally recognized as a minimal size for an α-helix to penetrate plasma membrane, which could indicate that the peptides are unable to penetrate the membrane as monomers. After a correct orientation and a minimal concentration, peptide is thought to induce membrane permeabilization.[39] As shown for V13-K, self-association appears essential to induce activity.[16] However, additional mechanisms, such as membrane depolarization, may be involved.

KK14 and analogues were either little or not haemolytic, suggesting that the activity of the peptides was quenched by the salt in PBS. Their low amphipathicity (0,175-0,184) of the peptides has also been correlated to a low haemolytic activity of the peptides. The presence of the polar residue on the hydrophobic face of the peptides, particularly the predicted helical-rich KK14(R), limits a high amphipathicity and is generally used to lower the haemolytic properties of AMPs.[51] However, KK14(Dip), kk14 and KK14(R) showed toxicity towards colonic cell lines Caco-2 and macrophages RAW 264.7. The percentage viability of macrophages after incubation with the peptides KK14(Dip), kk14 and KK14(R) at 200 μg/ml was 29.8%, 13.5%, and 5.8%, respectively. The phagocytic macrophage cells initiate the immune response, since they are some of the first cells to be exposed to invading microorganisms. When exposed to inflammatory stimuli, they secrete low molecular weight cytokines which possess a chemotaxis role among immune and non-immune cells and which mediate the unleashing of an effective immune response. The toxicity of these peptides may deregulate the secretion of the numerous cytokines, which will have a detrimental effect on the inflammatory response. Interestingly, a 13-mer peptide (KKFFRAWWRRFLK-NH_2), with a higher amphipathicity (0.729), showed higher cytotoxicity (data not published). Aggregation of KK14 and KK14(Dip) with serum proteins might explain the absence of activity, although interaction of molecules with the fungal membrane might limit the activity of these two peptides.[39] The increase in net charge and/or the helicity content of the peptide are thought to explain the cytotoxicity of KK14(R). Indeed, perfect helices are usually more cytotoxic than less structured peptide. However, the toxicity of KK14(R) must be limited by its sensitivity to proteases in contrast to kk14, which retained its antifungal activity after trypsin treatment. The use of D-enantiomers are common ways to improve the resistance of peptides to proteolytic digestion.[39,52] Trypsin usually cleaves preferentially at the C-terminal of Arg or Lys, which are protected in D-form of residues. The proteolytic resistance of kk14 is a drawback for its application as food preservative, Because of its potential stability in the gut environment and cytotoxicity.

In conclusion, the novel peptide KK14 inhibited the growth of food spoilage fungi and presented essential stability and safety parameters to be considered as a food preservative. However, as highlighted by a challenge test in an apple juice inoculated with *A. niger*, its application might be limited by the presence of other food ingredients, such as sugars. Interactions between peptide and food components, such as lipids, proteins, and sugars are a common hurdle to the food applications of AMPs. As such, the preincubation of the AMP dermaseptin S4 derivatives in apple juice hampered by 2- to 8-fold its activity against *Escherichia coli* O157:H7 compared with standard incubation conditions.

Peptide-based strategies have been developed to respond to the increase of invasive fungal diseases over the past 50 years while preventing the undesirable side effects of current prolonged treatments and resistance to the common antifungal agent amphotericin B. These modifications such as cyclization,[35] backbone modification (eg, β-peptides) or delivery modes (eg, liposomes, phosphate groups) aim to improve the stability and the half-life of small peptides. Peptide-based vaccine strategies against major fungal pathogens[53] and the development of peptibodies[54-57] are also considered. In this study, similar peptide-based strategies were used to generate KK14 derivatives with enhanced abilities to inhibit the food contaminants, which are reportedly involved in invasive fungal diseases. Thus, the D-enantiomer kk14, because of its trypsin resistance, could represent an interesting candidate for therapeutic applications, where proteolytic sensitivity of small peptides is a major issue. Although an extended characterization of its antifungal spectrum and in vivo studies are necessary, this study highlights the potential use of KK14 and its derivatives in therapeutic application or food preservation.

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