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Natural Antifungal Peptides/Proteins as Model for Novel Food Preservatives

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Abstract: A large range of ingredients for food and food products are subject to fungal contamination, which is a major cause of destruction of crops, exposure of animals and humans 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 for food 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 to commonly used food processes, 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.

Keywords: antimicrobial peptides, food preservative, food spoilage, peptide design, plants

Introduction

Fungi (molds and yeasts) play a major role in the food industry. They are involved, for example, in the manufacturing 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 one in 10 people worldwide every year (WHO [World Health Organization], 2015). Foodborne diseases are defined as a “result from the ingestion of contaminated foods and food products and include a broad group of illnesses caused by parasites, chemicals, and pathogens which contaminate food at different points in the food production and preparation process” (WHO, 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 (Stenmarck, Jensen, Qusted, & Moates, 2016). This loss must be added to another

third of the food production wasted every year, which represents 1.3 billion tons 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 to 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, Shattuck, Altieri, Herren, & Gliessman, 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 food-spoilage pathogens occurs over the range of pH 4 to 8, whereas spoilage yeasts and molds can survive and expand in the pH range of 2 to 5 (Booth & Stratford, 2003). In addition to health issues, due to the production of mycotoxins, fungi lead to deterioration of flavors and appearance (Oliveira, Mauch, Jacob, Waters, & Arendt, 2012). Mold and yeast spoilage are a serious issue of baking and beverage industries.

Losses of bakery products due to mold spoilage vary between 1% and 5%, 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*, and

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Table 1–Main fungal contaminants of bakery products and product defects.

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

Table 2–Main fungal contaminant of beverages and product defects.

Spoilage agents		Properties of colony and product defects
Moulds	<i>Aspergillus</i> spp. <i>Cladosporium sphaerospermum</i> <i>Fusarium oxysporum</i> <i>Eupenicillium brefeldianum</i> <i>Mucor</i> spp. <i>Neosartorya fischeri</i> <i>Penicillium glabrum</i> <i>Rhizopus stolonifer</i> <i>Talaromyces flavus</i> <i>Mucor</i> spp.	Mycelial white to grey mats, discoloration, swollen packages Musty, stale
	<i>Aureobasidium pullulans</i> <i>Candida</i> spp. <i>Cryptococcus</i> spp. <i>Debaryomyces</i> spp. <i>Dekkera</i> spp. <i>Kluyveromyces</i> spp. <i>Pichia</i> spp. <i>Rhodotorula glutinis</i> <i>Saccharomyces</i> spp. <i>Schizosaccharomyces pombe</i> <i>Zygosaccharomyces</i> spp.	Haze, clouds, surface films, swollen packages Abundant CO ₂ production Yeasty, aldehyde, vinegar, pineapple note flavours

Clostridium botulinum have been implicated in baking products foodborne illnesses, mold and yeast growth is by far the major factor limiting shelf life of high and intermediate bakery products (Smith, Daifas, El-khoury, Koukoutsis, & El-khoury, 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 (Battay, Duffy, & Schaffner, 2002). Among 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 common molds involved in bread spoilage (Tables 1 and 2). 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 colonization 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, mold and yeast spoilage cause undesirable odors (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 & 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 CO₂ production, turbidity, and the production of unpleasant flavors (Hernández et al., 2018). *Dekkera bruxellensis*, *Debaryomyces hansenii*, *Issatchenkia orientalis*, *Kloeckera apiculata*, *Pichia membranifaciens*, *Zygosaccharomyces bisporus*, *Saccharomyces* spp., and *Schizosaccharomyces pombe*, are commonly associated with soft drinks spoilage (Pitt & 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, Alvarenga, Sant'Ana, & Sabaa Srur, 2016). However, most molds

are aerobic and therefore inhibited in low oxygen tension environment. The presence of visible mycelium, discoloration, off-flavors 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 (Hernández et al., 2018; Kregiel, 2015; Pitt & 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 mold growth on baking products also include physical treatments (for example, UV light or IR radiation; Melini & Melini, 2018). Drinks containing fruit juices and teas, containing no chemical preservatives, are usually pasteurized at temperatures below 90 °C. The combination of low pH and heat treatment greatly 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, and 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 induces 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, Barreiro, Morales, & Ferreira, 2014).

The most common used preservatives of baking industry are propionic and sorbic acid (and respective salts). Their efficacy as mold inhibitor has been shown, especially against *Aspergillus niger* and *Penicillium* species (Melini & Melini, 2018; Saranraj & Geetha, 2012). The levels of sorbate used in bakery products ranges from 0.001% to 0.3% 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 flavor. 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 to 203), benzoates (E 210 to 219), and dimethyl dicarbonate (DMDC) (E 242) are permitted in ready-to-drink beverages in Europe. Benzoic acid (E210) occurs naturally in certain fruits (for example, 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 behavior (Beezhold and others 2014). DMDC and sulfites 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 mold 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, & Mor, 2006). Plants, animals, and microorganisms are potential sources of natural antimicrobials (Gyawali & Ibrahim, 2014). Several natural antimicrobials are recognized as safe and could represent a new alternative to traditional chemical preservatives. However, a limited bioactivity spectrum and/or adverse changes in organoleptic characteristics have been noticed with these natural antimicrobials (Ahmed, Singh, Kour, & Gupta, 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.

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 (Cederlund, Gudmundsson, & Agerberth, 2011; Ganz, 2003). Antimicrobial peptides are small, mostly cationic, peptides widespread within the immune system of the different prokaryotic and eukaryotic kingdoms (Adem Bahar & Ren, 2013; Beisswenger & Bals, 2005; Hassan, Kjos, Nes, Diep, & Lotfipour, 2012; Wiesner & Vilcinskas, 2010). These molecules have been the focus of recent reviews, especially their antimicrobial action (Jenssen, Hamill, & Hancock, 2006; Siedler, Balti, & Neves, 2019; Wiesner & Vilcinskas, 2010), their involvement in inflammatory diseases (Arias et al., 2017; Felício, Silva, Gonçalves, Santos, & Franco, 2017), and their immunomodulatory activity (Hancock, Haney, & Gill, 2016). The term AMP is commonly used to design antimicrobial peptides, for example, 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 developing novel specific antifungal and antibacterial drugs against emerging treatment-resistant infections (Matejuk et al. 2010) and also on a

biotechnological use of AMP genes to create genetically modified crops (Lacerda, Vasconcelos, Pelegrini, & Grossi de Sa, 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; CS $\alpha\beta$ peptides composed 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 and random structured small peptides. Other classifications based on covalent bondings, hydrophobicity, net charge, or targets have been proposed to improve peptide design (Wang, 2015). The peptide net charge is controlled intrinsically by the presence of polar, positively (Arg, Lys, His) or negatively (Glu, Gln, Asp) charged residues. A positive net charge (cationicity) is generally associated with a strong interaction with the negatively charged cell 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 of the cell membrane.

In this review, AFPs are presented on basis of their origin with a particular interest on AFPs from plants, whose extracts are more likely to be accepted for food applications. In addition, most of AFPs isolated from plants have been shown to be noncytotoxic, unlike peptides isolated from invertebrates.

The diversity of AFPs is also characterized by different sensitivities to salt concentrations, pH variation, and heat treatment. The comparison of peptide amino acid sequences and secondary structures has led to a better understanding of requirements for antifungal activity (Shagaghi et al., 2018). Thus, efficient peptides with improved characteristics, such as solubility, stability, or toxicity, have been developed in a time-effective manner. Currently, approximately 1200 antifungal peptides can be identified in different databases (antimicrobial peptide database apd3, collection of antimicrobial peptides camp, data repository of antimicrobial peptides dramp), and many others are reported in patents recently submitted.

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 new food preservatives. Peptides and proteins that are active only against *C. albicans* were deliberately omitted as these have previously been thoroughly described by others (De Lucca & Walsh, 1999; Desbois, Tschorner, & Coote, 2011; Jenssen et al., 2006; Matejuk et al., 2011). In addition, many AFPs, particularly those originating from insect venom, are not discussed due to a too high hemolytic activity/cytotoxicity, making them irrelevant as food preservatives.

Plant Sources of AFPs

Due to environmental stresses and constant threats of pathogen attacks, plants have adapted by developing a broad range of defense tools. Facing pathogen invasion, plants are able to generate pathogenesis-related (PR) proteins in different plant organs. At least 17 different families have been created to group these PR proteins, which possess a wide range of defense-related properties (Christensen et al., 2002; van Loon & van Strien, 1999). Several of these families, such as plant defensins (PR-12), thionins

(PR-13), and lipid transfer proteins (PR-14), display broad antimicrobial activities (Selitrennikoff, 2001) and belong to the class of AMP. The sequences and structural motifs of the different plant AFP families are presented in Table 3 and Figure 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, Del Sarto, Silva, Franco, & Grossi-De-Sa, 2011). They also share mechanisms of antifungal activity, such as the ability of binding to specific domains on the fungal plasma membrane (Thevissen, Francois, Aerts, & Cammue, 2005; Thevissen, Ferket, François, & Cammue, 2003). The main mechanisms of plant AFPs are summarized in Figure 2. In addition to their broad antifungal activities (presented in Table 4), the antibacterial activity of AMPs from plants (Nawrot et al., 2014; Tam, Wang, Wong, & Tan, 2015), and fruits (Meneguetti et al., 2017) has been reviewed elsewhere and will not be discussed further in the current review.

Thaumatococcus-like proteins

Antifungal thaumatococcus-like proteins (TLPs) are produced by diverse plant species such as barley (Van Der Weerden, Bleackley, & Anderson, 2013), and chestnut (Garcia-Casado et al., 2000). **Thaumatococcus** is a 22-kDa protein, stabilized by eight 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 to 26 kDa proteins, with 16 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. and *S. cerevisiae*. **Osmotin** and **zeamatin**, expressed in tobacco and maize, respectively, are among the most well described TLPs. They provide protection toward abiotic stresses and invading phytopathogens. Osmotin inhibits the growth of *S. cerevisiae* (IC_{50} = 0.3 μ M; Yun et al., 1998), *Fusarium solani*, and *Fusarium oxysporum* (IC_{50} = 67 μ g/mL; De Freitas et al., 2011) by using different mechanisms depending on the fungal targets. TLPs' 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* (Yun et al., 1997). Unlike zeamatin, which binds to β -glucans, osmotin docks specifically on cell wall phosphomannans to facilitate diffusion across the cell wall and subsequent cell damage (Ibeas et al., 2001). TLPs demonstrate variable activities against fungal species, which has been attributed to specific composition of fungal cell wall and membrane. In addition to the low concentration required for their activity, the structure of TL proteins imparts a great stability to heat- and proteolytic treatment of under varied pH (Roberts & Selitrennikoff, 1990; Smole, Bublin, Radauer, Ebner, & Breiteneder, 2008).

Thionins

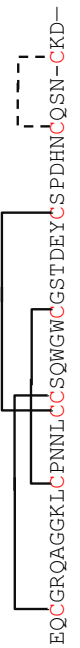
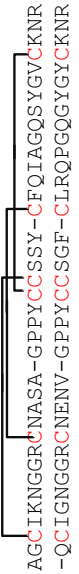
Thionins are isolated from various plant species including barley and cowpea. All thionins are present in almost every crucial plant tissue from endosperm to leaves. These ubiquitous small proteins of around 5 kDa contain a high proportion of Arg, Lys, and Cys residues, and are positively charged at neutral pH. They are

Table 3—Families and sequences of antifungal peptides/proteins isolated from plant sources (obtained from apd3) and discussed here. Solid lines indicate permanent disulfide bonds found in the peptide group. Stippled lines represent additional disulfide bond, which is specific to a peptide.

Peptide group	Peptides	Sequence
Thaumatin-like proteins (TLPs)	Thaumatococin	ATFEIVNRCSYTVWAAASKGDAALDAGRQLNSGESWTINVEPGTKGGKIWARTDCYF
	Osmotin	ATIEVRNNCPYTVWAASTPI-----GGRRLLDRGQTWVINAPPGTKMARVWGRTDCNF
Thionins	Viscotocin A3	 KSCCPNTTGRNLYNACRLTGAP-RPTCAKLSGCKIISGSTCPSPDYPK
	Crambin	TTCCPSIVARSNFVCRLLPGTP-EALCATYTGCIIPGATCPGDYAN
	Pth-St1	RNCESL-SHRFK---GFCRDSNC
	α-purothionin	KSCCRTTLGRNLYNLCPSRG-A--GKLCSTVCRCKLTSFLSCPCKGFPK
Defensins	NaD1	---REC-KTESNTFPGIITKPPCRKAIS---EKFTDG-HISKI---LRRCLCTKPC
	Rs-AFP2	--QKLC-QRPSGTWSGVCNNNAKNCQIRLE--KARHG-SCNYVFPAAHKCIYFPC
	MsDef1	---RTC-ENLADKYRGCFSG--CDTHCTTKE--NAVSG-RCRDD---FRCWCCKRC
		 -MTCCGVQGNLAQCIG-FLQK-GGVVPPSCCTGVKNILNSRRTTADRRAYCSCLKAAAGAVRGINPNNAEALPGKGVNIYKISTBTNSIN -VTCGQIQGVVNCILP-YLQN--RGPLRCCTGVKIDLLKCKTPHERRKSCRCVKTAANTIKIGIDFGKTAGLSGVCVKIPEIISPSVDCKVK ALSCTVSGNLAACTIG-YLTQ--GFLPRGCTGTNLNNMARTTDDRQAACRLVGAANAFPTLNAARAAGLPKACGVNIYKISTBTNSIN
Snakins	StSN2	YKKIDCGACCAARCRLLSSRPRLCNRACTGTCCARCNVPPPGTSGNTETCPYASLTTHGNKRKCP
	Snakin-like	LQIDCNACGACRLLSSRPRLCKRACGTCCQRCNCPVPGTSGNQEMCPYASLTTRGGKRKCP
2S albumins*	<i>C. annuum</i>	P-QCPCSQQFQQAQLRSCQS
	<i>B. napus</i> (napin)	PFRIKCRKEFQQAQHLR-ACQQ
	<i>R. sativus</i>	P-R-QRCQKEFQQAQHLR-ACQR
	Pf2	-PSERCRRQMGGQDFE - - - - -
	Pe-AFP1	QSERFEQQMQGQDF-SHDERFLSQAA PFRIKKRQGPFERP

(Continued)

Table 3--Continued.

Peptide group	Peptides	Sequence
Puroindolines*	PIN-a PIN-b	AQCPVETKLS-CRNYLLDRCSMTKDFVTWRWKWKGGCQELLGE-CCSRLGQMPQCRCNIIQGSIQGDLGGTFGQDRASKVIQFAKNILPPRCNQGPCCNI SQCPQERPKLSSCKDYVMERCFTMKDFPVTPPT-KWKKGCCHEVREKCKKQLSQIA PQCRCDSTRRVTQGRLLGGFLGIWRGEVFKQLQRAQSLPSKCNMGADCKF
Hevein-like peptides	Hevein	 EQCGRQAGGKLCNNLCCSQWGCSTDEY CSPDHN CQSN-CKD-
	Pn-AMP1	QQCGRQASGRLLCGNRLCCSQWGYCGSTASYCG--AGCQSQ-CRS-
	WAMP-1	QRCGDQARGAKCPNCLCCGKYGFCGSGDAYCG-AGSCQSQ-CRGC
	vH1	FQCGRQAGGARCSNGLCCSQFGYCGSTPPYCG-AGCQSQ-CA---
	GAFP	PTCSVLGDFK-CNPGRCCSKINYCGAC YQWRFGLT PAR
	EAFP-1	QTCASRCPR-PCNAGLCCSIYGYCGSGNAYCG-AGNCR CQ-CRG--
Knottins	PAFP-S Mj-AMP1	 AGCIKNGGRCNASA-GPPYCCSSY-CFQIAGQSYGYCKNR -QCIKNGGRCNENV-GPPYCCSGF-CLRQPGQGYGYCKNR
Cyclotides	Kalata B1 Circulin A	 GLPVCGETDVGGT(-NT--PGCTDSWPFV)TRN G-IPCGESCVWIPC-ISAALGCSCKNKVCYRN
α-Hairpinins	SmAMP-X2 ECAMP1	---VDPDVRAYCKHC-MSTRGDQ--ARKICESVCMRQD----- -----GSGRGS CRSQC-MRRHEDEPWRVQECVSQCRRRRGGGD-
	<i>Impatiens balsamina</i> peptides Ib-AMP1 Ib-AMP3	QWGRRCFGWGFGRRIQVVRWD -YRHRCCAWGPGRKYCKRWC

* : only large subunit was represented without disulphide bonds.

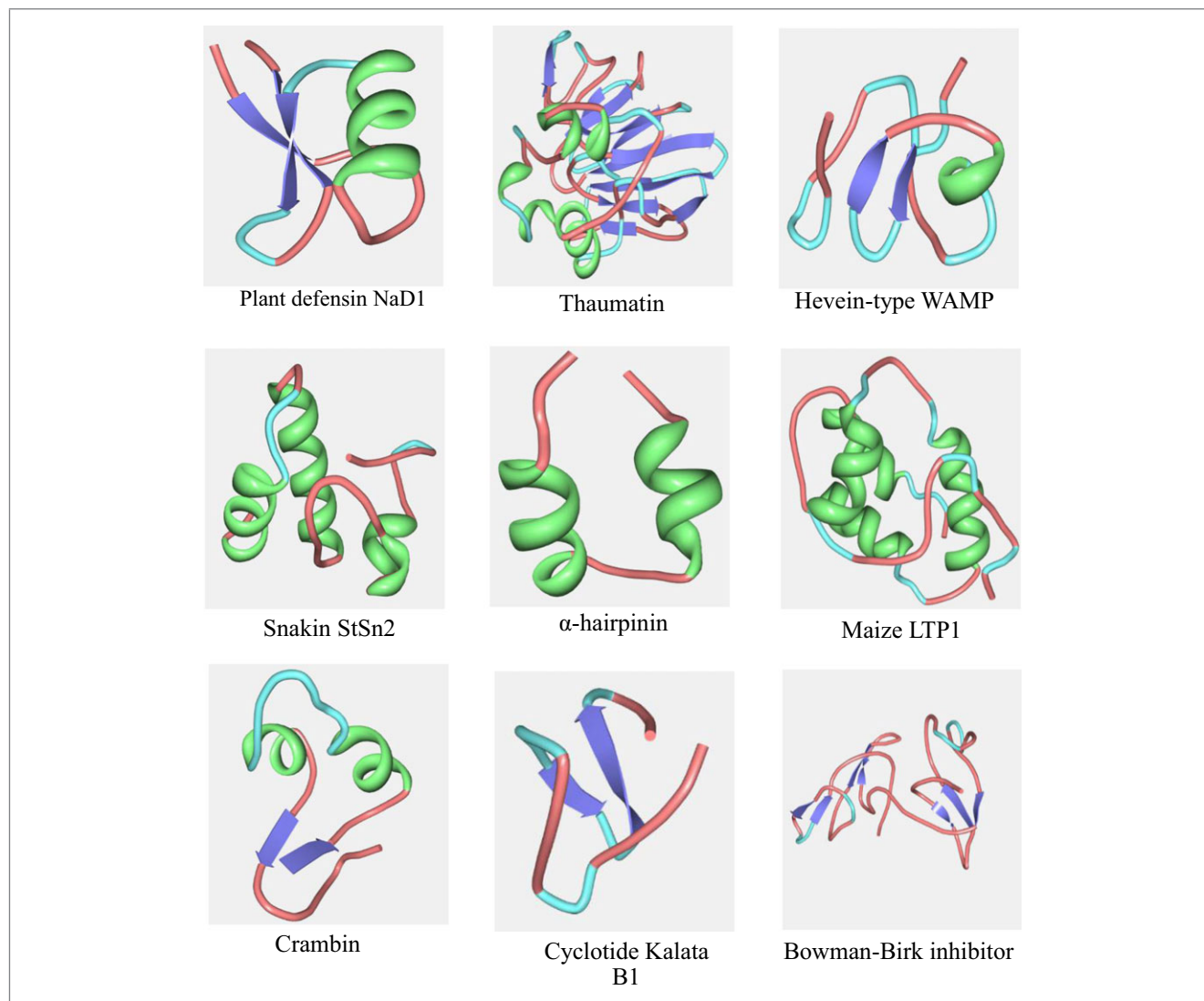


Figure 1—Structural diversity of plant derived antifungal peptides/proteins. Secondary structures are represented by green α -helices, purple arrows as β -strands, red random coil and blue turns. The 3D representations were from RCSB (<http://www.rcsb.org/>) and modified via ProteinWorks.

divided into two 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. Thionins consist of two antiparallel α -helices and one antiparallel double-stranded β -sheet connected by three or four 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 elements, possibly leading to lysis of the fungal cell membrane (De Lucca, Cleveland, & Wedge, 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 and inhibits acidification of the medium, ultimately 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 considered safe (Srikanth & Chen, 2016). 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, & 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.

Defensins

Plant defensins belong to the largest group of plant AMPs. These Cys-rich peptides (45 to 54 amino acids), with a mass of approximately 5 kDa, possess a characteristic CS α β motif, which comprises a β -sheet made of three strands, one 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 (Terras, Schoofs et al., 1992). However, unlike mammalian defensins, plant defensins possess a fourth disulfide bond joining the two termini, making 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). Sequence homology

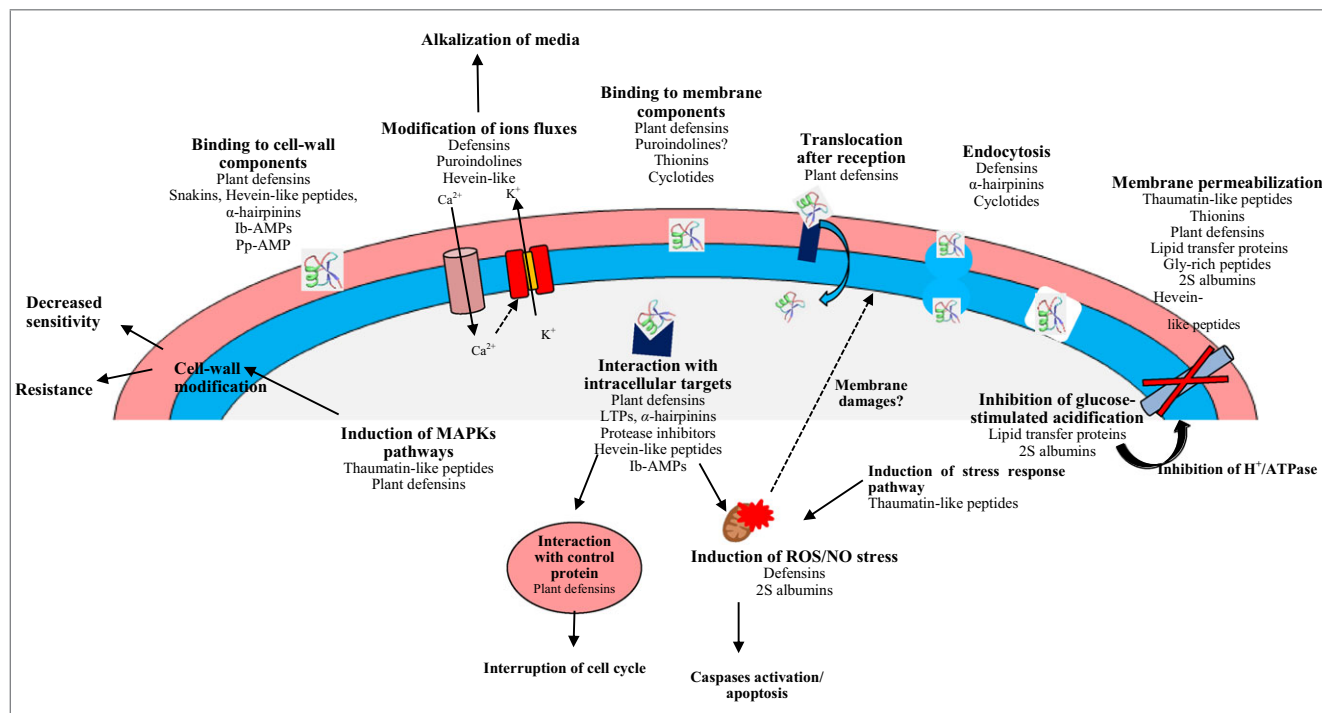


Figure 2–Diversity of mechanisms used by natural antifungal peptides isolated from plants. This representation is a modification of the figure (and described mechanisms) presented in the review by Van Der Weerden et al. (2013).

of plant defensins from different species is limited to a number of characteristically conserved residues: eight Cys, two Gly, one Glu, and one 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, & 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, & Wang, 2009). Although they are active mainly against phytopathogens, there is an increasing interest to combat fungal infection and biofilms formation from *C. albicans* in humans (Cools, Struyfs, Cammue, & Thevissen, 2017).

The radish defensin **Rs-AFP2** is one of the most well-characterized defensins. It is active against a broad range of filamentous fungi (Terras, Goderis et al., 1992). Rs-AFP2 binds to the sphingolipid glucosylceramide (GlcCer), permeabilizing the fungal membrane, and causing a Ca^{2+} influx and K^{+} efflux, which results in alkalization of the growth media (Vriens, Cammue, & 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 GlcCer to convey its antifungal activity against *Fusarium graminearum*. MsDef1 interacts with the fungal membrane, blocks L-type calcium channels, and activates MAPK signalling in *F. graminearum*.

MIP2C, a different sphingolipid than GlcCer, is also the binding site of the plant defensin **DmAMP1**, isolated from *Dahlia merckii*. DmAMP1 causes a Ca^{2+} influx, a K^{+} efflux, and alkalization of the growth media. *Fusarium* species are particularly sensitive to the tomato defensin, **TPP3**, and the tobacco defensin, **NaD1** (MIC = 1 μM) (Baxter et al., 2015; Van Der Weerden, Hancock, & Anderson, 2010). TPP3 permeabilizes the fungal membrane

after binding to phosphatidylinositol (4,5)-bisphosphate (PIP2) (Baxter et al., 2015). Following interaction with the fungal cell wall, NaD1 enters the hyphae via membrane permeabilization and kills by inducing 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 toward *Candida* spp., *Kluyveromyces marxianus*, and *S. cerevisiae* (Games et al., 2008; Salas, Badillo-Corona, Ramírez-Sotelo, & Oliver-Salvador, 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 have intracellular targets. For example, the antifungal activity of PsD1 is based on the halting of the 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, Bleackley, Anderson, & Lowe, 2018). Nevertheless, mutants of *S. cerevisiae* show resistance to the defensin **HsAFP1**. The action of this peptide requires a properly working yeast respiratory chain (Aerts et al., 2011; Thevissen, Kristensen, Thomma, Cammue, & François, 2007) and *S. cerevisiae* can generate spontaneous respiration-deficient mutants.

Plant defensins can generally maintain their activity in extreme temperatures, pH, and 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 the presence of KCl and CaCl_2 , respectively, whereas the inhibitory effect of Ms-Def1 is reduced in the 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 tissue. Land plants such as radish, barley, or onion contain

Table 4–Antifungal peptides/proteins isolated from plant sources. English (if common name) and *Latin* names of the peptides are specified.

Peptide group	Peptides	Source	Antifungal activity	References
Defensins	TPP3 Rs-AFP2 MsDef1 DmAMP1 NaD1 PvD1	Tomato, <i>Solanum lycopersicum</i> Radish, <i>Raphanus sativus</i> Alfalfa, <i>Medicago sativa</i> <i>Dahlia merkii</i> Tobacco, <i>Nicotiana tabacum</i> <i>Phaseolus vulgaris</i> cv. <i>Perola</i>	<i>Candida</i> spp. <i>Aspergillus</i> spp., <i>S. cerevisiae</i> <i>Fusarium</i> spp. <i>Fusarium</i> spp. <i>Fusarium</i> spp. <i>Candida</i> spp., <i>K. marxianus</i> , <i>S. cerevisiae</i>	Baxter et al., 2015 Terras, Schoofs et al., 1992; Thevissen et al., 1999 Sagaram et al., 2011 van der Weerden & Anderson, 2013 van der Weerden, 2010 Games et al., 2008; Salas et al., 2015
Thaumatococin-like proteins (TLPs)	Osmotin Zeamatin	Tobacco, <i>Nicotiana tabacum</i> Maize, <i>Zea mays</i>	<i>S. cerevisiae</i> , <i>F. solani</i> <i>S. cerevisiae</i> , <i>F. solani</i>	Abad et al., 1996
Thionins	Pth-St1 Viscotoxin A3 and B CaThi	Potato, <i>Solanum tuberosum</i> Mistletoe, <i>Viscum album</i> Chili Pepper, <i>Capsicum annuum</i>	<i>Fusarium solani</i> <i>F. solani</i> <i>Candida</i> spp. <i>S. cerevisiae</i>	M. Moreno, Segura, & Garcia-olmedo, 1994 Giudici et al., 2006 Taveira et al., 2016
LTPs	Ca-LTP1 Bc-nsLTP Ha-AP10	Chili Pepper, <i>Capsicum annuum</i> Mustard, <i>Brassica campestris</i> Sunflower, <i>Helianthus annuus</i>	<i>F. oxysporum</i> , <i>C. tropicalis</i> <i>F. oxysporum</i> <i>F. solani</i>	Diz et al., 2011 Lin et al., 2007 Regente & De La Canal, 2000
2S albumin	Pf2, Pef Bn-2S Ca-Alb	Passion fruit, <i>Passiflora edulis</i> <i>Brassica napus</i> <i>Capsicum annuum</i>	<i>F. oxysporum</i> <i>F. culmorum</i> , <i>F. oxysporum</i>	Pelegri et al., 2006 Ngai & Ng, 2004
Unclassified peptides <i>Impatiens balsamina</i> peptides (Ib-AMPs) Shepherins	Ib-AMP1-4 Shepherin1 and 2	<i>Impatiens balsamina</i> Shepherd's purse, <i>Capsella bursa-pastoris</i>	<i>F. culmorum</i> , <i>P. digitatum</i> <i>C. tropicalis</i> , <i>S. cerevisiae</i>	Thevissen, Francois, Sijtsma et al., 2005; Wang et al., 2009 Park et al., 2000; Remuzgo et al., 2014
Snakins	StSN1-2 Snakin Z Sn2	Potato, <i>Solanum tuberosum</i> Jujuba fruits Tomato, <i>Solanum lycopersicum</i>	<i>Fusarium</i> spp., <i>A. flavus</i> <i>A. niger</i> <i>S. cerevisiae</i>	Berrocal-Lobo et al., 2002 Daneshmand et al., 2013 Herbel et al., 2015
Knottins Cyclotides*	Mj AMP2 PAFP-s Kalata B1	Marvel-of-peru, <i>Mirabilis jalapa</i> American pokeweed, <i>Phytolacca americana</i> <i>Oldenlandia affinis</i>	<i>F. oxysporum</i> , <i>B. cinerea</i> <i>F. oxysporum</i> , <i>F. graminearum</i> <i>C. tropicalis</i> , <i>C. kefir</i>	Cammue et al., 1992; Gao et al., 2001 Tam et al., 1999 Tam et al., 1999
Hairpinins	Sm-AMP-X2 EcAMP-1-3	Common chickweed, <i>Stellaria media</i> Barley grass, <i>Echinochloa crus-galli</i>	<i>A. niger</i> , <i>F. oxysporum</i> <i>Alternaria alternata</i> , <i>A. niger</i>	Slavokhotova et al., 2014 Utkina et al., 2013
Hevein-like peptides	GAFF WAMP-1a and -b Vaccatides EAFP1-2 Pn-AMP1	Ginkgo, <i>Ginkgo biloba</i> Wheat, <i>Triticum aestivum</i> <i>Vaccaria hispanica</i>	<i>F. graminearum</i> , <i>F. moniliforme</i> <i>Fusarium</i> spp.	Wong et al., 2016, 2017 Odintsova et al., 2009 Huang et al., 2002
Puroindolines	PIN-A PIN-B	Wheat, <i>Triticum aestivum</i> Barley, <i>Hordeum vulgare</i>	<i>S. cerevisiae</i> , <i>F. culmorum</i> , <i>F. graminearum</i>	Charnet et al., 2003
7S globulins	Vicillin-like peptides MiAMP2c-3	<i>Capsicum baccatum</i> Macadamia nut, <i>Macadamia integrifolia</i>	<i>S. cerevisiae</i> <i>S. cerevisiae</i>	Marcus et al., 1999 Ribeiro et al., 2007
Gly-rich peptides Protease inhibitors (PIs)	Cc-GRP Kunitz -type Bowban-birk (BBIs)	Coffee, <i>Coffea canephora</i> Potato <i>Solanum tuberosum</i>	<i>F. oxysporum</i> <i>S. cerevisiae</i>	Zottich et al., 2013 Kim et al., 2009; Pesoti et al., 2015

*Sub-group of Knottins.

LTPs, in most tissues, either in seeds or leaves (Nawrot et al., 2014). LTPs are also important allergens in fruits or nuts (Egger, Hauser, Mari, Ferreira, & Gadermaier, 2010). In addition to their lipid carrier function, LTPs display antifungal activity. The two sub-families of LTPs, LTP1 and LTP2 have a molecular mass of 9 and 7 kDa, respectively. These proteins are made of four to five helices stabilized by four disulfide bonds and exhibit 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** (Regente & De La Canal, 2000). However, the mechanism is not fully elucidated. **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) (Cruz et al., 2010; Diz et al., 2011). Ca-LTP1 has been shown to use different mechanisms. For example, disruption of the fungal membrane was discovered in *C.*

tropicalis, and the ability to inhibit growth medium acidification was pinpointed when testing its activity against *S. cerevisiae* (Diz et al., 2011). Finally, 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 six 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 toward several phytopathogens such as *Fusarium* spp. and *A. niger* (IC₅₀ = 1 to 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, & Ebrahimi, 2013). Another 66-residue **snakin-2** (**Sn2**) found in tomato shows a

broad-spectrum activity, including against *S. cerevisiae* (MIC = 4.25 μ M). The mechanism underlying the bioactivity of Sn2 includes its agglomeration into single yeast cells prior to killing (Herbel, Schäfer, & 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 did not exhibit membrane disruptive effect on liposomes. There is still a lack of knowledge concerning 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 μ M and 100% at 35 μ M.

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. The coffee seeds Gly-rich peptide, **Cc-GRP**, (Zottich et al., 2013) inhibits the mold *F. oxysporum* and a broad range of yeasts (Mousavi & Hotta, 2005). It also alters the permeability of *F. oxysporum* hyphal membrane (Zottich et al., 2013) and prevents colony formation in yeasts (Mangeon, Junqueira, & Sachetto-Martins, 2010). This peptide seems to inhibit *F. oxysporum* via several mechanisms, as revealed by its ability to target the fungal cell wall as well as the nucleus. Although poorly studied regard to their antifungal activity or stability, new Gly-rich peptides are regularly discovered (Nawrot et al., 2014; Tam et al., 2018).

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 by exhibiting antifungal activity (Agizzio et al., 2003, 2006), as shown for 2S albumins isolated from *Malva parviflora* and radish (*Raphanus sativus*) seeds. The 2S albumins **Pf2** and **Pe-AFP1**, identified in seeds of *Passiflora edulis f. flavicarpa*, show activity against the molds *Aspergillus fumigatus*, *F. oxysporum*, and the yeast *S. cerevisiae* (Menegueti et al., 2017; Pelegrini et al., 2006). 2S Albumins are synthesized as a polypeptide of 18 to 21 kDa in response to pathogens, and cleaved into two peptides as a posttranslational modification (PTM). These two peptides represent the small (approximately 4 kDa) and large (approximately kDa) chain and present similar characteristics to other AFPs, such as richness in cationic residues and presence of disulfide bonds.

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 was identified for the 2S albumin, **Ca-Alb**, isolated from *Capsicum annuum* (Ribeiro et al., 2012). Cells treated with the 2S albumin **Pf-Alb** exhibit an apoptotic-like behavior, 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, which limits their use in food preservation (Moreno & Clemente, 2008).

The presence of eight 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 (Ribeiro et al., 2012). Among the 2S albumin family, **napins** show high water solubility, which is an interesting property for food applications. Napins have been first isolated from *Brassica napus* (rapeseed) and several napin-like peptides have been identified (Ngai & 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. Puroindolines show activity 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, Molle, Marion, Rousset, & Lullien-Pellerin, 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 the total seed proteins. Cysteine-rich peptides from 7S globulins are capable of inhibiting potential pathogens around the germinating seed (Marcus, Green, Goulter, & Manners, 1999). **MiAMP2c**, a homologous to 7S globulins, inhibits the growth of *S. cerevisiae* at a concentration of 50 μ g/mL (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 degradation of membranes and H^+ -pumps are associated with their antifungal properties (Bard et al., 2014; Ribeiro et al., 2007). However, the chitin-binding ability of 7S globulins does not appear to be a prerequisite since some 7S globulins can inhibit the growth of *S. cerevisiae* (Ribeiro et al., 2007).

Hevein-like peptides

Hevein-like peptides are named after the peptide hevein, due to amino acid sequence and structure similarities. Hevein is a small 4.7-kDa protein isolated from the rubber tree *Hevea brasiliensis*. It is characterized by a high content of Cys residues and chitin-binding abilities (Van Parijs, Broekaert, Goldstein, & Peumans, 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. They are divided in three subfamilies based on their number of Cys residues (6, 8, or 10), with eight 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 alkalization of the media, have been shown for the hevein-like peptide **GAFP**. Following interaction with chitin, this peptide, isolated from the leaves of *Ginkgo biloba*, inhibits several *Fusarium* species, namely *F. graminearum*, *Fusarium moniliforme* (Huang, Xie, & 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 to 30 $\mu\text{g/mL}$ (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 basicity (pI 12.02) and heat-stability. Moreover, the hevein-like peptides, **vaccatides**, from *Vaccaria hispanica*, maintain their antifungal activity after heat-treatment and under 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 (Huang et al., 2002).

Knottins

Plant knottins are part of a superfamily of peptides of around 30 residues. Knottins, most of which are linear, typically contain three conserved disulfide bonds, which form a cystine knot. However, their Cys motifs, and associated secondary and tertiary structures, differ among 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 **PAFP-S** pokeweed (*Phytolacca americana*) (Cammue et al., 1992; Gao et al., 2001). PAFP-S consists of an antiparallel three-strand β -sheet 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). Cyclotides are a sub-family of the knottins and known for their high stability. These circular peptides of 28 to 37 residues have been identified in bacteria, plants, and animals. A head-to-tail cyclical backbone and three intramolecular disulfide bonds confer them a supplementary stability. Cyclotides are classified into three subfamilies: Moebius cyclotides, which contain a 180° backbone twist, the bracelets cyclotides and hybrid Moebius cyclotides, also named kalatafirst isolated from *Oldenlandia affinis* (Pelegri, Quirino, & Franco, 2007). Coffee plants contain four cyclotides (kalata, circulin A and B, and cyclopsychotride) with activity against *Candida kefyr* (MIC 14.0 to 29.0 μM) and *C. tropicalis* (MIC 21.4 to 56.5 μM ; Tam, Lu, Yang, & Chiu, 1999). Membrane interactions between cyclotides and phospholipids, and a cell penetrating behavior, may be involved in the various biological activities (Greenwood, Daly, Brown, Stow, & Craik, 2007), but their mechanism of action remains unknown. The tricyclic-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)X₃C(2)X_nC(3)X₃C(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 (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* (Odintsova et al., 2009; Slavokhova et al., 2014). Antifungal activity of a barnyard grass *Echinochloa crus-gali* peptide, termed **Ec-AMP1-3**, has been demonstrated against several phytopathogens ($IC_{50} = 1$ to 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

PIs 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 defense 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**) (Kim et al., 2009; Pesoti et al., 2015). BBIs are cysteine-rich proteins of 1.5 to 20 kDa, with up to seven disulfide bonds, that possess inhibitory activities against proteases (Fernandez et al., 2007). **Kunitz-type inhibitors**, on the other hand, are 18 to 24 kDa peptides containing two disulfide 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 (Wang & 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 & 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 **Impatiens balsamina peptides (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-AMP1-4** are active against a broad range of fungal phytopathogens, including the mold *F. culmorum* ($IC_{50} = 1$ to 2 $\mu\text{g/mL}$) and the yeasts *Pichia pastoris* and *S. cerevisiae* (Thevissen, Francois, Sijtsma 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 & Anderson, 2013). However, the mechanism of action of Ib-AMPs is not clearly identified but involves binding to the cell wall.

Shepherdins are plant AFPs with a high basic character (net charge +8). Due to the presence of multiple histidine residues (Park et al., 2000) and the repetition of a Gly-Gly-His sequence motif, shepherdins have unique properties. **Shepherdin 1** and **Shepherdin 2**, isolated from the shepherd's purse, *Capsella bursa-pastoris* shepherdins, are active against *C. tropicalis* and *S. cerevisiae* but inactive against filamentous fungi (Park et al., 2000; Remuzgo et al., 2014). The mechanism underlying shepherdin's yeast inhibitory activity has not yet been identified. Other unclassified AFPs (not listed in Table 4) include peptides isolated from the Japanese bamboo, *Phyllostachys pubescens* (**Pp-AMP**) (Fujimura, Ideguchi, Minami, Watanabe, & Tadera, 2005), and 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. MiAMP1 is a 76-amino acids basic peptide, which has a particular Greek key motif that has also been found in yeast killer toxin.

Other Sources of AFPs

Plants have developed a specific defense 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 stabilized 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 (Faruck, Yusof, & Chowdhury, 2016; Jenssen et al., 2006; Lohner & Leber, 2016; Matejuk et al., 2011; Van Der Weerden & Anderson, 2013).

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 (Table 5).

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 against *S. cerevisiae* and *P. digitatum* than the parental protein (Fernandes & Carter, 2017; Kanyshkova, Buneva, & Nevinsky, 2001). Bovine and human lactoferricin adopt different folded structures, stabilized by disulfide bonds. The difference in the content of 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 to 32 μ g/mL; Lee et al., 2003). Several analogues of bovine cathelicidin-derived peptides, called **bovine myeloid antimicrobial peptide (BMAP)**, are effective inhibitors of *Candida* spp., *S. cerevisiae*, and the molds *Aspergillus* spp. and *Penicillium* spp. (MIC 2 to 32 μ M) (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 in food products is limited due to hemolytic activity at high concentrations, they are used as lead structures for less hemolytic peptides, such as CP-11 (Chan, Prenner, & 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 three categories based on their structural properties and cysteine spacing: α -, β -, and θ -defensins (Lehrer & Lu, 2012; Silva, Gonçalves, & Santos, 2014). β -Defensins contain 38 to 47 amino acids, with a disulfide linkage motif C1-C5, C2-C4, and C3-C6. Human β -defensins are active against *Candida* spp., including *C. tropicalis* and *Candida krusei*, with a MIC between 2 and 15 μ g/mL (Joly et al. 2004). The mechanisms involved in the antifungal activity of defensins remain to be elucidated.

Table 5—Antifungal peptides/proteins isolated from vertebrate sources.

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

It is suggested that the mechanism does not involve membrane disruption, as highlighted by calcein-release assays, calcein being retained by cells with intact membranes. 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 as a possible mode of action for β -defensins in *S. cerevisiae*.

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 human β -defensin 3 retains its activity against *F. culmorum* in the 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 toward fungi, especially against *Candida* species, including food-related species (Nikawa, Jin, Fukushima, Makihira, & Hamada, 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 heat-shock membrane protein Ssa2p, **histatin 5 (Hst 5)** is internalized and alters the integrity of the mitochondrial membrane (Puri & Edgerton, 2014). 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 mammals' liver and, hence, belong to the liver-expressed antimicrobial peptide (LEAP) family. Among these peptides, **hepcidins**, such as Hcp20/Hcp25 are active against *A. niger* and *S. cerevisiae* (Park, Valore, Waring, & Ganz, 2001).

Frog skin contains an important arsenal of AMPs, including brevinins and temporins. Brevins and temporins belong to the families of peptides identified in *Rana* frog species (Pál, Abraham, Sonnevend, Jumaa, & Conlon, 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 and 26 amino acids in length and are enriched in Gly and Ser residues. Magainins have antifungal activity against *C. tropicalis* (MIC, 12.5 $\mu\text{g/mL}$), *C. krusei* (MIC 12.5 to 25.0 $\mu\text{g/mL}$) 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 three. They have been identified in the skin of tree frogs of the genus *Phyllomedusa*. Dermaseptins are cidal to pathogenic fungi including yeasts and the mold *A. fumigatus* (De Lucca & Walsh, 1999; Morton et al., 2007). **Other AFPs from frog**, active against *Candida* spp. include bombinins, buforins, aureins, citropin 1.1. (Kamysz et al., 2006; Simmaco, Kreil, & Barra, 2009; Souza et al., 2013).

Invertebrate sources

Due to the absence of adaptive immune system, invertebrates need a potent first line of defense 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 5).

Thanatin is an inducible and nonhemolytic peptide of 21 residues isolated from the spined soldier bug *Podisus maculiventris*. This peptide has a single disulfide bond that forms a high positively charged C-terminal loop. Thanatin is fungicidal against several species, including the mold *F. culmorum* at a concentration below micromolar level, and *A. fumigatus* (MIC 10 to 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 molds (for example, *F. oxysporum*, MIC < 5 μM) and recent studies have revealed its anti-yeast activity (*S. cerevisiae*, MIC, 12 μM ; Zhang & 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 molds such as *Fusarium* spp. and *A. fumigatus*, and the yeast *P. pastoris* (Lamberty et al., 2001). Heliomicin's mechanism of action is analogous to that of 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, Bachelet, Raman, Rosengaus, & Sasisekharan, 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 (*Bombyx mori*). 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 to 100 $\mu\text{g/mL}$), and *F. oxysporum* and *F. moniliforme* (MIC, 12.5 $\mu\text{g/mL}$; De Lucca et al., 1998). 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 Arg-rich peptides called protegrins, which are isolated from porcine leukocytes (Silva, Daffre, & 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 two disulfide bonds. Its amphipathic nature is correlated to its ability to disrupt lamellar vesicles and suggests membrane permeabilization as its 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 known to inhibit *Fusarium* spp. or *Candida kefyr*, but the mechanism is still unknown. The presence of two disulfide bonds is thought to give tachyplesins resistance to low pH and high temperature.

Marine natural products have raised a great interest as novel antimicrobials (Choudhary, Naughton, Mont, Dobson, & Rai, 2017). **Penaeidins** are chitin-binding AFPs produced by the marine invertebrates, penaeid shrimps. These 47- to 63-residue peptides do not have a well-defined skeleton, but possess an N-terminal domain rich in Pro and an α -helical C-terminal domain comprising three disulfide bonds. Penaeidins appear to increase the hyphal branching of *Aspergillus* spp. and *F. oxysporum* (Destoumieux et al., 2000) and inhibit sporulation of molds.

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

The termite *Pseudacanthotermes spiniger* stores in its hemocytes the antifungal peptide **spinigerin**. Unstructured in an aqueous environment, this 25-residue peptide acquires an amphipathic α -helix under 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).

Microbial sources

Eukaryotic and prokaryotic microorganisms can produce AFPs and are known to play a competitive role in ecological niches (Table 5). Most of these peptides are isolated from mold and yeast species but are not related to AFPs. However, **fungal defensins** include plectasin from *Pseudoplectanina nigrella* and eurocin from *Eurotium amstelodami* (Silva et al., 2014). The molds *Aspergillus giganteus* and *Penicillium chrysogenum* are, respectively, able to secrete the antifungal proteins termed AFP and PAF (Silva et al., 2014). These proteins are active against *Fusarium* spp. and *A. fumigatus*, and are also able to inhibit the environmental mold species *Zygomycetes* sp. The peptide AnAFP produced by, for example, *A. niger*, shows anti-yeast activity (Silva et al., 2014). The antifungal mechanisms of AFPs from fungi are well characterized. These fungal AFPs act via interaction with receptors on the cell wall/membrane and induce signaling 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 or food applications is limited due to an extreme hemotoxicity (and not listed in Table 5). However, analogues of these peptides with low hemolytic activity have been created as will be described in more details later. The fungal lipopeptides, aureobasidins, produced by *Aureobasidium pullulans* are cyclic lipophilic depsipeptides composed of eight residues and one α -hydroxyacid (Takesako et al., 1993). Several members of this family are active

against *Candida* spp. (for example, aureobasidin A with a MIC of 0.05 to 0.2 $\mu\text{g}/\text{mL}$).

Killer toxins are small protein-based toxins secreted by so-called "killer" yeasts, 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, Ruiz, Alonso, Marquina, & Santos, 2017; Hatoum, Labrie, & Fliss, 2012; Izgu & Altinbay, 2004). 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, San Mauro, Bravo, & Marquina, 2009; Soares & 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, Corazzi, Turchetti, Buratta, & Martini, 2004). These toxins are able to withstand heat treatment and are active over a wide pH range. (Albergaria & Arneborg, 2016). However, yeast killer toxins usually possess high MIC values (250 to 1,000 $\mu\text{g}/\text{mL}$) against yeast species, which limits their use in food applications. Interestingly, a single mold, *Ustilago maydis*, is able to produce the killer toxin, KP4, which exhibits its fungistatic activity by blocking Ca^{2+} channels, and thereby inhibits Ca^{2+} -regulated pathways.

Some bacteria are also a good source of AFPs, either derived from ribosomal activity or from enzymatic cleavage of proteins derived from the growth environment (McNair, Siedler, Vinther, Hansen, & Neves, 2018; Siedler et al., 2019). 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 to 25 $\mu\text{g}/\text{mL}$) and *A. fumigatus* (MIC 5 to 40 $\mu\text{g}/\text{mL}$) (Sorensen, Kim, & Takemoto, 1996). The bacterium *Streptomyces tendae* is also able to produce the chitin-synthesis inhibitors, nikkomycins (R. K. Li & Rinaldi, 1999), and the non-bacteriocin protein AFP1, which are both active against the yeast *S. cerevisiae*. Nikkomycins are not active against filamentous fungi (Freitas et al., 2005). Several bacterial species express specific peptides with antifungal activity, such as cepacidines and flagellin (Lim, Suh, Kim, & Hyun, 1994; 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* and *Penicillium chrysogenum* (Gupta & 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 (Beauvais, Latgé, Beauvais, & Latgé, 2018). *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, Hassan, Bakar, & Saari, 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. Hence, the degradation of these peptides by proteases of the intestinal tract, when used as food additive, would limit their activity against intestinal flora and potential adverse effects on health. 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 eight peptides, which inhibit the bread spoilage fungus, *P. roqueforti*, with an 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, Cassone, Coda, & Gobbetti, 2011). The different mechanisms underlying the activity of LAB against fungal food spoilage have been recently reviewed by Siedler et al. (2019).

Mode of Action

Figure 3 summarizes the diversity of targets and mechanisms used by several AFPs to combat fungal pathogens. The antifungal activity of AFPs is generally peptide and species dependent. In addition, structural differences between yeasts and molds are usually cited as an explanation for differences in sensitivity to a particular peptide (Erwig & Gow, 2016). The internal environment of yeasts and molds is protected by a cell wall and a lipid membrane, each of which has distinctive differences (Figure 3). The cell wall has a protective role by limiting access to the cell membrane, and a functional role in cell mechanisms such as adhesion and signaling. The outer layer of fungal cell wall 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 of mannose residues, whereas the cell wall of molds can also contain glucose or galactose. The second cell wall 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 certain AFPs. Another characteristic element fungal membrane 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, & Thevissen, 2013; Hegedüs & Marx, 2013; Rautenbach, Troskie, & Vosloo, 2016; van der Weerden & 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 & Gorr, 2017; Nawrot et al., 2014; Sani & Separovic, 2016). These models are generally based on the activity of AMPs against bacterial membranes, but similar mechanisms are thought to occur within fungal membranes. AFPs' activity generally involves membrane-related mechanisms, but inhibition of enzymes or synthesis of cell wall components has been shown. Moreover, some peptides are able to induce the formation of ROS (Thevissen, Terras, & Broekaert, 1999) and damage nucleic acids. The combination of several mechanisms is

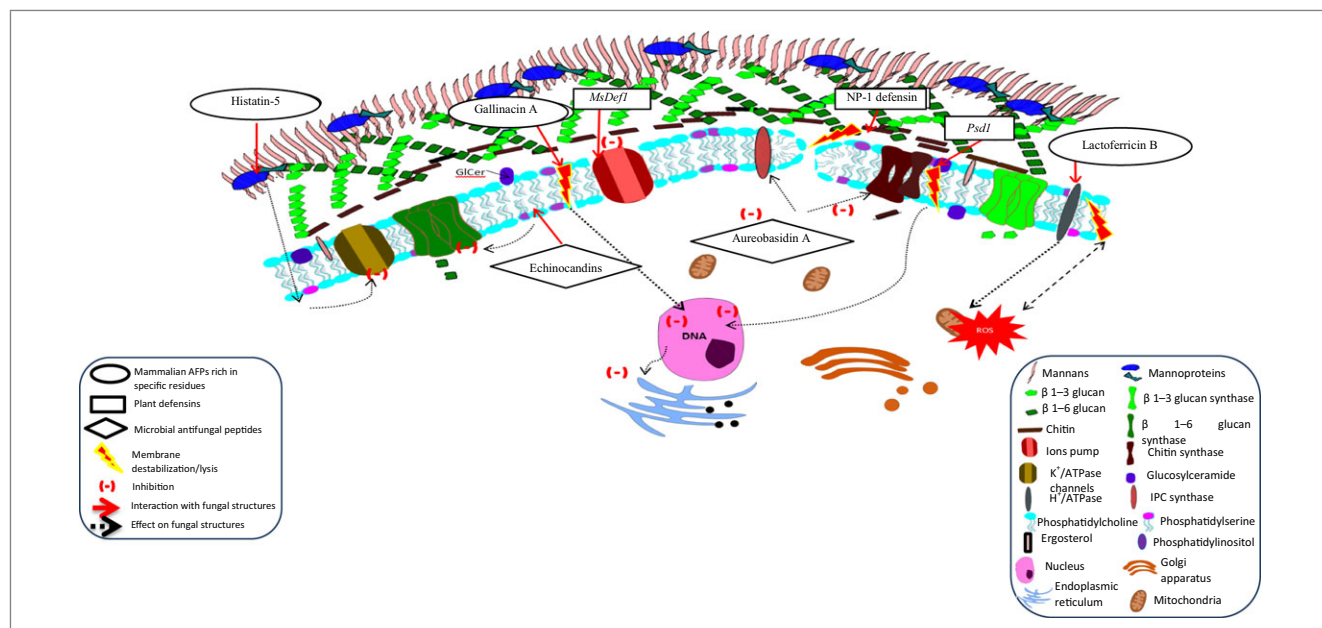


Figure 3–Diversity of fungal targets and antifungal mechanism of AFP derived from mammalian, plant, insect, and microbial sources. Histatin-5 (His-rich peptide) binds to Ssa1/2 protein, is internalized, and affects the activity of K^+ /ATPase channels; Gallinacin A (Arg- and Lys-rich peptide) binds to the phospholipid membrane, inducing membrane lysis; the peptide is then internalized and inhibits DNA synthesis and protein synthesis. Lactoferricin B (Trp-rich peptide) binds to the lipid bilayer or an 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 GICer, 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 et al., 2016).

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 signaling cascades, leading to cell death (Puri & Edgerton, 2014).

In comparison to traditional fungal inhibitors, such as azoles or polyenes, the plurality of mechanisms of action of AFPs against molds reduces the risk of developing resistance (Prema & Pruthvi, 2012). However, several fungi are able to withstand AFPs and develop resistance via the activation of signaling pathways related to cell wall modification or alkaline pH stress response (Kwon et al., 2016; Van Der Weerden et al., 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, among which poor solubility, poor resistance to environmental factors, and potential toxicity are conspicuous. A better understanding of the structural requirements for antifungal activity should enable such issues to be overcome. In addition, the development of novel synthesis technologies facilitates the design of synthetic peptides with an increased resistance to salt or serum, which can be produced in economically viable amounts.

Many AFPs presented in this review possess a broad spectrum of antifungal activity, against both molds 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 *C. 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 & Gomes, 2009). Although it shows strong effect on the growth of certain fungi, the radish defensin Rs-AFP2 is inactive at 100 $\mu\text{g/mL}$ against other species (Urdangarín, Norero, Broekaert, & De La Canal, 2000).

Unnatural Peptides

Natural peptides are a rich source of inspiration for the design of novel synthetic bioactive peptides, suitable for numerous applications (Bondaryk, Staniszevska, Zielińska, & Urbańczyk-Lipkowska, 2017). In recent years, different studies have focused on the biological activity of peptide fragments derived from precursor proteins. These latent peptides are called cryptides and can be derived from natural cleavage of proteins (for example, milk caseins degradation into peptides by LAB) or *in vitro* proteolysis (Iavarone et al. 2018). Many natural cryptides have been shown to display antifungal activities, lactoferricin (Lfcin) being one of the most studied (Fernandes & Carter, 2017). Synthetic derivatives, based on natural cryptides, can be prepared via chemistry methods, such as Fmoc chemistry. For instance, the hexapeptide (RRWQWR-NH₂) based on lactoferricin B, is active toward molds such as *P. digitatum*, via membrane permeabilization (Lopez-Garcia, Marcos, Abad, & Perez-Paya, 2004).

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, Wei,

Smith, Mashhoon, & Bobek, 2003). Examples of derivatives are presented in Table 8, including peptides from the cathelicidin family (for example, IB-367 (Barchiesi et al., 2007), omiganan KR-20 (Wang, 2008; Wiesner & Vilcinskas, 2010)), insect AFPs (for example, 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 (with an increased net charge or hydrophobicity, for example) 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 instability.

In addition to these natural-based derivatives, many synthetic peptides have been constructed via *de novo* synthesis (Table 6). Among these, PAF26 is a six-residue peptide with the sequence Ac-rkkwfw-NH₂, 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 (kkkkkkkkkaafaawaafaa-NH₂), and showed an MIC between 16 and 64 $\mu\text{g/mL}$ against *Candida* spp. (Burrows et al., 2006). The design that includes a high number of cationic and hydrophobic residues has been used in the construction of the Trp-rich peptide, Pac 525 (Wang et al., 2009), or (KW)n based-peptides (Gopal, Na, Seo, & Park, 2012), which are active toward *Candida* spp. and *S. cerevisiae*. An exhaustive list of antifungal cryptides and *de novo* synthetic peptides has been reviewed previously (Ciociola et al., 2016), hence only examples are given in Table 8.

For larger peptides, the use of recombinant organisms is more appropriate despite a low yield of production and the necessity of using strains that 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 posttranslational modifications, 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, & Kirti, 2008), has been overexpressed in tobacco. Several other defensins, expressed in recombinant plants and fruits presenting PTMs such as disulfide bonds, show similar activity to the native peptides (rPsd1, rTvD1) and pH

Table 6—Antifungal peptides isolated from invertebrate sources.

Peptide group	Peptides	Source	Antifungal activity	References
Tachyplesin/ polyphemusin	Tachyplesin-1	Asian horseshoe crab, <i>Tachypleus tridentatus</i>	<i>C. kefyi</i> , <i>C. tropicalis</i> <i>F. culmorum</i> , <i>F. oxysporum</i> , <i>A. fumigatus</i>	Nakamura et al., 1988; Rosa & Barracco, 2010
	Gomesin	Tarantula spider, <i>Acanthoscurria gomesiana</i>	<i>A. brassicola</i> , <i>A. fumigatus</i> , <i>Fusarium</i> spp., <i>S. cerevisiae</i>	Silva et al., 2000
Insect defensins	Drosomycin	Fruit fly, <i>Drosophila melanogaster</i>	<i>Fusarium</i> spp., <i>Candida</i> spp., <i>Aspergillus</i> spp.	Zhang & Zhu, 2009
	Termicin/Spinigerin	Termite, <i>P. spiniger</i>	<i>F. culmorum</i> , <i>S. cerevisiae</i> , <i>F. oxysporum</i>	Lamberty et al., 2001
Thanatin	Thanatin	Spiner soldier bug, <i>Podisus maculiventris</i>	<i>F. culmorum</i> , <i>F. oxysporum</i> , <i>A. fumigatus</i>	Fehlbaum et al., 1996; Koch et al., 2012
Cecropins	Cecropin A	Giant silk moth, <i>Hyalopora cecropia</i>	<i>F. oxysporum</i> , <i>F. solani</i>	DeLucca et al., 1997
Ponericin	Ponericin G1-6	Ant, <i>Pachycondyla goeldii</i>	<i>S. cerevisiae</i>	Orivel et al., 2001
Knottin-like	Tachystatin	Crab	<i>Pichia pastoris</i>	Van Der Weerden et al., 2013
Insect venom	Mastoparan	Wasp, <i>Sphodromantis viridis</i>	<i>F. culmorum</i> , <i>S. cerevisiae</i>	Moerman et al. 2002
	Mastoparan-S		<i>A. niger</i> , <i>A. fumigatus</i>	Zare-Zardini et al., 2015
Scorpion NDBP	Opisthoporin-1	South-African scorpion	<i>F. culmorum</i> , <i>S. cerevisiae</i>	Moerman et al. 2002
Long chain peptide	BmKbpb2	<i>Opisthophthalmus carinatus</i>	<i>F. culmorum</i>	Zeng et al. 2012
Short AMP		Chinese scorpion <i>M. martensii</i>		

Table 7—Antifungal peptides/proteins isolated from microbial sources.

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

Table 8—Examples of antifungal cryptides and *de novo* peptides.

Peptide	Source	Antifungal activity	References
MUC-7 12-mer	MUC-7	<i>C. krusei</i>	Situ et al., 2003
MUC-7 20-mer		<i>S. cerevisiae</i>	
P-113	Histatin-5 fragment	<i>C. kefyi</i> , <i>C. krusei</i> , <i>C. parapsilosis</i> , <i>C. tropicalis</i>	Rothstein et al., 2001
KR-20	Human cathelicidin LL-37	<i>A. niger</i>	Wang, 2008
Temporin-SHf Analogues	Temporin-SHf: FFFLSRIF-NH ₂	<i>C. tropicalis</i>	André et al., 2015
D4E1	Cecropin A: FKLRAKIKVRLRAKIKL	<i>Aspergillus</i> spp., <i>Fusarium</i> spp.	De Lucca et al., 1998
K18M	thanatin (8-21): KVPPIIYCNRRTGKQCRM	<i>F. culmorum</i>	Fehlbaum et al., 1996
IB-367	Synthetic protegrin-1 derivative	<i>C. krusei</i> , <i>C. tropicalis</i> , <i>C. parapsilosis</i>	Barchiesi et al., 2007
Omiganan	Synthetic indolicidin derivative	<i>C. krusei</i> , <i>C. parapsilosis</i> , <i>C. tropicalis</i>	Rozek et al., 2003; Wiesner & Vilcinskas, 2010
CP10A/CP11		<i>F. solani</i> , <i>F. oxysporum</i>	Gopal et al., 2012
(KW)n/RWn	Lys (or Arg)- and Trp-rich	<i>Aspergillus</i> spp.	Jiang et al., 2009
D-V13K	<i>De novo</i> peptide	<i>Rhizomucor</i>	
Pac525	Trp-rich peptide: Ac-KWRRWVRWI-NH ₂	<i>C. krusei</i> , <i>C. parapsilosis</i> , <i>C. tropicalis</i> , <i>S. cerevisiae</i>	Wang et al., 2009
kaxins	dF21-10K: kkkkkkkkkaafaawaafaa-NH ₂	<i>C. krusei</i> , <i>C. parapsilosis</i> , <i>C. tropicalis</i>	Burrows et al., 2006
PAF26/PAF32	Ac-rkkwfw-NH ₂	<i>Penicillium</i> spp., <i>F. oxysporum</i> , <i>S. cerevisiae</i>	López-García et al., 2000; Muñoz et al., 2007

and heat stability (Larentis et al., 2004; Meneguetti et al., 2017).

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 (**cationicity**) net charge, the **hydrophobicity**, and the distribution of the residues and associated **structure**, **length**, and **amphipathicity** (Sagaram, Pandurangi, Kaur, Smith, & Shah, 2011; Yount & Yeaman, 2004).

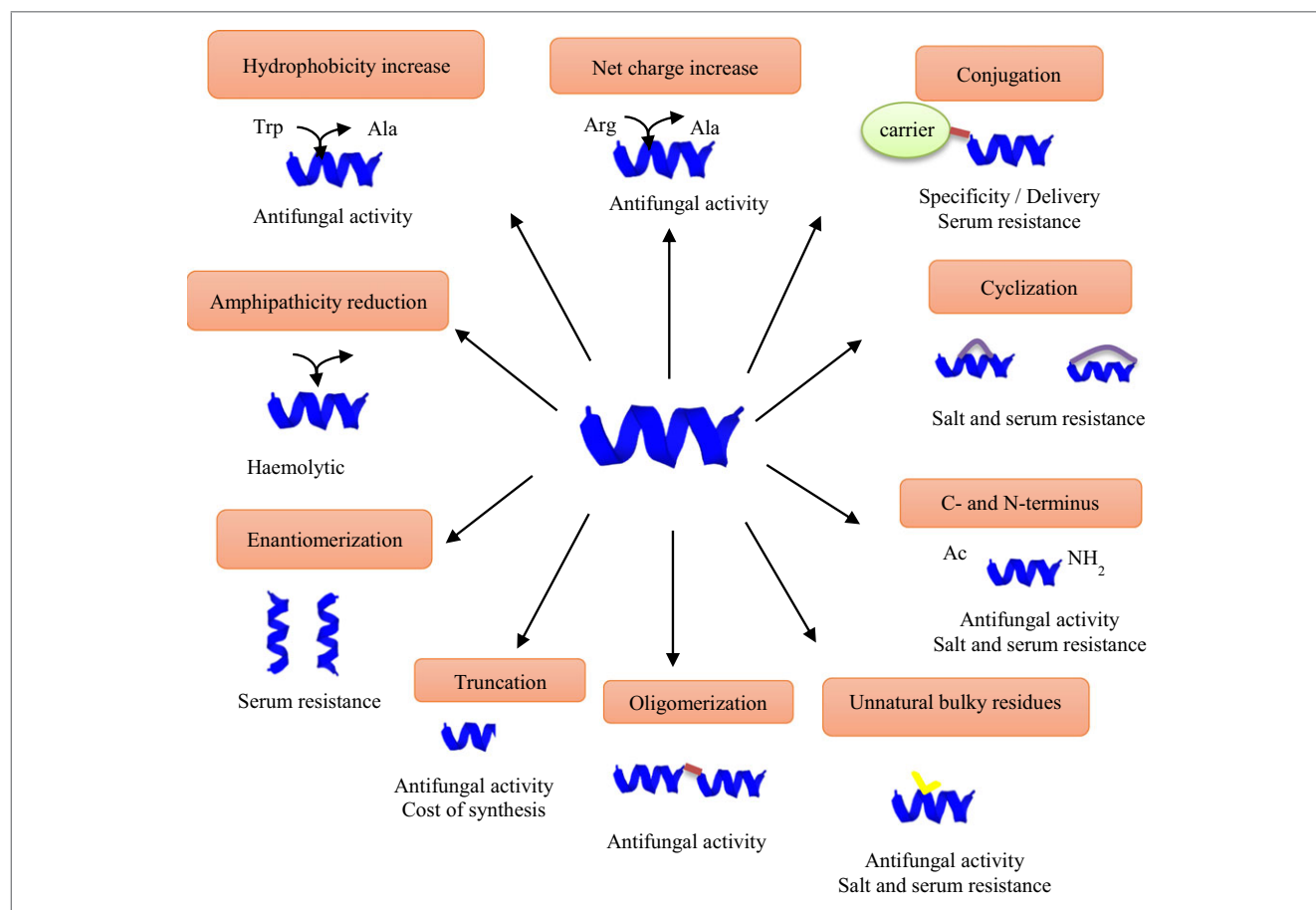


Figure 4—Common modifications used in AMP design and the properties it usually affects. For example, acylation and amidation of a peptide sequence are generally used to improve the resistance of a native peptide. Amino acid substitution is a common modification to highlight the role of a specific residue or to improve the antimicrobial properties of a peptide.

These properties can be modified in the design of optimized peptides (see Figure 4), including shorter sequences, higher stability to heat treatment and resistance in high salt or serum concentrations.

The first barrier faced by AFPs is the fungal cell wall. AFPs can either interact with the fungal cell receptors and/or are taken up to fungal membrane via transporters (Rautenbach et al., 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 penetration of the hydrophobic elements of the peptide into the bilayer. Hence, helicity, amphipathicity, and structure of the peptide are clearly involved in the potency of AFPs (Liu, Fang, & Wu, 2013). The following paragraphs will highlight the role of these parameters in the antifungal activity of peptides and how these parameters can be taken into account when designing potent new AFPs (Table 9).

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.

Increasing the number of basic residues in antifungal peptides, often improves their antifungal activity (André et al., 2015; Jin et al., 2016; Lum et al., 2015). In fungal or yeasts cell walls/membranes, this interaction with AMPs involves negatively charged components such as glycopospholipids (GPLs), sphingolipids, and mannoproteins (Figure 3). Thus, the insertion of Arg residues into the D-hexapeptide rktwfw-NH₂, increases its activity against *P. digitatum* (López-García, Gonzalez-Candelas, Perez-Paya, & Marcos, 2000). A similar modification has been shown to enhance the activity of a temporin-Shf analog against *S. cerevisiae* (André et al., 2015). On the contrary, the replacement of these basic residues in the hexapeptide has led to a loss of antifungal activity PAF26 (De Brucker et al., 2014; Muñoz, López-García, Pérez-Payá, & Marcos, 2007). 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 (Aliste, MacCallum, & Tieleman, 2003; Falla & Hancock, 1997). The replacement of Lys residues in the defensin TPP3 increases the solubility of this AFP, but 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 problematic (Gopal et al., 2012; Guo, Pan, Xiao, Chen, & Jiang, 2013). Due to their more dispersed charge and associated stability than Lys residues, the interaction of Arg residues with membrane surface via hydrogen

Table 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	Baxter et al., 2015
Arg → Lys	Tritrpticin	Loss of activity	Schibli et al., 2006
Arg→Lys	granulysin	Lower hemolytic activity	Li et al., 2005
Arg/Lys on hydrophobic face	Cathelicidin-BF derivatives	Disruption of α -helix	Jin et al., 2016
Leu11/Tyr18 ↔ Lys7/Lys14	RIVQRIKKWLLKWKGLGY	Lower hemolytic activity	Jin et al., 2016
Asn → Lys	KU2:	Increased hydrophobic moment	Lum et al., 2015
Val→Lys (non-polar face)	GIWKKWIKKWLNVLNLF-NH ₂	Higher hemolytic activity	
Lys → His	DDA-DL6K6	Lower hydrophobicity	
Insertion of Trp	(rD5) of HMWK	Lower antifungal and hemolytic activity	Sharma et al., 2010
Trp/Phe → Ala	PAF26	Higher activity at pH < 5.5	Sonesson et al., 2011
Addition of hydrophobic residues	PAF26	Higher activity	
Asn → Trp	HP (2-20): AKKVFKRLEKLFSKIQNDK-NH ₂	Increased salt resistance	Muñoz et al., 2007
Val10 → Trp10	KU1	Lower activity	Muñoz et al., 2007
Trp→ Phe	Tritrpticin	Higher activity	
Ala → Leu	DV13-K	Higher activity against <i>Ascomycetes</i> only	Lee et al., 2013
Val → Ile/Leu	GNU5	Higher hemolytic activity	Lum et al., 2015
Glu → Pro	Anal3(HP(2-20))	Lower hemolytic activity	Schibli et al., 2006
Truncation	PMAP-36	Increased hydrophobicity	Jiang et al., 2009
KW repetition	(KW) _n motif	Higher activity against <i>Ascomycetes</i> only	
D-enantiomers	Kaxin F17-6K	Higher hemolytic activity	Kim et al., 2014
D-enantiomers	Polybia MPI	Salt resistance	
D-enantiomers	Ac-RKTWFW-NH ₂	Higher activity	Lee et al., 2013
D-enantiomers	Ib-AMPs	Kink in helical structure	
Gly→Aic or Cha	Dnal	Higher activity	
Addition of bulky β -naphthylalanine	Ac-KKWRKWLA KK-NH ₂	Lower hydrophobicity	Lyu et al., 2016
Linearization	Arasin-1	Lower hemolytic activity	
Cyclization	RRWQWR	Increased cationicity and hydrophobicity	Gopal et al., 2013
Cyclization	Indolicidin	Higher activity	
Cyclization	granulysin	Similar or higher activity	Burrows et al., 2006
Cyclization	Magainin-2	Increased stability	Zhao et al., 2016
Acetylation	Lactoferrin	Similar activity	
Acetylation	RRWQWR (Lfcin derivative)	Acquisition of antifungal activity	López-García et al., 2000
Amidation	P-113	Higher activity	Thevisen, Francois, Sijtsma et al., 2005
Amidation	Trp and Arg-rich analogs	Acquisition of candidacidal activity	Grieco et al., 2013
Acetylation and amidation	RRWQWR	Improved salt resistance	Chu et al., 2013
Lauric acid	O3TR	Similar antifungal activity	Paulsen et al., 2013
Double repetition	PAF26	Serum stability	Nguyen et al., 2010; Rozek et al., 2003
AGPA hinge for concatemerization	PAF26	Similar antifungal activity	Falla & Hancock, 1997
Conjugation	Hp-magainin hybrid	Higher hemolytic activity	Li et al., 2005
		Lower hemolytic activity	Unger et al., 2001
		Lower activity	Pan et al., 2007
		Slight serum resistance	Pan et al., 2007
		Higher activity	Rothstein et al., 2001
		Decreased salt resistance	Chan et al., 2006
		Higher serum resistance	Pan et al., 2007
		Higher activity	Thery et al., 2018
		Hemolytic activity	
		Higher activity	López-García et al., 2015
		Lower solubility and proteolytic resistance	
		Higher activity	López-García et al., 2015
		Maintain of resistance	
		Higher activity	Park et al., 2004

bonds is favored. This preferential interaction facilitates the entry of Arg-rich peptides into the hydrophobic environment (Gopal et al., 2009). Cation- π interactions also make the introduction of Arg residues energetically more favorable. Thus, it is assumed that the presence of Arg, rather than Lys, improves the antifungal activity but also increases the hemotoxicity (Schibli, Nguyen, Kernaghan, Rekdal, & Vogel, 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 shown that the correlation between a net charge increase and the antimicrobial activity reached a threshold (Lyu, Yang, Lyu, Dong, & Shan, 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 twofold (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, Sundriyal, Wangoo, Tegge, & Jain, 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 recognized as another important parameter, as the AFP is not expected to bind to the membranes in absence of hydrophobic residues. Lee, Raman, Gellman, Lynn, and Palecek (2014) have showed the positive correlation between the hydrophobicity of 1,4- β -helical peptides and their antifungal activity (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 (Muñoz et al., 2007; Sonesson, Nordahl, Malmsten, & Schmidtchen, 2011; Van Der Kraan et al., 2005). 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 (Fernández-Vidal et al. 2008).

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 is crucial. The presence of Trp rather than Phe favors interactions between AFPs side chains and facilitates the penetration into the target organism membrane (Jin et al., 2016). The insertion of a Trp residue on the hydrophilic face of a helix generally 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 nonpolar 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, Seo, Song, & Park, 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 Tritipicin (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 (Avrahami & Shai, 2004; Kim, Jang, Kim, & Cho, 2014), salt resistance (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 nonpolar residues, as described in

Figure 5, is also expressed as a polar angle for α -helices (Fernández-Vidal, Jayasingh, Ladokhin, & White, 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 strongly with increasing hemolytic activity (Gautier, Douguet, Antonny, & Drin, 2008; Hollmann et al., 2016; Figure 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 & 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.

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 (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 to 20) analog has been shown to increase its antifungal activity twofold against several contaminants, such as *S. cerevisiae* (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 seven to eight 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 (Lyu et al., 2016; Situ et al., 2003). 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 (Gopal et al., 2013; Lyu et al., 2016).

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,

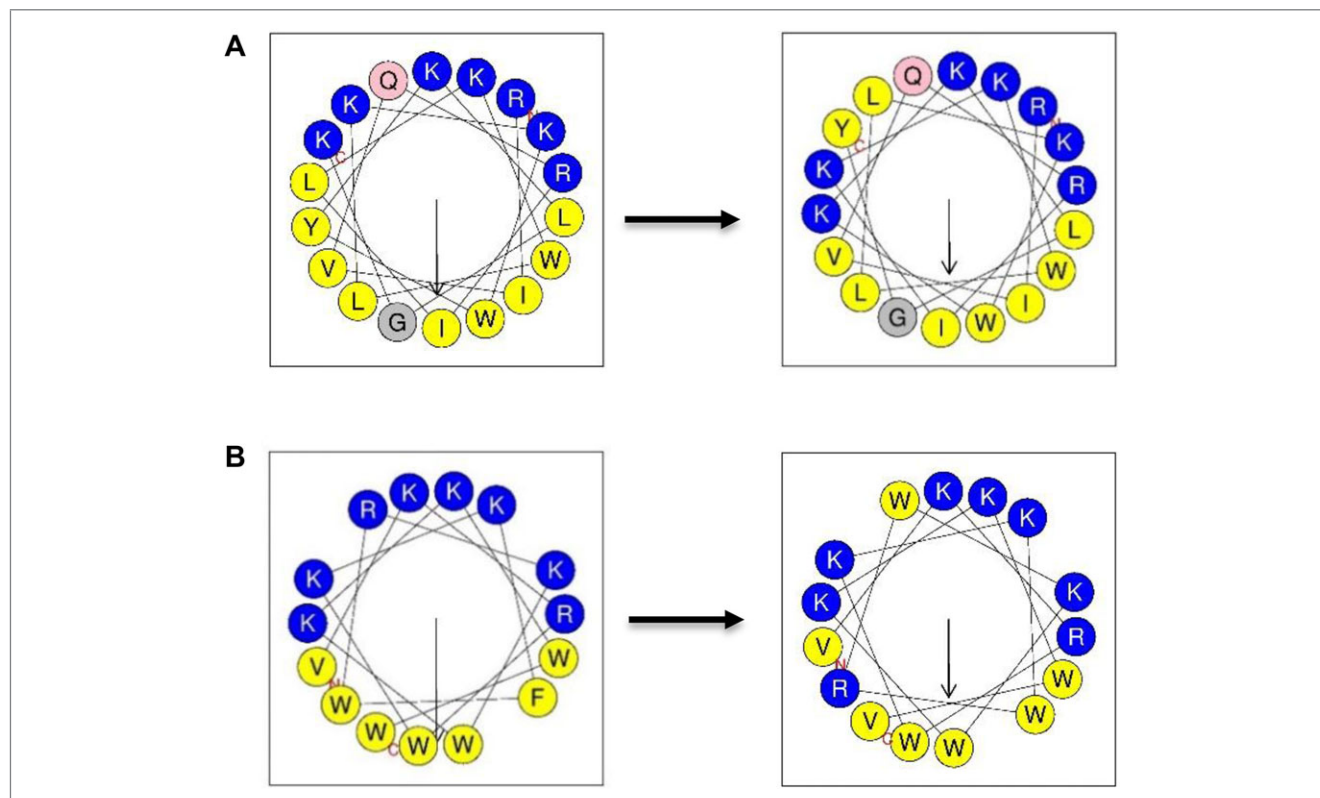


Figure 5—Helical wheels of novel peptides highlighting: (A) the reduced amphipathicity in the peptides reduces the hydrophobic moment, leading to a lower haemolytic activity. (B) In cathelicidin-BF derivatives, the substitutions lead to a lower HM but higher hydrophobicity, making the peptide more haemolytic. 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://heliquet.ipmc.cnrs.fr/> (Gautier et al., 2008).

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.

Computer-Based Methods for Designing AMPs

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 modeling, 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. These methods are the “traditional” methods to elaborate new peptides and, although widely used by microbiologists to highlight peptide properties, are quite incomplete. 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. (2011).

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 study highlighted the correlation between hydrophobicity and the hydrophobic moment and the activity of the peptides (Blondelle & 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 (for example, PhytAMP focuses only on plants AMPs), are presented in a review by Wang (2015). The most complete database, APD3, has recently been updated (access address: <http://aps.unmc.edu/AP/main.php>) (G. Wang, Li, & 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 & Yeaman, 2004) or structural patterns (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 (Jenssen, 2011).

Use of PTMs in AMP Design

Mimicking natural of peptides PTMs has several objectives, including reducing their toxicity, and increasing their serum stability and resistance to proteolytic digestion. In nature, AMPs are exposed to different PTMs and the use of PTMs for peptide design has been reviewed recently (Wang et al., 2015). Here, only the most common modifications used in the design of AFPs are discussed (Table 7). The commonly used modifications in peptide design and the subsequent studied properties are presented in Figure 4.

Peptidomimetics

Peptidomimetics are compounds whose main elements mimic a peptide structure, which generally arise from the modification of an existing peptide. Residues of natural peptides are usually present in the L-enantiomeric form. However, D-isomers of residues can be found in AFPs after PTM, such as in bacterial lipopeptides or bombinins (Simmaco, Kreil, & 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, & 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 two- to eightfold 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 (Thevisen, Francois, Sijtsma et al., 2005). A complete enantiomerization (Figure 4), which maintains the structure of the native peptide, is required to avoid loss of activity, as shown for polybia-MPI (Zhao et al., 2016). Another method to enhance stability is to modify the “common” backbone of peptides, via the design of oligomers or an unnatural backbone, for instance. These modifications generally increase the therapeutic index and reduce the cytotoxic activity of peptides. These peptidomimetics include peptoids (N-substituted glycine oligomers), β -peptides, acrylamide oligomers, phenylene ethynyls, and sulfono- γ -AA peptides. Their design has been reviewed recently (Bolarinwa, Nimmagadda, Su, & Cai, 2017; Galetti, Cirigliano, Cabrera, & Ramírez, 2012; Mojsoska & Jenssen, 2015; She et al., 2017).

These nonpeptidic analogs 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, Rawat, Kumar, Kumar, & Pasha, 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 -dihydrophenylalanine (Δ Phe), allows the stabilization of secondary structures and increases antimicrobial properties (Maurya et al., 2013). The replacement of Trp (Wang et al., 2009) 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 (Figure 4). The synthesis of disulfide bridges generally makes the development of synthetic peptides more complex.

In several cases, the antifungal activity is maintained in spite of the absence or 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 its 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 mold, 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 analog (Rodziewicz-Motowidło et al., 2010) or hydrophobicity of HBD-1 (Schroeder et al., 2011), favoring 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 (Li et al., 2005). Cyclization increases the hemolytic activity of the linear peptide RRWWRF whereas it decreases the hemolytic activity of magainin 2 (Unger, Oren, & 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, Rozek, Patrzykat, & Hancock, 2001; Rozek, Powers, Friedrich, & Hancock, 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 analogs (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 (C- and N-terminus tagging)

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 (Figure 4). 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 (Torrent, Andreu, Nogués, & Boix, 2011; Wang, 2014). 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 & 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 (Lavery, McLaughlin, Shaw, Gorman, & Gilmore, 2010; Thery, O'Callaghan, O'Brien, & Arendt, 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. Unlike oligomerization, conjugation does not only involve the repetition of same amino acid motifs. These advances have been recently reviewed by Reinhardt and Neundorff (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 (Figure 4). This issue has been resolved through the incorporation of the AGPA sequence "hinge" between the repeats (Lopez-Garcia et al., 2015). The creation of hybrids of different antifungal peptides, for example, HP(2-9)-magainin 2(1-12), also increases the potency compared to the single peptides alone (Park, Lee, & Hahm, 2004).

Food Applications of AFPs

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. 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 targeted fungi developing resistance. The nonnative expression of several plant defensins in plants conferred resistance toward phytopathogenic fungi. Thus, Rs-AFP2 and the hevein Pn-AMP were expressed in tobacco or a TL from *Ocimum basilicum* in *Arabidopsis*. Osmotin was also used to confer resistance to different fruits and vegetables against pathogenic mold such as *A. niger* (Anil Kumar, Hima Kumari, Shravan Kumar, Mohanalatha, & Kavi Kishor, 2015). AMPs appear to be more active than weak acids commonly used as food preservatives, which are active at concentrations of 1 to 10 mM against yeasts (Bell, Etchells, & Borg, 1959), but also inhibited filamentous fungi (18

to 236 $\mu\text{g/mL}$). In addition, these acids are highly active in a narrower range of pH (Razavi-Rohani & 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 & 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, Costa, Conte, & Del Nobile, 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, Pandit, Gaikwad, & Kövics, 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 two- to eightfold 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 lactoferrin, 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 to 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 & 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 (Yun, Kong, Chung, Kim, & Kong, 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 active packaging, as demonstrated for several bacteriocins (Malhotra, Keshwani, & Kharkwal, 2015; Rydlo et al., 2006).

Large-scale production of AFPs for food applications

The lack of an optimized process for the production of AFPs and the limited amount of studies on the effects of natural AFPs in food restrict their application (Tajkarimi & Ibrahim, 2012). Seeds are a convenient source that is commonly used for isolation of plant AFPs (Table 3). However, the yield of plant AFPs from seeds is generally as low as 14 to 30 mg/kg (De Zélicourt et al., 2007; Terras, Schoofs 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 et al., 2009). An increase of the level of AMPs has

been observed in plant species during development (Grosbois, Guerbet, & Kader, 1989; Kovaleva, Krynsky, Gout, & Gout, 2011; Terras et al., 1995), after wounding, or in presence of signaling molecules (Thomma et al., 1998; Vriens et al., 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, in the future, be economically more viable compared to natural extraction or production of recombinant (natural) peptides (see section “Unnatural Residues”).

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, & Gargouri, 2010), have been shown to maintain their activity in the presence of highly concentrated salt solutions. Recombinant ZmD32 retained activity against a range of fungal species in media containing elevated concentrations of salt. In addition, ZmD32 was active against (Kerenga et al., 2019). 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 (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 (Park et al., 2004). The replacement of Trp or His residues with bulky amino acids, such as β -naphthylalanine in the peptide P-113, 113 (Wang et al., 2009), makes the peptide resistant to up to 150 mM NaCl (Yu et al., 2011). Other methods, such as end-to-end cyclization (Yu, Lehrer, & Tam, 2000), creation of dimers, or hybridization of peptides can also improve the salt resistance (Han, Jyoti, Song, & Jang, 2016).

Effect of temperature, pH, and proteolysis

Heat treatment has always been a process employed to prevent food contamination (for example, pasteurization). 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 (Chan, Wong, Fang, Pan, & Ng, 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, Klein, Vo, & Wimley, 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 (Lin et al., 2007), are also resistant to high temperature, although a slight loss of activity can occur at temperatures higher than 80 to 90 °C, when the structure is irreversibly changed (Gallarate, Battaglia, Peira, & Trotta, 2011). Killer toxins produced by yeasts are usually active within a narrow range of pH and rapidly inactivated at temperatures higher than 40 to 50 °C (Soares & Sato, 2000; Wilson & 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 (Kim et al., 2014; Lin & Ng, 2009; Zhao et al., 2013), 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 (Corbo et al., 2009).

Cytotoxicity

Most plant AFPs are recognized as being nontoxic to mammalian cells due to their preferential interaction with microbe-specific targets, such as ergosterol or GlcCer, that are absent on mammalian cells (Lohner & 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 & Schimmel, 1997; Matsuzaki, 2009). The insertion of PTMs, the modification of peptide backbone, and disruption of α -helical structures are common methods employed to decrease the toxicity of AFPs.

Conclusion

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, highlights 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-labeled, 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 (for example, lactoferrin), or plant extracts containing highly stable peptides, such as brazzein, appears to be the best compromise.

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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.

Nomenclature

AFP	antifungal peptide (otherwise stated)
Ala A	alanine
AMP	antimicrobial peptide
ANNs	artificial neural networks
Arg R	arginine
Asn N	asparagine
Asp D	aspartic acid
Cys C	cysteine
DT	decision tree
FDA	Food and Drug administration
Gln Q	glutamine
Glu E	glutamic acid
Gly G	glycine
Gly-rich	peptides glycine-rich peptides
HB	hydrogen bonding
His H	histidine
HM	hydrophobic moment
Ib-AMP	<i>Impatiens balsamina</i> antimicrobial peptides
Ile I	isoleucine
Leu L	leucine
LTP	lipid transfer protein
Lys K	lysine
Met M	methionine
Phe F	phenylalanine
Pro P	proline
PR-protein	pathogenesis-related protein
PTM	posttranslational modification

Pyl O	pyrrolysine
QSAR	quantitative structure-activity-relationship
Sec U	selenocysteine
Ser S	serine
Thr T	threonine
TLP	thaumatin like protein
Trp W	tryptophan
Tyr Y	tyrosine
Val V	valine
WHO	World Health Organization

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