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Thibaut Thery, Elke K. Arendt

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

4 Thibaut Thery¹ and Elke K. Arendt^{1,2*}

6 ¹School of Food and Nutritional Sciences, University College Cork, Ireland

7 ² APC	Microbiome I	nstitute. U	University	College	Cork,	Ireland
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9 <u>E-mail: e.arendt@ucc.ie</u>. T : +353-21-490-3000. F: +353 21 490 2064.

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11 ABSTRACT

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13 Plant defensins are small, cysteine-rich antimicrobial peptides of the immune system found in 14 several organs during plant development. A synthetic peptide, KT43C, a linear analogue of the native Cp-thionin II found in cowpea seeds, was evaluated for its antifungal potential. It 15 16 was found that KT43C displayed antifungal activity against Fusarium culmorum, Penicillium expansum and Aspergillus niger. Like native plant defensins, KT43C showed thermostability 17 up to 100°C and cation sensitivity. The synthetic peptide decreased the fungal growth without 18 19 inducing morphogenic changes in the fungal hyphae. Non-inhibitory concentrations of the 20 peptide induced permeabilization of the fungal membrane. In addition, high concentrations of KT43C induced the production of reactive oxygen species in the granulated cytoplasm. To 21 22 investigate potential applications, the peptide was used as an additive in the preparation of 23 dough which did not contain yeast. This peptide delayed the development of fungal growth in the dough by 2 days. Furthermore, KT43C did not induce red blood cell lysis up to a 24 concentration of 200 µg.ml⁻¹. These results highlight the potential for the use of synthetic 25 26 antimicrobial defensins for shelf-life extension of food products.

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28 Keywords : cowpea thionin ; linear defensin ; antifungal activity ; dough

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⁸ * Author to whom correspondence should be addressed:

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36 INTRODUCTION

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38 Plants have developed an ancient and complex defense strategy through their immune system 39 to combat pathogens and abiotic stresses (de Beer and Vivier, 2011; Lacerda et al., 2014). 40 Among their many defense systems, the production of cationic antimicrobial peptides 41 (cAMPs) is a major contributor to plant resistance to phytopathogens, thanks to their broad 42 spectrum of activity (Stotz et al., 2009). Plant AMPs have been divided into several 43 categories based on their amino acid structure, sequence identity or tertiary structure (Nawrot 44 et al., 2014). Among these AMPs, plant defensins were first discovered in the seeds of wheat 45 and barley (Colilla et al., 1990; Mendez et al., 1990). Plant defensins can be divided into 46 three groups : defensins leading to morphogenic changes in the fungal hyphae, defensins 47 causing reduction of hyphae without morphogenic changes, and defensins without antifungal 48 activity (Broekaert et al., n.d.). The expression of plant defensin genes has been reported to 49 be increased in response to pathogens, which supports the idea that these peptides constitute a 50 major defence mechanism (Garcia-Olmedo et al., 1998). In addition, the localization of the plant defensins in different plant organs and tissues, with a preferential cell-wall location in 51 52 epidermal cells (Lacerda et al., 2014), is highly consistent with a defensive role. The defensins also play a role in the protection against insects, abiotic stress and metal tolerance 53 54 (Carvalho and Gomes, 2009).

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

The mode of action of several plant defensins has been extensively studied and different mechanistic steps have been identified but are yet not completely understood (Thevissen et al., 2004; Thomma et al., 2002). In many cases, it involves the recognition and binding to a cell wall (e.g., glucosylceramide) or membrane (e.g., phosphatidic acid) receptor (14). Then, plant defensins are either internalized into the fungal cell or stay at the surface of the membrane and induce several mechanisms: activation of enzyme pathways, modification of for fluxes, inhibition of conidial germination or induction of apoptosis. In contrast to

69 mammalian and insect defensins, the insertion of plant defensins in the fungal membrane via 70 pore-formation is uncommon and the mechanism of translocation of the peptides has not yet 71 been identified (14). Plant defensins are generally non-cytotoxic to mammalian and plant cells (Thevissen et al., 2004). However, some plant defensins have been reported to display 72 Ca²⁺ channel inhibition, anticancer activity and mitogenic activity towards mammalian cell 73 74 lines (3, 15). The toxicity of defensins and cAMPs in general has been attributed to the 75 presence of disulfide bonds, their high hydrophobicity and amphipathicity (Hollmann et al., 76 2016; Jenssen et al., 2006). Consequently, the design of linear analogues of AMPs and defensins showing antimicrobial properties has gained strong interest. For example, a linear 77 78 variant of human β -defensin 3 has been shown to partially retain its antimicrobial activity, without any toxicity towards human erythrocytes and conjunctiva epithelial cells (Liu et al., 79 80 2008).

81 Fungal spoilage is a major issue in cereal-based foods and in the wider food industry. In 82 addition to economic losses due to unpleasant flavours and appearance, mould has the 83 potential to cause health issues due to the production of mycotoxins (Pitt and Hocking, 2012). Among the different cereal-based products, chilled dough is recognized as a convenient 84 solution to the consumer's desire for oven-fresh baked products. Despite the chilled 85 86 conditions, bacterial and fungal growth can appear during the storage of this dough. In order 87 to prevent spoilage, a range of preservatives are used, but the overuse of these artificial preservatives has a negative image with the consumer. Alternative solutions to such 88 89 preservatives, including natural antimicrobial peptides such as nisin, are already in use but 90 their application is still challenging, mainly due to their narrow spectrum of activity 91 (Dielbandhoesing et al., 1998). In contrast, due to their natural role in plant protection, 92 defensing properties, such as inhibition of a broad range of microorganisms 93 and this can be exploited in food applications.

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

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101 MATERIAL AND METHODS

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103 Defensin

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105 A synthetic peptide (purity >70%), named KT43C (5.2 kDa), a linear analogue of the plant 106 defensin Cp-thionin II, was synthesized by and purchased from GLBiochem, Shanghai, 107 China. Synthesis was made by solid-phase method using Fmoc chemistry, as described by the 108 manufacturer. Fmoc group was removed by treating the resin with a mix of piperidine/DMF 109 (20/80). A MALDI-TOF mass spectrometry (SHIMADZU AXIMA Assurance) was performed by GLBiochem to confirm the stability of the peptide after synthesis (see 110 Supplementary information). The lyophilized peptide was reconstituted in 0.1% acetic acid 111 112 (pH 3.5) and stored in vials at different concentrations at -20°C.

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114 Fungal strains

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

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122 Fungal growth inhibition assay

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124 To determine the antifungal activity of the synthetic peptide, the fungal growth was followed 125 in a microtiter plate assay as described previously by van der Weerden et al. (Van Der 126 Weerden et al., 2008) with some modifications. Briefly, fungal spores were collected from mature colonies grown for 72 h on potato dextrose agar (PDA) (Sigma-Aldrich) plates at 127 128 25°C, counted on a haematocytometer and inoculated in synthetic nutrient-poor broth. Fungal 129 spores were then diluted in half- strength potato broth (1/2 PDB) (Sigma-Aldrich) to obtain a final concentration of 1×10^4 spores.ml⁻¹. Spore suspensions (80 µl) were added to the wells of 130 131 a 96-well microtiter plate.

132 Filter-sterilized synthetic peptides (20 cl) at concentrations ranging from 5 μ g.ml⁻¹ to 200

133 μ g.ml⁻¹ (final concentration) were then added to the fungal cells. Fungal growth was assessed

134 by measuring the absorbance at 595 nm for 96 h at 25°C (Multiskan TM, Thermo Scientific).

135 Cell suspensions in 0.1% acetic acid (in ½ PDB, pH=5.0) were used as negative controls. 136 Each test was performed in triplicate. 137 The inhibition of fungal growth by KT43C was then examined on PDA plates. The same 138 solutions were prepared as described above and after 24 h incubation at 25°C, 50 µl were 139 spot-inoculated on PDA plates. Pictures of the plates were taken at 24 h intervals for a period 140 of three days. 141 142 *MIC/IC*₅₀ determination 143 144 After 96 h of incubation at 25°C, the MIC was read as the lowest concentration of KT43C resulting in complete inhibition of fungal growth. The required concentration to inhibit 50% 145 of the growth (IC_{50}) was determined by non-linear regression, using the software Graph 146 147 PRISM (GraphPad Software, Inc., La Jolla, CA) with the microplate reader data. 148 149 Thermal stability of KT43C 150 In order to investigate the thermal stability of KT43C, a peptide solution was heated at 100°C 151 for 15 min. After cooling at RT, the activity of KT43C (MIC = $20 \mu \text{g.ml}^{-1}$) was measured 152 against F. culmorum in a 96-well microtiter assay as described above. 153 154 Effect of cations on the antifungal activity of KT43C 155 156 To determine the effect of cations on KT43C activity, an antifungal assay was performed in 157 different salt solutions. Fungal spores $(1 \times 10^4 \text{ spores.ml}^{-1})$ were inoculated in medium 158 containing either 100 mM NaCl, 50 mM KCl, 5 mM CaCl₂ or 5 mM MgCl₂, as described 159 previously (Terras et al., 1992). After the addition of the peptide, the inhibitory activity of 160 KT43C (concentration = 20 μ g.ml⁻¹) was measured against *F. culmorum* in a 96-well 161

- 162 microtiter assay as described above.
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- 164 Characterization of the antifungal activity

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166 To visualize the action of KT43C against *F. culmorum* spores, the peptide, at concentrations 167 of 5 and 20 μ g.ml⁻¹, was added to ½ PDB inoculated with fungal spores. After 24 h, 500 μ l of 168 each solution was freeze-dried for further analysis. Five hundred μ l of 70% glycerol was

added to the solutions to avoid damage due to freeze drying. The lyophilized samples were
then analysed using a JEOL scanning electron microscope type 5510 (JEOL, Tokyo, Japan),
as described by Oliveira et al. (Oliveira et al., 2012), with some modifications. Briefly, fungal
mycelium was mounted onto plain aluminium stubs using carbon double surface adhesive
and coated with a 7 nm gold layer using a Gold Sputter Coater (BIO-RAD Polaron Division,
SEM coating system, England) and observed under a constant accelerating voltage of 5 kV.

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176 Membrane permeabilzation assay

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

F. culmorum hyphae were grown in $\frac{1}{2}$ PDB from a suspension of 1×10^4 spores.ml⁻¹ for 18 h 181 at 25°C. The hyphae were then incubated with 10, 20 and 50 µg. ml⁻¹ of KT43C at room 182 183 temperature with gentle agitation. SYTOX® Green was added (final concentration of 0.5 µM) and the hyphae were allowed to stand for 10 min. Fluorescence of hyphae was measured 184 using a fluospectrophotometer (Varioskan® LUX reader) for 2 h with excitation and 185 emission wavelengths of 488 and 538 nm, respectively, or visualized using a fluorescence 186 microscope (Olympus Fluoview) (excitation wavelength 460-490 nm). Solutions of hyphae 187 188 without peptides or with 1% Triton X-100 (Sigma-Aldrich) were used as negative and positive control, respectively. For each concentration of KT43C, the experiment was 189 190 performed in triplicate.

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192 Induction of reactive oxygen species (ROS) assay

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194 The detection of ROS was performed as described by van der Weerden et al. (Van Der 195 Weerden et al., 2008) with some modifications. F. culmorum hyphae were grown as 196 described above and then treated with KT43C or water for 12 h. Following the incubation, the hyphae was incubated with 10 µg.ml⁻¹ dihydrorhodamine 123 (Sigma-Aldrich) for 2 h 197 198 followed by extensive washing with 0.6 M KCl. Fluorescence of hyphae was measured using 199 a fluospectrophotometer with excitation and emission wavelengths of 488 and 538 nm 200 respectively, or visualized using a fluorescence microscope (Olympus) (excitation 201 wavelength 460-490 nm). Solutions of hyphae without peptides or with 1% Triton X-100

202 (Sigma-Aldrich) were used as negative and positive control, respectively. For each203 concentration of KT43C, the experiment was performed in triplicate.

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205 Circular dichroism

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

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214 Chilled dough shelf life

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Dough of 10 g was prepared according to a standard recipe (without yeast) consisting of 63% (w/w) flour, 1.5% (w/w) sugar, 3% (w/w) fat and 0.3% (w/w) salt. A solution of 20 μ g.ml⁻¹ KT43C in sterile distilled water was inoculated with 1x10² spores.ml⁻¹ (final concentration) of *F. culmorum* and added to the mix. Water, without peptide, was used as control. After preparation, the dough was packed into sealed plastic bags and filter pipette tips were inserted into the bag to allow gas exchange. Dough was stored at 4°C. The shelf life of the dough was monitored over 14 days, as described by Dal Bello et al. (Dal Bello et al., 2007).

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224 Fungal contamination in the dough

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226 Fungal contamination of dough was estimated by measuring the ergosterol concentration as described by Jedličková et al. (Jedlickova et al., 2008) with some modifications. Briefly, 10 g 227 228 of ground dough was treated with 50 mL of methanol in a closed vessel and was shaken for 229 30 min. Then, 25 mL of cleaned extract was placed into a test tube and 3 g of KOH was 230 added and stirred until dissolution of KOH. Ten mL of n-hexane was then added and the 231 solution was incubated at 65°C in a water bath for 30 min. After cooling at RT, 5 ml of 232 distilled water were added and the hexane layer was placed into a beaker. The extraction 233 process using hexane was repeated three times using 10 mL of the solvent. The extracts were then pooled and evaporated until dry in a vacuum dryer (Scanvac, Labogene) and redissolved 234 235 in 5 mL of methanol. The extracted samples were analyzed using HPLC with a DAD. The

mobile phase was methanol, and 1.0 mL/min was the optimal flow rate. The column
temperature was held at 25 °C. A control was made with the addition of standard ergosterol
(Sigma-Aldrich) into fresh dough. A 95% recovery was obtained with the standard ergosterol.

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240 Haemolysis assay

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242 KT43C was assayed for its ability to induce haemoglobin release from fresh defibrinated 243 sheep erythrocytes as described by Laverty et al. (Laverty et al., 2010). Fresh sheep red blood 244 cells (Thermo Fisher Scientific) were washed three times with equal volumes of PBS. After centrifugation for 15 min at 900g, erythrocytes were resuspended to 4% (v/v) in PBS. 245 Eythrocyte suspension (80 µL) was added to the wells of a 96-well microtiter plate, followed 246 247 by the addition of 20 µl of KT43C in PBS at different concentrations. After incubation at 37°C for 1 h, the suspension was clarified by centrifugation at 1,000g for 10 min. Supernatant 248 was transferred to a fresh 96-well microtiter plate and haemoglobin release was measured 249 250 spectrophotometrically at 405 nm. Erythrocytes were treated with 0.1% Triton X-100 (in 251 PBS) and PBS alone as positive and negative control, respectively. The percentage 252 haemolysis was calculated as described by Laverty et al. (Laverty et al., 2010) :

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254 % Haemolysis =
$$\frac{(Abs405 \ peptide \ treatment) - (Abs405 \ PBS)}{(Abs405 \ 0.1\% \ Triton \ X - 100) - (Abs \ 405 \ PBS)}$$

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256 The release of haemoglobin was determined for six replicates.

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258 Statistical analysis

259 Statistical analysis was carried out using Microsoft XLSTAT Version 2015.5.01. (Adinsoft 260 Inc, New York, USA). Standard deviations were calculated for absorbance values at each 261 concentration of KT43C based on triplicates, except for the haemolysis assay for which six 262 replicates were used.

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

267 in haemolysis activity for each concentration of KT43C in comparison to the negative268 control.

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270 Results

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272 Antifungal activity of KT43C

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274 The growth of spores of F. culmorum, P. expansum and A. niger was followed in presence of different concentrations of the peptide KT43C, in a microtiter plate assay. After 96 h, KT43C 275 showed the highest antifungal activity against F. culmorum with an IC₅₀ of 12.5 μ g.ml⁻¹ and a 276 MIC of 20 µg.ml⁻¹ (Fig.1A). Growth of A. niger was delayed but the MIC, after 96 h, was not 277 apparent within the concentration range of the peptide used in the study (up to 200 μ g.ml⁻¹) 278 279 (Fig.1B). The growth of *P. expansum* was only inhibited with a peptide concentration of 200 µg.ml⁻¹ (Fig.1C). The inhibition of fungal growth was controlled on PDA plates (Fig. 2). 280 KT43C inhibited fungal growth without induction of morphogenic changes in F. culmorum 281 hyphae, e.g. branching, as observed with scanning electron microscopy (Fig. 3). 282

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284 Effect of heat and salt on the antifungal activity of KT43C

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

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

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294 *Mode of action of KT43C*

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To identify some characteristics of the mode of action of KT43C, a SYTOX® Green uptake assay was performed on *F. culmorum* with different concentrations of KT43C. The peptide induced permeabilization of *F. culmorum* hyphae at a concentration of 10 μ g.ml⁻¹ and higher (Fig. 5). Permeabilized hyphae had significant cytoplasmic granulation at higher

300 concentrations. However, KT43C-induced permeabilization appeared to be required for301 inhibition but was not sufficient to cause cell death.

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

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309 Circular dichroism

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311 Native defensins possess a characteristic CS $\alpha\beta$ motif, in which tertiary structures, such as an 312 alpha helix and antiparallel β -sheet are stabilized by disulfide bonds. In aqueous solutions, in 313 the absence of disulfide bonds, the linear analogues of defensins present random 314 conformations and therefore are thought to be more flexible. In contrast, in trifluoroethanol or 315 SDS micelles (a membrane-mimicking model), the analogues could adopt structured 316 conformations.

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

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- 326 Application of KT43C in dough
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328 Spoilage fungi, such as *Fusarium* spp. in wheat or barley, are ubiquitous in cereal 329 environments. Although the baking step essentially kills spoilage fungi, the presence of heat-330 stable mycotoxins remains a potential health issue. The protection of raw material or a 331 microbiologically unstable cereal-based product against fungal growth is therefore an 332 absolute necessity.

333 The synthetic peptide, KT43C, was used as an ingredient in the preparation of bread dough $(20 \ \mu g.ml^{-1})$, based on total flour weight. The monitoring of the shelf life of chilled dough, 334 335 previously inoculated with F. culmorum spores is presented in Figure 8. For 14 days, dough 336 was monitored each day and classified into different categories based on the percentage of 337 fungal contamination present on the surface. The development of fungal colonies on the 338 treated dough was delayed by 2 days compared to the dough without peptide. To quantify the 339 fungal contamination, the concentration of ergosterol in the dough was determined. After 14 340 days of storage, the level of ergosterol in the control dough was twice as high as in the treated dough (17.5 \pm 3.5 and 7.6 \pm 0.9 μ g.ml⁻¹, respectively). The synthetic defensin was thus 341 shown to be an efficient tool against fungal spoilage of dough. In addition, the thermostability 342 343 of KT43C could allow its exploitation as a novel preservative in baked goods.

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345 Haemolysis assay

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

353

354 Discussion

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Previous studies have highlighted the antifungal (Carvalho et al., 2001) and antibacterial 356 357 (Franco et al., 2006) activity of antimicrobial peptides isolated from cowpea seeds, such as the defensin, Cp-thionin II as described by Franco et al. (Franco et al., 2006). In this study, a 358 359 synthetic linear analogue of Cp-thionin II, namely KT43C, exhibited antifungal activity 360 against F. culmorum, P. expansum and A. niger (Fig. 1); while KT43C has been previously shown to inhibit Gram-positive bacteria (Kraszewska et al., 2016), to our knowledge, the 361 362 antifungal activity of cowpea antimicrobial peptides or derivatives has never been described for the fungi studied here. A synthetic linear analogue of Cp-thionin II, namely KT43C, 363 364 exhibited antifungal activity against F. culmorum, P. expansum and A. niger (Fig. 1). Despite the absence of disulfide bonds in its linear structure, KT43C displays antifungal and 365

366 antibacterial activity. Disulfide bonds are determinants of defensins' integrity and have been

367 reported to increase the antimicrobial activity (Jenssen et al., 2006). However, some linear derivatives of AMPs (Wu and Hancock, 1999) and defensins (Liu et al., 2008) are able to 368 369 retain partial or complete antimicrobial activity after removal of the disulfide bonds. Other 370 parameters, such as net charge, hydrophobicity, amphipathicity and flexibity, are essential for 371 the antimicrobial activity of AMPs. Although disulfide bridges are not essential for the 372 antimicrobial and antifungal activity of Cp-thionin II, the presence of free cysteine residues 373 may modify the hydrophobicity of the peptide and then change its activity. However, this 374 effect seems unclear for linear analogues of defensins (Liu et al., 2008; Nagano et al., 1999).

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

381 KT43C displayed antifungal activity against F. culmorum, A. niger and P. expansum. These 382 three fungal species belong to the same subdivision, Pezizomycotina, but F. culmorum 383 belongs the class of *Sordariomycetes* while *P. expansum* and *A. niger* both belong to the class of Eurotiomycetes. Although the mechanistic action of KT43C on these fungi may have 384 385 similarities, differences in cell/wall composition or fungal physiology between these species 386 may be pertinent to the mode of action of KT43C and its antifungal potency. Differences in 387 the mode of action of the plant defensin MtDef4 against Neurospora crassa and F. 388 graminearum have been described by El-Mounadi et al. (El-Mounadi et al., 2016).

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

394 After heat treatment, KT43C retained its antifungal activity against *F. culmorum*. The heat 395 stability of KT43C has also previously been shown regarding its antibacterial potency 396 (Kraszewska et al., 2016). Terras et al. (Terras et al., 1992) and Broekaert et al. (Broekaert et 397 al., n.d.) have reported the thermal stability of defensins from radish and other plant species. 398 The role of disulfide bonds in defensins stabilization was highlighted by Terras et al. (Terras 399 et al., 1992). KT43C appeared unstructured in aqueous solutions but adopts an α -helical 400 conformation in a membrane environment (Fig. 7). The random conformations and flexibility

401 of the peptide could protect from thermal denaturation. The adoption of a structured 402 conformation in presence of SDS may indicate possible interactions between KT43C and a 403 membrane-mimic environment. This new conformation could be related to its antifungal 404 activity (Domingues et al., 2008; Liu et al., 2008).

- 405 The presence of ions, especially divalent cations, has been proven to decrease the antifungal 406 activity of native plant defensins (Vriens et al., 2014). The antifungal activity of this linear 407 analogue of Cp-thionin II was demonstrated to be also affected by the presence of cations 408 (Fig. 4). Kraszewska et al. (Kraszewska et al., 2016) reported that the peptide keeps its 409 antibacterial activity in the presence of NaCl, up to 50 mM, but loses it at 100 mM. The loss 410 of activity in presence of cations is a common feature for plant defensins and AMPs linear 411 derivatives in general (Adem Bahar and Ren, 2013; Vriens et al., 2014). This effect is due to 412 the weakening of electrostatic interactions between the cationic peptides and the negatively 413 charged membrane of microbial cells (Wu et al., 2003). Other potential effects may include structural changes in the peptide (Oard and Karki, 2006), or stabilization of the microbial 414 415 membrane by cations (Thevissen et al., 1999).
- 416 Plant defensins (Vriens et al., 2014) and linear AMPs (Domingues et al., 2015; Liu et al., 2008) have been shown to present several modes of action. Among these different 417 418 mechanisms, permeabilization of the fungal membrane has been described as a secondary 419 effect of plant defensin action (Vriens et al., 2014), but is primarily involved in the 420 antimicrobial activity of linear peptides (Bechinger and Lohner, 2006). Like native plant 421 defensins, such as NaD1 (Van Der Weerden et al., 2010) or linear antimicrobial peptides 422 (Van Der Weerden et al., 2010), KT43C causes fungal membrane permeabilization of F. 423 culmorum at the MIC (Fig. 5), but at a slower rate of action than the native peptide (data not 424 shown). The time difference can be explained by the formation of oligomers of the synthetic 425 peptide or the necessity to reach a sufficient concentration of peptide at the surface of fungal membrane (Thevissen et al., 2004). Another hypothesis would be a limited mobility of 426 aggregates of KT43C that tend to form into the bilayer environment because of changes in 427 428 hydrophobicity, as described for tachyplesin (Han and Lee, 2015).
- At high concentrations, KT43C induces a high production of ROS in *Fusarium* hyphae (Fig. 6). The generation of ROS is involved in mechanisms related to oxidative stress and damage, leading generally to cell-death. The interaction with intracellular targets and the overproduction of ROS in the fungal cytoplasm has been highlighted for several defensins (Vriens et al., 2014) and linear AMPs (Huang et al., 2010). In addition, a model, involving pore-formation and intracellular target strategy, has already been considered for several linear

AMPs (Mason et al., 2007). Like KT43C, ROS production with NaD1 was only observed at
concentrations greater than the MIC, even when membrane permeabilization was observed,
suggesting a partial role for oxidative stress in fungal inhibition. (Hayes et al., 2013).

438 A major issue with the use of cAMPs in pharmaceuticals or food applications is their 439 potential toxicity towards mammalian cells. The reduction of hydrophobicity and the absence 440 of disulfide bridges in linear derivatives have been pointed out as key elements in reducing 441 their cytotoxicity (Liu et al., 2008). KT43C did not induce red blood cells lysis in the range of 442 concentration used for the antifungal assays. Due to the presence of cholesterol, mammalian 443 cell membranes have been shown to be less sensitive to destabilization by linear cationic AMPs than fungal membranes (containing mostly ergosterol) (Mason et al., 2007). Another 444 445 study from our group has shown that the synthetic cationic peptide OOWW-NH2 is inactive against gut Caco-2 cell lines (Thery et al., 2018). The cytotoxicity of plant defensins has 446 447 already been proven to be low, even negligible (Thevissen et al., 2004). In addition, Liu et al. (Liu et al., 2008) showed that a linear analogue of hBD-3 displayed lower cytotoxicity 448 449 compared to the native form of HBD-3. The decreased cytotoxicity towards mammalian cells 450 of linear derivatives of AMPs and defensins has been attributed to the removal of the 451 disulfide bridges, decreasing the overall hydrophobicity (Liu et al., 2008).

KT43C (20 µg.ml⁻¹) was used as an ingredient in the preparation of chilled dough and 452 453 delayed the growth of F. culmorum by 2 days in a challenge test. The use of natural (Lucera et al., 2012; Rai et al., 2016; Rydlo et al., 2006) and synthetic (Appendini and Hotchkiss, 454 2000; Thery et al., 2018) AMPs to prevent spoilage of food products has been reported. Thus, 455 456 the synthetic analogue of the human β -defensin 3 protects bread against environmental contaminants, with a shelf-life extension of 3 days (Thery et al., 2016). Although the 457 concentration of KT43C used in this test was the MIC against F. culmorum, the conidial 458 459 germination was not completely inhibited. The presence of other dough ingredients and 460 proteases resulting from the preparation process may affect the antifungal action of the 461 peptide. The sensitivity of AMPs to proteolytic digestion is a major concern for a potential 462 use as food additive to avoid further action once in the intestinal system.

In this study, a synthetic linear analogue of the Cp-thionin II, showed antifungal activity against common fungal contaminants of cereal and cereal-based products. The analogue defensin, KT43C, presented similar characteristics to native plant defensins, such as heat stability and cation sensitivity. The synthetic peptide appears to be an efficient tool against

467	fungal contamination of dough. Given its harmlessness towards erythrocytes, KT43C may
468	represent a novel alternative to commonly used chemical preservatives in the baking industry.
469	
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471	
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Name	Source	Sequence	Identity	Activity	Function	Reference
Linear peptide KT43C	Synthetic peptide	KTCMTKKEGWGRCLID TTCAHSCRKYGYMGG KCQGITRRCYCLLNC	-	F. culmorum A. niger P. expansum		This study
Cp-thionin II	Vigna unguiculata	KT <u>C</u> MTKKEGWGR <u>C</u> LID TT <u>C</u> AHS <u>C</u> RKYGYMGG K <u>C</u> QGITRR <u>CYC</u> LLN <u>C</u>	100%	Gram-positive S. aureus Gram-negative E. coli P. syringae	γ-thionin	(Franco et al., 2006)
Cp-thionin	Vigna unguiculata	RV <u>C</u> ESQSHGFKGA <u>C</u> TG DHN <u>C</u> ALV <u>C</u> RNEGFSGG N <u>C</u> RGFRRR <u>C</u> F <u>C</u> TLK <u>C</u>	42%	Unknown	Trypsin inhibitor	(Melo et al., 2002)
VrD1	Vigna angularis	RT <u>C</u> MIKKEGWGK <u>C</u> LID TT <u>C</u> AHS <u>C</u> KNRGYIGGN <u>C</u> KGMTRT <u>C</u> Y <u>C</u> LVN <u>C</u>	73%	Insecticidal	α-amylase inhibitor	(Liu et al., 2006)

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

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



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



 $0 \,\mu g.ml^{-1}$

100 µg.ml⁻¹¹

 $200 \ \mu g.ml^{-1}$

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



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









b)



Fig. 5 SYTOX® Green uptake assay. a) After 18 h of growth, *F. culmorum* hyphae were treated for 24 h with KT43C (0, 10, 20 and 50 μ g.ml⁻¹). Permeabilization of the fungal membrane was determined by fluorescence with SYTOX® Green (excitation 438 nm,

emission 538 nm) and correlated to the percentage of inhibition. Values are the mean of three different experiments. b) *F. culmorum* hyphae were observed with a confocal laser scanning microscope, with an excitation wavelength 460-490 nm. Left: no KT43C; middle: $20 \mu g.ml^{-1}$ KT43C; right: $50 \mu g.ml^{-1}$. High concentrations of KT43C induced permeabilization of fungal membrane, highlighted by fluorescence of the dye, and granulation of the fungal cytoplasm a)



b)

Fig. 6 Detection of reactive oxygen species (ROS) production. a) After 24 h of growth, *F. culmorum* hyphae were treated for 12 h with KT43C (0, 10, 20 and 50 μ g.ml⁻¹). Production of ROS was determined by fluorescence of DHR 123 (excitation 438 nm, emission 538 nm),

and correlated to the percentage of inhibition. Each value is the mean of triplicates b) *F*. *culmorum* hyphae were observed with a confocal laser scanning microscope, with an excitation wavelength 460-490 nm. Left: no KT43C ; right : $50 \mu \text{g.ml}^{-1}$



Fig. 7 CD spectra of KT43C in presence of DIW (solid line) or 20 mM SDS/DIW (dashed line). In DIW, the absence of crossover and the minimum at ~200 nm indicate unstructured conformation with transient β -hairpin. In SDS, KT43C adopts a more structured conformation with the presence of α -helical and β -hairpin conformers



Fig. 8 Shelf life of chilled dough inoculated with *F. culmorum* $(1x10^2 \text{ spores.ml}^{-1})$. The development of fungal mycelium was monitored over 14 days and the dough was classified

according to the percentage of contamination (from A (0% contamination) to E (50% contamination)). a) Control dough; b) Treated dough with KT43C ($20 \ \mu g.ml^{-1}$). Three doughs were used in each experiment

Pictures of fungal contamination on chilled dough after 14 days of storage are presented with the charts

Highlights

- A novel synthetic antimicrobial peptide inhibits fungal spoilage
- The structure and the mode of action of the synthetic peptide are characterized
- The synthetic peptide extends the shelf life of dough

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