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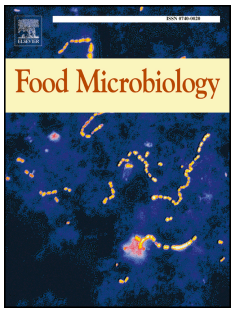

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Thibaut Thery, Elke K. Arendt

PII: S0740-0020(17)30481-1

DOI: 10.1016/j.fm.2018.01.006

Reference: YFMIC 2930

To appear in: *Food Microbiology*

Received Date: 23 May 2017

Revised Date: 21 December 2017

Accepted Date: 5 January 2018

Please cite this article as: Thery, T., Arendt, E.K., Antifungal activity of synthetic cowpea defensin Cp-thionin II and its application in dough, *Food Microbiology* (2018), doi: 10.1016/j.fm.2018.01.006.

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

Thibaut Thery[1] and Elke K. Arendt[1,2*]

[1]School of Food and Nutritional Sciences, University College Cork, Ireland
[2]APC Microbiome Institute. University College Cork, Ireland
* Author to whom correspondence should be addressed:

E-mail: e.arendt@ucc.ie. T : +353-21-490-3000. F: +353 21 490 2064.

ABSTRACT

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

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

INTRODUCTION

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

Plant defensins are cysteine-rich cationic peptides of 5-8 kDa whose mature domain comprises 45 to 54 amino acid residues, with an isoelectric point around 9. The global fold of plant defensins consists of a cysteine-stabilized motif (CSα/β) made up of an α-helix and a triple-stranded β-sheet stabilized by four disulfide bridges (C1-C8/C2-C5/C3-C6/C4-C7) (Lay et al., 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 ion fluxes, inhibition of conidial germination or induction of apoptosis. In contrast to

mammalian and insect defensins, the insertion of plant defensins in the fungal membrane via pore-formation is uncommon and the mechanism of translocation of the peptides has not yet been identified (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 Ca^{2+} channel inhibition, anticancer activity and mitogenic activity towards mammalian cell lines (3, 15). The toxicity of defensins and cAMPs in general has been attributed to the presence of disulfide bonds, their high hydrophobicity and amphipathicity (Hollmann et al., 2016; Jenssen et al., 2006). Consequently, the design of linear analogues of AMPs and defensins showing antimicrobial properties has gained strong interest. For example, a linear variant of human β-defensin 3 has been shown to partially retain its antimicrobial activity, without any toxicity towards human erythrocytes and conjunctiva epithelial cells (Liu et al., 2008).

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

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

MATERIAL AND METHODS

Defensin

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

Fungal strains

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

Fungal growth inhibition assay

To determine the antifungal activity of the synthetic peptide, the fungal growth was followed in a microtiter plate assay as described previously by van der Weerden et al. (Van Der Weerden et al., 2008) with some modifications. Briefly, fungal spores were collected from mature colonies grown for 72 h on potato dextrose agar (PDA) (Sigma-Aldrich) plates at 25°C, counted on a haematocytometer and inoculated in synthetic nutrient-poor broth. Fungal spores were then diluted in half- strength potato broth (1/2 PDB) (Sigma-Aldrich) to obtain a final concentration of $1x10^4$ spores.ml^{-1}. Spore suspensions (80 µl) were added to the wells of a 96-well microtiter plate.

Filter-sterilized synthetic peptides (20 cl) at concentrations ranging from 5 µg.ml^{-1} to 200 µg.ml^{-1} (final concentration) were then added to the fungal cells. Fungal growth was assessed by measuring the absorbance at 595 nm for 96 h at 25°C (Multiskan TM, Thermo Scientific).

Cell suspensions in 0.1% acetic acid (in ½ PDB, pH=5.0) were used as negative controls. Each test was performed in triplicate.

The inhibition of fungal growth by KT43C was then examined on PDA plates. The same solutions were prepared as described above and after 24 h incubation at 25°C, 50 µl were spot-inoculated on PDA plates. Pictures of the plates were taken at 24 h intervals for a period of three days.

MIC/IC_{50} determination

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

Thermal stability of KT43C

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

Effect of cations on the antifungal activity of KT43C

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

Characterization of the antifungal activity

To visualize the action of KT43C against *F. culmorum* spores, the peptide, at concentrations of 5 and 20 µg.ml^{-1}, was added to ½ PDB inoculated with fungal spores. After 24 h, 500 µl of each solution was freeze-dried for further analysis. Five hundred µl of 70% glycerol was

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

Membrane permeabilzation assay

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

F. culmorum hyphae were grown in ½ PDB from a suspension of $1x10^4$ spores.ml^{-1} for 18 h at 25ºC. The hyphae were then incubated with 10, 20 and 50 µg. ml^{-1} of KT43C at room temperature with gentle agitation. SYTOX® Green was added (final concentration of 0.5 µM) and the hyphae were allowed to stand for 10 min. Fluorescence of hyphae was measured using a fluospectrophotometer (Varioskan® LUX reader) for 2 h with excitation and emission wavelengths of 488 and 538 nm, respectively, or visualized using a fluorescence microscope (Olympus Fluoview) (excitation wavelength 460-490 nm). Solutions of hyphae without peptides or with 1% Triton X-100 (Sigma-Aldrich) were used as negative and positive control, respectively. For each concentration of KT43C, the experiment was performed in triplicate.

Induction of reactive oxygen species (ROS) assay

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

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

Circular dichroism

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

Chilled dough shelf life

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

Fungal contamination in the dough

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

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

Haemolysis assay

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

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

The release of haemoglobin was determined for six replicates.

Statistical analysis

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

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

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

Results

Antifungal activity of KT43C

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

Effect of heat and salt on the antifungal activity of KT43C

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

The effect of cations on the activity of KT43C against *F. culmorum* is presented in 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.

Mode of action of KT43C

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

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

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

Circular dichroism

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

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

Application of KT43C in dough

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

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

Haemolysis assay

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

Discussion

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

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

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

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

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

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

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

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

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

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

At high concentrations, KT43C induces a high production of ROS in *Fusarium* hyphae (Fig. 6). The generation of ROS is involved in mechanisms related to oxidative stress and damage, leading generally to cell-death. The interaction with intracellular targets and the overproduction of ROS in the fungal cytoplasm has been highlighted for several defensins (Vriens et al., 2014) and linear AMPs (Huang et al., 2010). In addition, a model, involving pore-formation and intracellular target strategy, has already been considered for several linear

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

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

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

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

fungal contamination of dough. Given its harmlessness towards erythrocytes, KT43C may represent a novel alternative to commonly used chemical preservatives in the baking industry.

Acknowledgements

This study was funded the Irish Department of Agriculture, Food and the Marine through the research program FIRM/RSF/CoFoRD (Reference: 13 F 462). The authors declare no conflict of interest

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636 peptides.

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

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

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

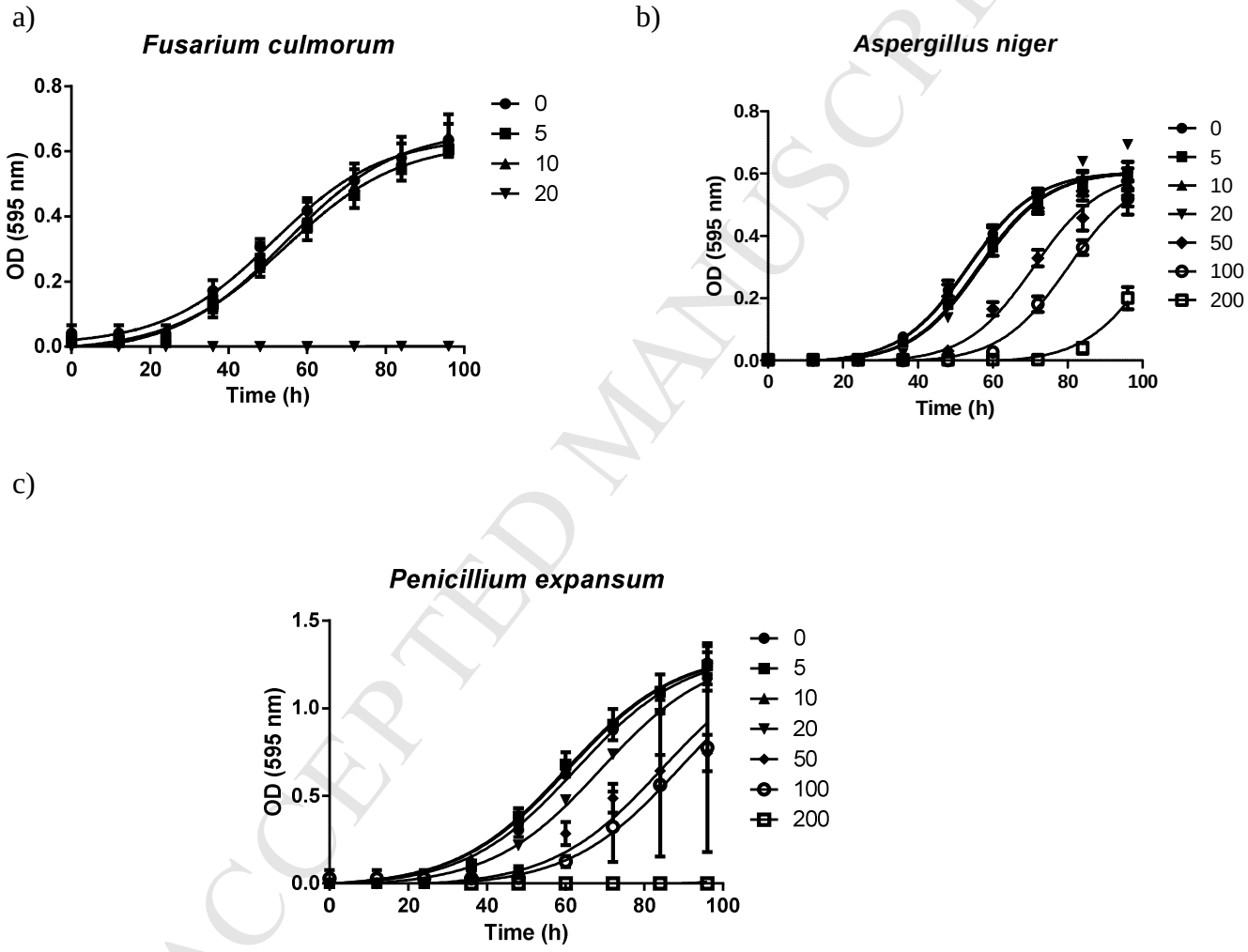
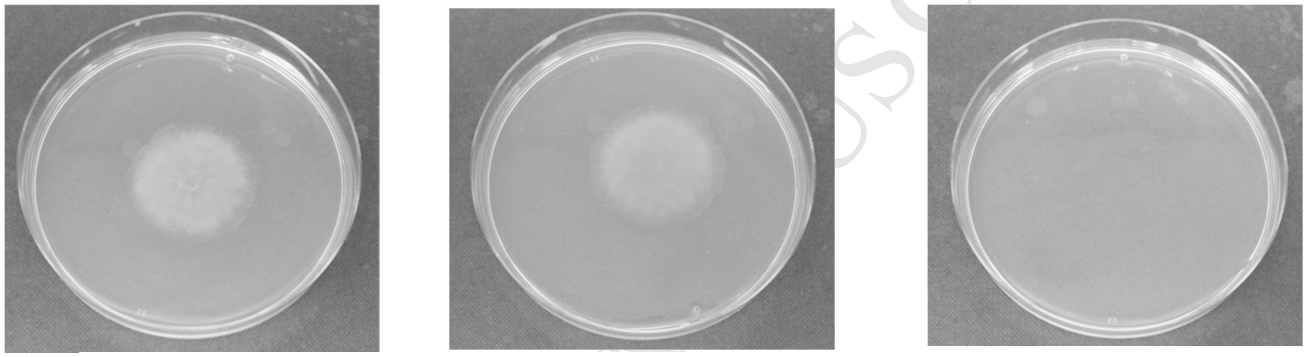


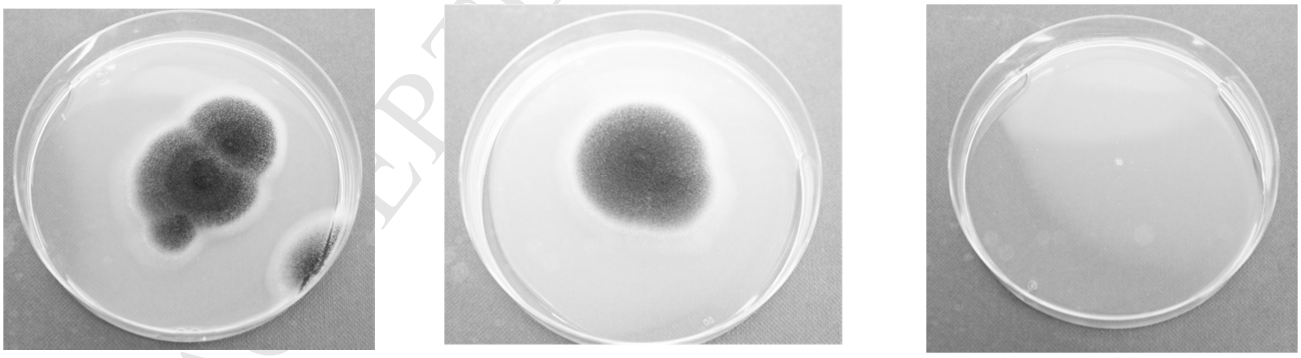
Fig. 1 Fungal growth inhibition by KT43C. Concentrations of KT43C are indicated in µg.ml^{-1}. (a) *Fusarium culmorum* FST 4.05, (b) *Aspergillus niger* FST 4.22, (c). *Penicillium expansum* FST4.21. All absorbance values are the mean of three replicates

a)



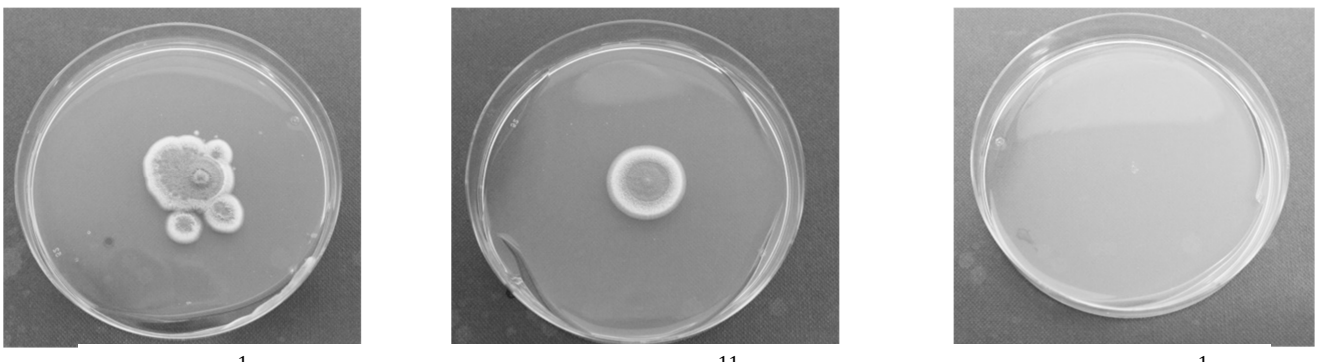
0 µg.ml^{-1} 10 µg.ml^{-1} 20 µg.ml^{-1}

b)



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

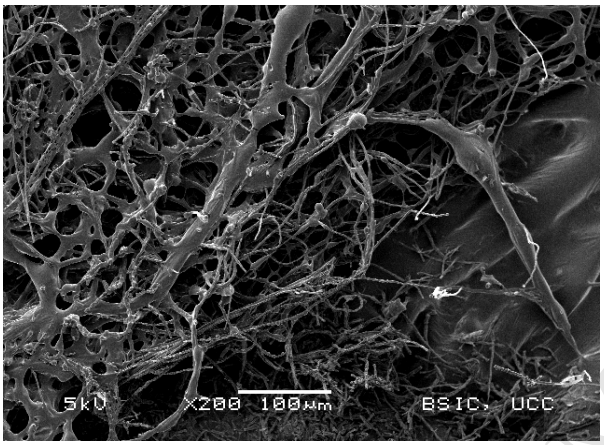
c)

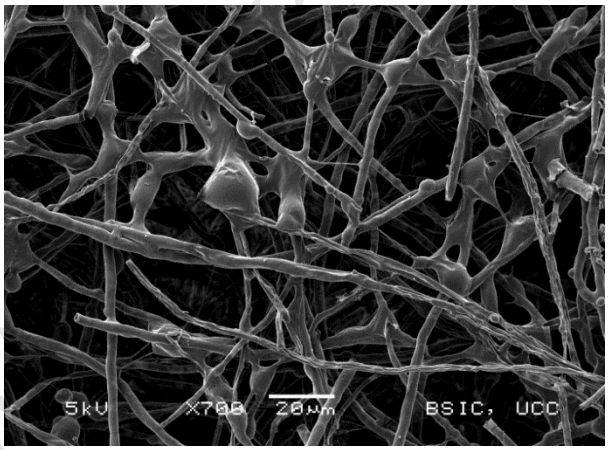


0 µg.ml^{-1} 100 µg.ml^{-11} 200 µg.ml^{-1}

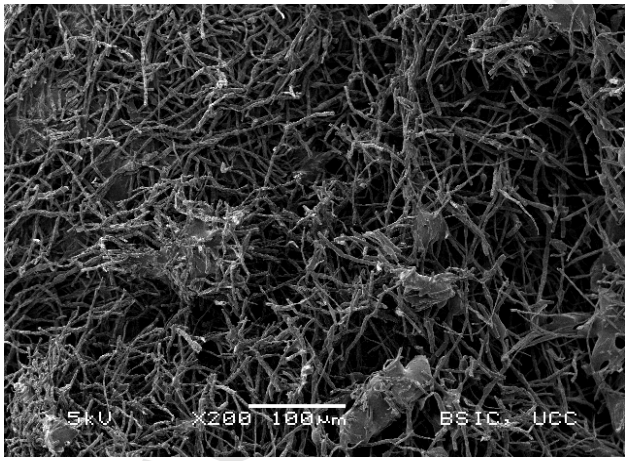
Fig. 2 Examination of fungal inhibition after 24 h of incubation in 1/2PDB with different concentrations of KT43C followed by 72 h of incubation on agar plates. (a) *F. culmorum* FST 4.05, (b) *A. niger* FST 4.22, (c). *P. expansum* FST4.21. The concentrations of KT43C are indicated below the pictures

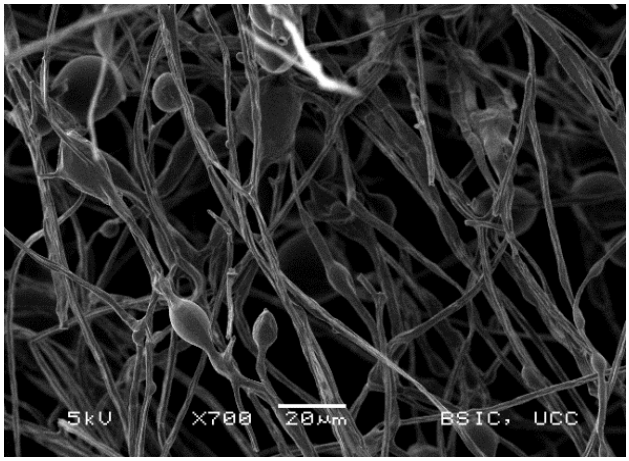
a)



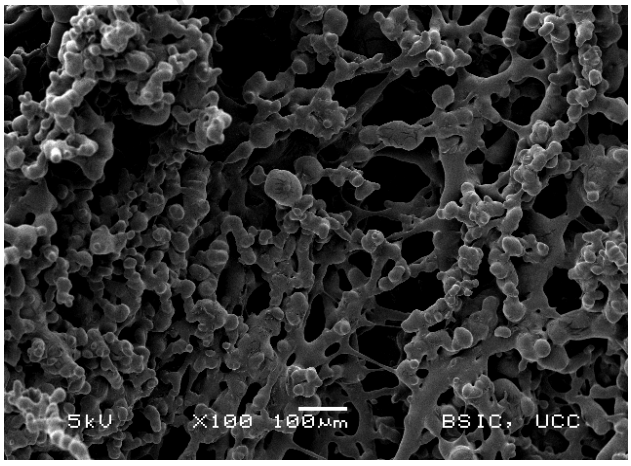


b)





c)



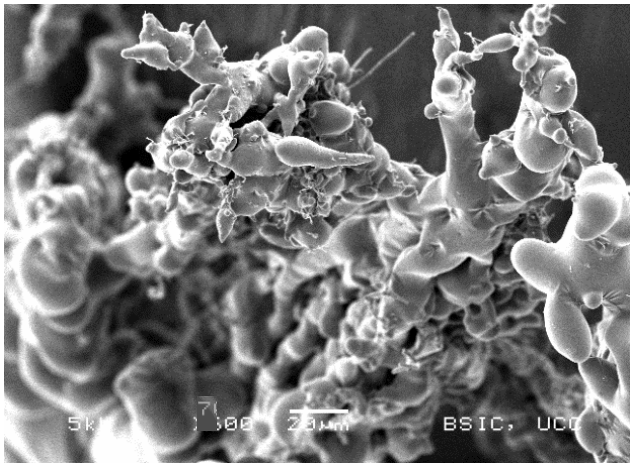


Fig. 3 Inhibition of *F. culmorum* germination in presence of KT43C observed with a scanning electron microscopy: a : Control ; b : 5 µg.ml^{-1} ; c : 20 µg.ml^{-1}. The magnifications are indicated on the pictures. The absence of conidial germination and mycelium are clearly visible with a concentration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

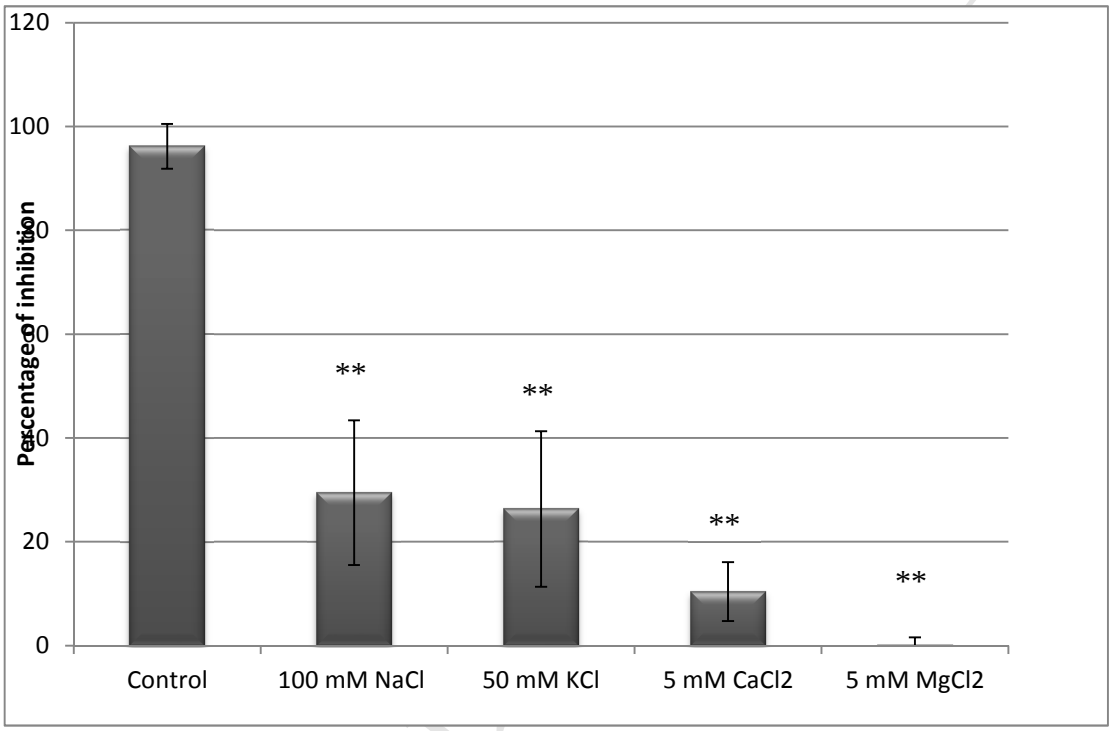
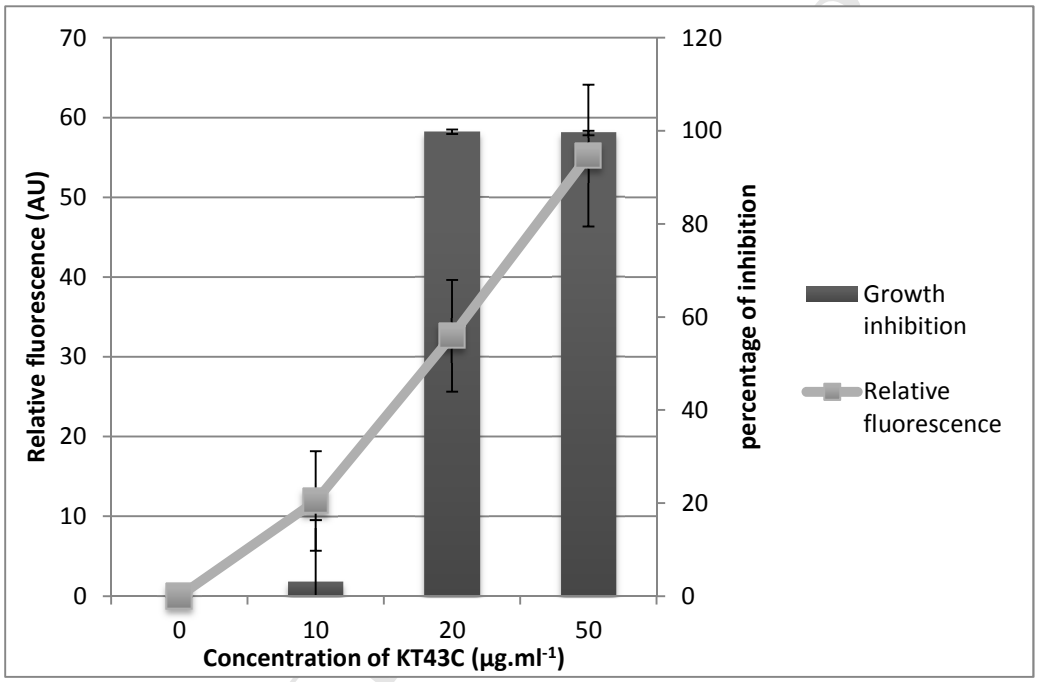


Fig. 4 Cation sensitivity of the synthetic cowpea defensin, KT43C (20 µg.ml^{-1}). Percentage of inhibition of *F. culmorum* growth after 96 h in growth medium in presence of different salts: **p<0.01, significant difference of the percentage of inhibition between the control and the tested media

a)



b)

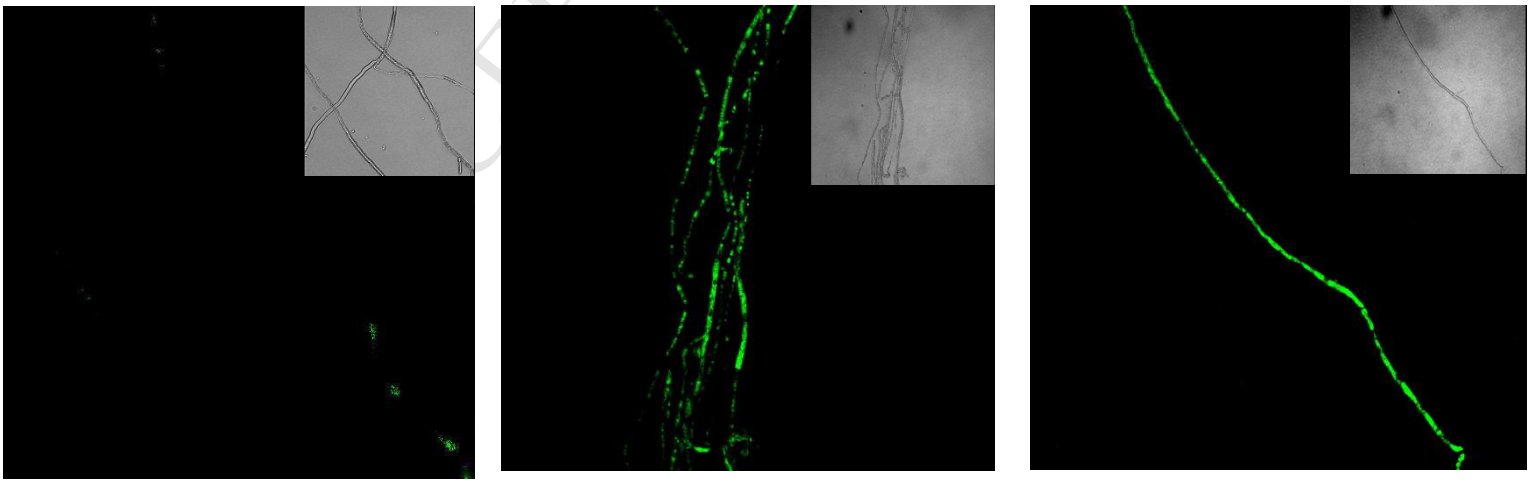
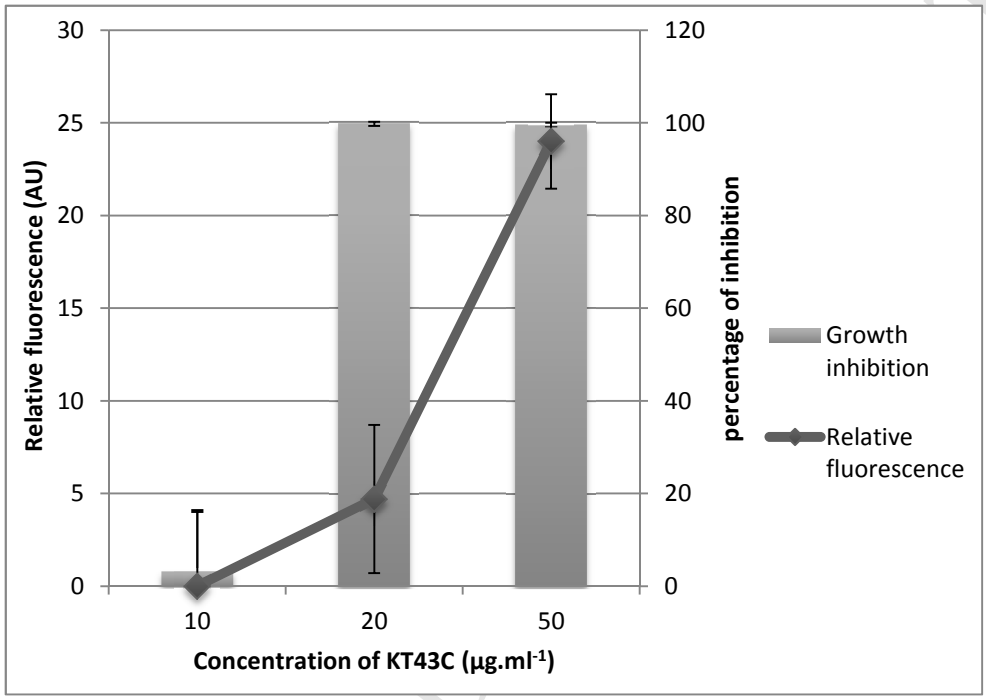


Fig. 5 SYTOX® Green uptake assay. a) After 18 h of growth, *F. culmorum* hyphae were treated for 24 h with KT43C (0, 10, 20 and 50 µg.ml^{-1}). Permeabilization of the fungal membrane was determined by fluorescence with SYTOX® Green (excitation 438 nm,

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 µg.ml^{-1} KT43C; right: 50 µg.ml^{-1}. High concentrations of KT43C induced permeabilization of fungal membrane, highlighted by fluorescence of the dye, and granulation of the fungal cytoplasm

a)



b)

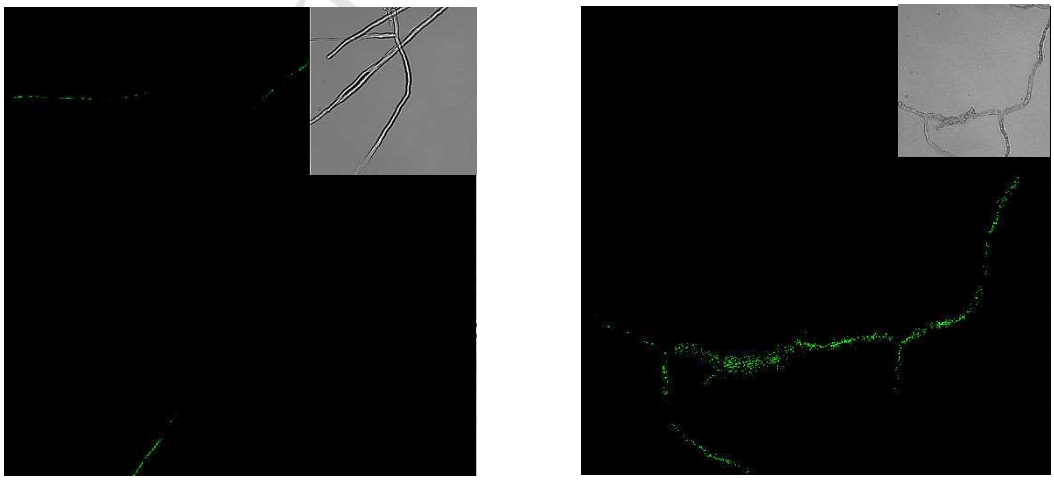


Fig. 6 Detection of reactive oxygen species (ROS) production. a) After 24 h of growth, *F. culmorum* hyphae were treated for 12 h with KT43C (0, 10, 20 and 50 µg.ml^{-1}). Production of ROS was determined by fluorescence of DHR 123 (excitation 438 nm, emission 538 nm),

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 µg.ml^{-1}

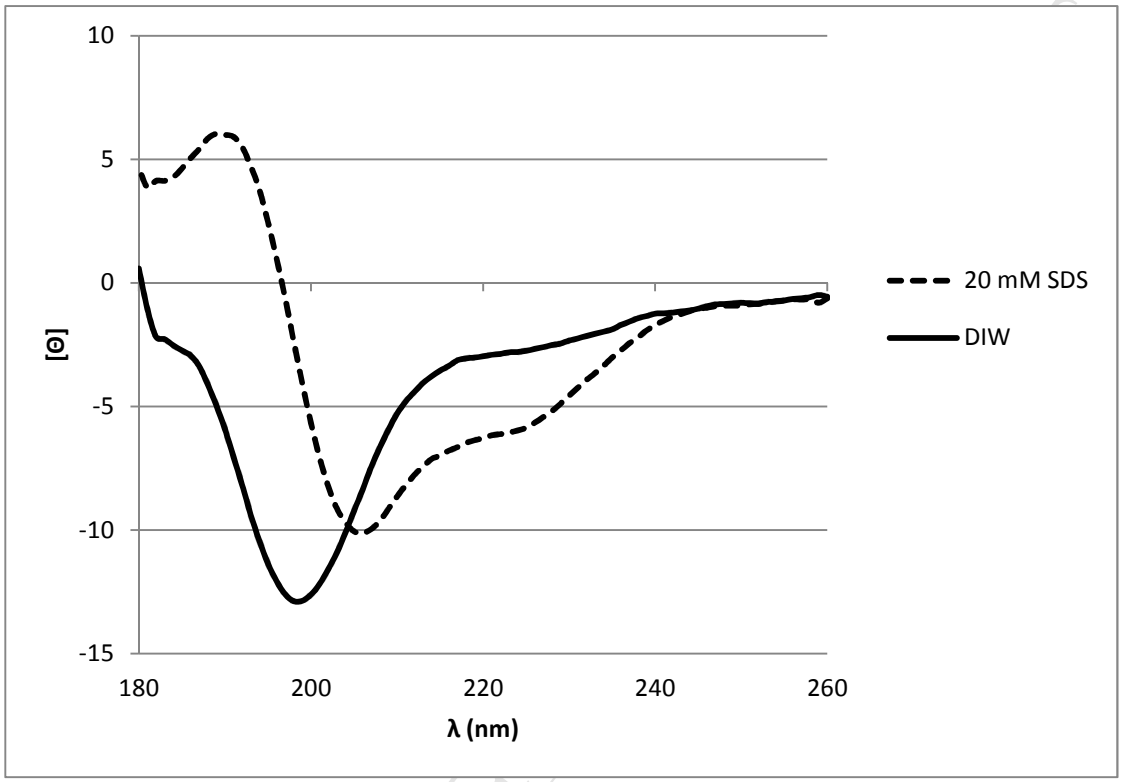
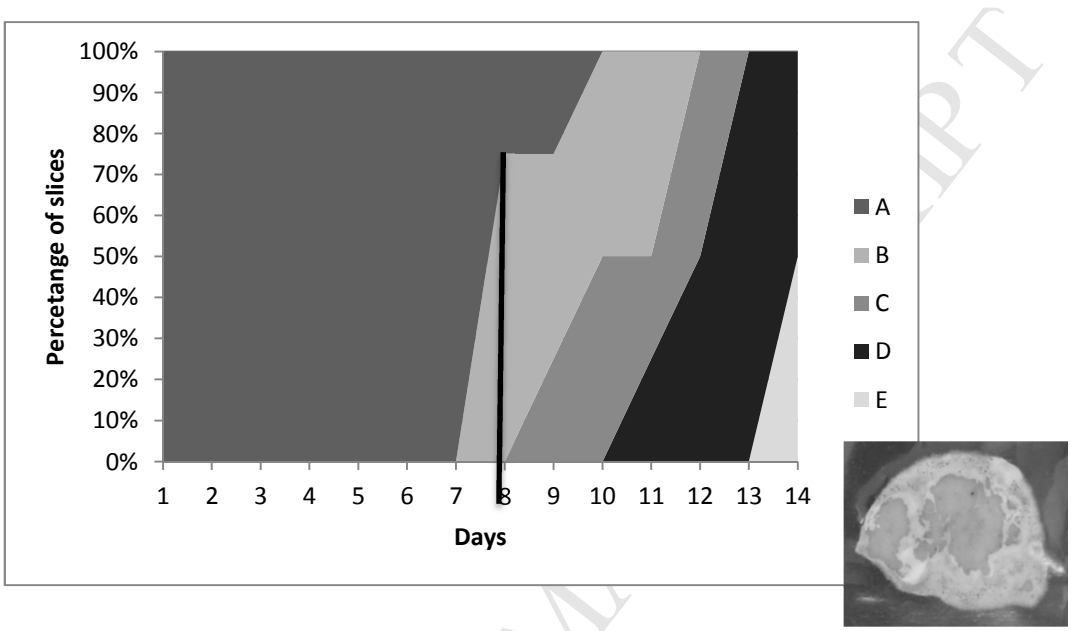


Fig. 7 CD spectra of KT43C in presence of DIW (solid line) or 20 mM SDS/DIW (dashed line). In DIW, the absence of crossover and the minimum at ~200 nm indicate unstructured conformation with transient β-hairpin. In SDS, KT43C adopts a more structured conformation with the presence of α–helical and β-hairpin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

a)



b)

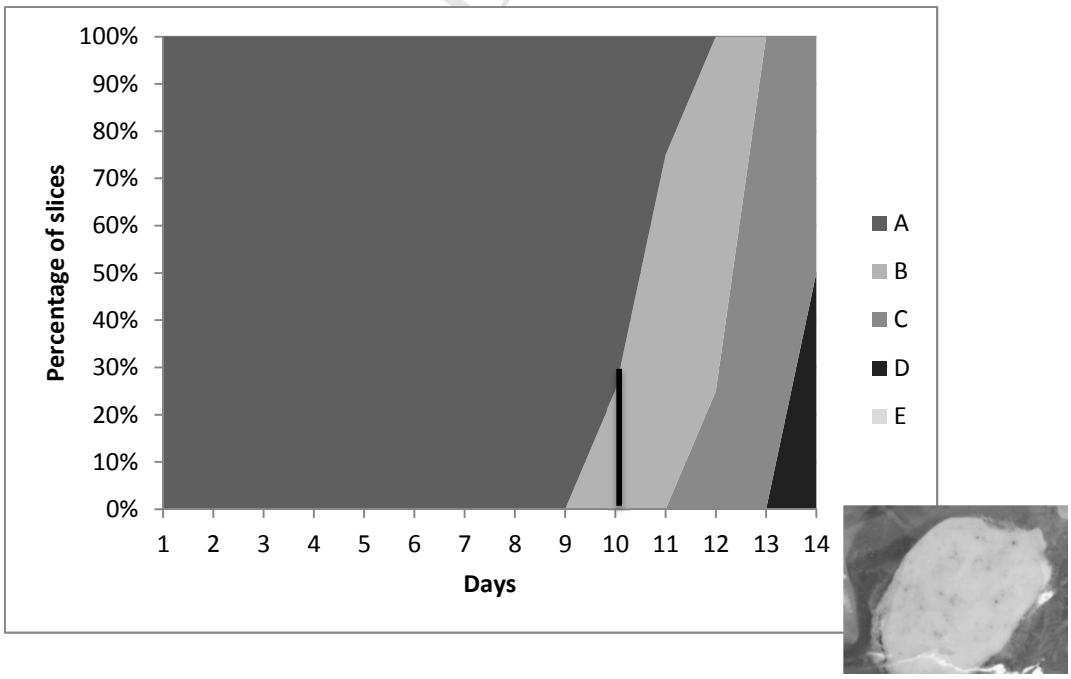


Fig. 8 Shelf life of chilled dough inoculated with *F. culmorum* ($1x10^2$ spores.ml^{-1}).The development of fungal mycelium was monitored over 14 days and the dough was classified

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

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