International Journal of Food Microbiology 300 (2019) 43–52

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Inhibitory effect of four novel synthetic peptides on food spoilage yeasts

Laila N. Shwaiki[a], Elke K. Arendt[a,b,*], Kieran M. Lynch[a], Thibaut L.C. Thery[a]

[a] School of Food and Nutritional Sciences, University College Cork, Cork, Ireland
[b] APC Microbiome Ireland, University College Cork, Cork, Ireland

ARTICLE INFO

Keywords:
Anti-yeast
Peptide
Preservative
Zygosaccharomyces
Synthetic

ABSTRACT

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

1. Introduction

Yeasts are a diverse group of microorganisms that have been exploited for the production of foods and beverages for centuries. In the food industry, yeasts are an essential tool for the manufacture of bread, beer and wine, amongst other products (Gil-Rodríguez et al., 2015; Hittinger et al., 2018). Despite their positive impact in the food and beverage sector, the negative impact of undesirable spoilage yeasts is a significant problem in food production and continue to cause unnecessary waste leading to major economic losses (Fleet, 2006). A target of spoilage yeasts includes dairy products (Ledenbach and Marshall, 2009), mayonnaises, salad dressings (Kurtzman et al., 1971), fruit juices (Tournas et al., 2006), soft drinks (Wareing and Davenport, 2005), alcoholic beverages (Jespersen and Jakobsen, 1996), and even chocolate (Ho et al., 2014). The most common spoilage yeasts found in food belong to the genera *Zygosaccharomyces*, *Pichia*, *Debaryomyces, Kluyveromyces, Candida* and *Saccharomyces* (Loureiro and Malfeito-Ferreira, 2003). Such food products spoiled by yeast include salad dressings (*Debaromyces*), soft drinks (*Zygosaccharomyces*), dairy products (*Kluyveromyces*), and alcoholic beverages (*Saccharomyces*). In order to prevent the growth of such spoilage yeast, several methods of preservation have been adopted by food industries, ranging from chemical to physical preservation techniques (Gould, 1996). However, spoilage by yeast still occurs.

The popularity of chemical preservatives has been decreasing due to consumers' demand for more natural foods which contain less chemicals. The exploitation and application of antimicrobial peptides (AMP) derived from animal, plant or microbial sources have great potential for addressing this desire for more natural methods of food preservation (Rai et al., 2016). AMPs are small proteins composed of 12 to 100 amino acid residues and are amphiphilic and generally positively charged; they are ubiquitous in animals, plants, insects and microorganisms (Jenssen et al., 2006; Mahlapuu et al., 2016). Exploiting natural AMPs to prevent the growth of spoilage microorganisms can be expensive and also difficult to apply in foods due to the fact that any type of peptide used for food application must be safe for ingestion. Designing novel, synthetic peptides, based on natural, native peptides, can be an alternative option for the application of peptides in foods (Mohamed et al., 2016). The aim of this study was to design a number of novel synthetic peptides and investigate their inhibitory activity

* Corresponding author at: School of Food and Nutritional Sciences, University College Cork, Cork, Ireland.
E-mail address: e.arendt@ucc.ie (E.K. Arendt).

https://doi.org/10.1016/j.ijfoodmicro.2019.04.005
Received 2 November 2018; Received in revised form 8 April 2019; Accepted 10 April 2019
Available online 13 April 2019

against yeast commonly found to cause food spoilage, namely, *Zygosaccharomyces* spp., (*Z. bailii* and *Z. rouxii*), *K. lactis, D. hansenii* and *S. cerevisiae*. The synthetic peptides were designed through the modification of one original peptide sequence. This peptide displayed a broad spectrum of activity against fungi, consisting of a short sequence, which can be advantageous for future reproduction at a low cost of synthesis. The design of the original starting peptide was based on sequences of different peptides with previously demonstrated activity against fungi. Thus, the attributes primarily hypothesised to be responsible for their antifungal activity were translated to, and exploited in the creation of this novel, original synthetic peptide. A hydrophobic centre, containing the hydrophobic residues Alanine (Ala), Tryptophan (Trp) and Proline (Pro) was chosen for the basis of this original peptide. In order to avoid a high cost of synthesis, the size of the peptide was limited to a short sequence of 14 amino acid residues. C-terminal amidation was incorporated in order to increase the peptides overall positive charge. Construction of a peptide with high haemolytic activity was avoided through the Lysine (Lys)/Arginine (Arg) and Trp/Phenylalanine (Phe) amino acids substitutions. The haemolytic activity is based on the measure of the peptides' ability to lyse erythrocytes (Ebbensgaard et al., 2018). Previous studies have shown Arg/Phe rich peptides to contain low haemolytic activity against red blood cells (Ebbensgaard et al., 2018; Strøm et al., 2002; Wessolowski et al., 2004).

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

2. Material and methods

2.1. Synthetic peptides used in this study

The starting peptide (KK-14) was assembled in conjunction with sequences of numerous other antifungal peptides shown to be effective. This peptide was used to generate 3 others by modifying its structure and were synthetized by GL Biochem (Shanghai) Ltd. The first modified peptide, termed Dip KK-14, was constructed by the addition of an unnatural ß-diphenylalanine residue as a substitution for Trp at position 8 (Trp8). The peptide termed, dKK-14, was generated by constructing a D-enantiomer of the KK-14 peptide, while the peptide, KK-14 (R10), was constructed via the replacement of the arginine residue with that of a proline. The sequences of the peptides are presented in Table 1. The purity of these peptides was > 90% as indicated by the supplier. Each peptide was resuspended in sterile distilled water and at a concentration of 2 mg/mL.

2.2. Yeast strains

Five different strains of yeast were used in this study. *Kluyveromyces lactis* ATCC56498, *Zygosaccharomyces bailii* Sa1403, *Zygosaccharomyces rouxii* ATCC14679, *Debaromyces hansenii* CBS2334 were obtained from DMSZ (Germany). *Saccharomyces cerevisiae* Baker's yeast was obtained from Puratos (Belgium). Each yeast was grown aerobically in Yeast Extract-Peptone-Dextrose (YPD; Sigma-Aldrich) agar at 25 °C. Stock cultures were maintained at −80 °C. Overnight incubation was performed in YPD broth at 25 °C under gentle agitation. All media and reagents used in this study were obtained from Sigma-Aldrich (MO, USA), unless otherwise stated.

2.3. Antifungal activity by disc diffusion assay

The susceptibility of the yeast strains to each peptide was demonstrated using the disc diffusion assay as outlined by Cai et al., 2016. Overnight cultures of each yeast were used to prepare a cell suspension of 5×10^6 CFU/mL. Cells were centrifuged at 1000*g* for 5 min and washed 3 times in sterile distilled water. A 1×10^5 cell suspension (1 mL) was added to 5 mL of 1% molten agarose (Serva, Heidelberg, Germany). This molten mixture was applied to YPD agar plates and allowed to set. Sterile paper discs, 6 mm in diameter were placed on the solidified agar and 50 µL of peptide was applied to the disc at a concentration of 1) the MIC (as determined in the broth microdilution assay) and 2) two-fold the MIC, for each peptide. The plates were then incubated at 25 °C for 3 days. Following incubation, the halo around the discs, representing the zone of inhibition, was measured in cm.

2.4. Antifungal assays and determination of minimum inhibitory concentration

For each yeast strain, the MIC for each peptide was determined using a broth microdilution method outlined by the National Committee for Clinical Laboratory Standards (NCCLS M-27A, NCCLS 2002). Suspensions of the yeast strains were prepared from overnight cultures and adjusted to 1×10^5 CFU/mL using a Marienfeld-Superior Haemocytometer. One hundred microliters of this suspension was added to a flat-bottomed 96-well microtitre plate (Sarstedt, Nümbrecht, Germany). Peptide (10 µL) was added into the first well of the plate at a concentration of 400 µg/mL and serially diluted to yield five different concentrations of each peptide (12.5 to 200 µg/mL). Ninety microliters of Yeast minimal media (YMM) [per 1 L; 0.8 g Complete Supplement Mixture (Formedium, Norfolk, United Kingdom) 6.5 g Yeast Nitrogen Base without amino acids (FORMEDIUM) and 10 g glucose] was added to each well to a total volume of 200 µL. Water, instead of peptide, was used as a control. The individual challenge experiment (yeast versus peptide) was performed on 2 different plates and in duplicate on each plate. Plates were incubated at 25 °C for 48 h with measurement of the optical density (600 nm) at 2 h intervals under gentle agitation (Multiskan FC Microplate Photometer, Thermo Scientific, MA, USA). The MIC was determined as the minimum concentration of peptide needed to inhibit the growth of the yeast. The peptides' ability to cause complete inhibition on the yeast was determined by subsequent

Table 1
Amino acid sequences of the four synthetic peptides applied in this study. The residues in bold and underlined represent the modifications to the original peptide.

Peptide	Amino Acid Sequence	
Original Synthetic Peptide (KK14)	KKFFRAWWAPRFLK-NH2	Lys-Lys-Phe-Phe-Arg-Ala-Trp-Trp-Ala-Pro-Arg-Phe-Leu-Lys-NH_2
Dip KK-14 Peptide	KKFFRAW**Dip**APRFLK-NH2	Lys-Lys-Phe-Phe-Arg-Ala-Trp-**Dip**-Ala-Pro-Arg-Phe-Leu-Lys-NH_2
dKK-14 Peptide	***(D-enantiomer)*** KKFFRAWWAPRFLK-NH2	Lys-Lys-Phe-Phe-Arg-Ala-Trp-Trp-Ala-Pro-Arg-Phe-Leu-Lys-NH_2
KK-14 (R10) Peptide	KKFFRAWWA**R**RFLK-NH2	Lys-Lys-Phe-Phe-Arg-Ala-Trp-Trp-Ala-**Arg**-Arg-Phe-Leu-Lys-NH_2

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

2.5. Effect of pH, salts and temperature on antifungal activity of peptides

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

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

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

2.6. Examination of membrane permeabilisation

Study of the peptides' mode of inhibitory action against the yeast, was examined by determining their potential to cause yeast cell membrane permeabilisation. Membrane permeabilisation is becoming increasingly recognised as a mechanism by which antimicrobial peptides target both yeast and both Gram positive and Gram negative bacterial cells (Dias et al., 2017; Lyu et al., 2016; Pérez-Peinado et al., 2018). This was tested against *S. cerevisiae,* a yeast frequently used in membrane permeabilisation studies of anti-yeast agents (Cools et al., 2017; Diz et al., 2006; Mayan, 2010). A yeast cell suspension of 10^6 CFU/mL was prepared from overnight cultures. Peptide (10 µL) was added into 90 µL of yeast cell suspension and incubated for 2 h at 25 °C, after which 5 µM of propidium iodide (PI) (SIGMA) was added. This solution was incubated at room temperature under dark conditions for 20 min, before being washed with YMM to remove unbound dye, centrifuged at 3000*g* for 5 min and applied to glass slides. Triton X-100 (0.1%) was used as the positive control as it is a known to cause membrane permeabilisation. PI was used for this assay since it can only penetrate cells with compromised plasma membranes and subsequently binds to nucleic acids. It will only fluoresce when bound, therefore enabling detection of whether membrane permeabilisation has occurred. The extent of permeabilisation was measured using a confocal laser scanning microscope (CLSM) (OLYMPUS FV3–259) at the maximal excitation (λ_{Ex}) and maximum emission (λ_{Em}) wavelengths of 535 nm and 617 nm, respectively.

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

2.7. Examination of reactive oxygen species generation

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

2.8. Application of peptides in food matrices

The antifungal effect of the peptides was investigated in two food matrices (i.e. a soft drink and mayonnaise) in which fungal spoilage can occur, in particular with *Z. bailii*, which was therefore used for this assay. For the soft drink (Fanta Orange, Coca-Cola, Ireland) a microtitre plate method, as applied for the antifungal assay was used. The yeast was inoculated in YPD for overnight culture and a 10^2 cfu/mL solution was prepared in filter sterilised Fanta Orange from this overnight culture. This low concentration of yeast was used to represent the number of cells commonly found to spoil products such as soda drinks. All four peptides were tested using the same conditions and concentrations as performed for the microdilution plate assay (minimum of 12.5 µg/mL and maximum 200 µg/mL). The optical density (600 nm) was monitored over 48 h at 25 °C. Controls consisted of Fanta Orange with 10^2 cfu/mL yeast in the absence of peptide, and Fanta Orange with no yeast. The ingredients of the Fanta Orange used for this application, as listed on the label, were as follows: carbonated water, sugar, orange fruit from concentrate (5%), citric acid, vegetable concentrates (carrot and pumpkin), natural orange flavouring with other natural flavourings, preservative (potassium sorbate), malic acid, acidity regulator (sodium citrate), sweeteners (acesulfame-K, aspartame), antioxidant (ascorbic acid), and stabilizer (guar gum). It also contained a source of phenylalanine. The pH of the Fanta Orange was recorded as 3.1; a pH lower than what was found for the YMM (pH 5.33).

The application of the peptides in mayonnaise (Hellman's, Unilever, United States) was also tested. The ingredients of the mayonnaise, as listed on the label, was as follows: Canola Oil, Water, Liquid Whole Egg, Vinegar, Liquid Yolk, Salt, Sugar, Spices, Concentrated Lemon Juice and Calcium Disodium EDTA. Overnight cultures of *Z. bailii* were added to mayonnaise at 10^2 cfu/mL in addition to peptide (12.5 to 200 µg/mL). This yeast, peptide and mayonnaise solution (100 µL) was spread onto YPD agar and incubated at 25 °C for 48 h.

2.9. Statistics

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

3. Results

3.1. Antifungal disc diffusion assay

The susceptibility of *Z. bailii,* a yeast already found be sensitive towards the peptides, was tested using a disc diffusion assay. The zones

Table 2
Z. bailii Sa1403 susceptibility in the disc diffusion assay. The table represents the degree of inhibition based on the measurement of the diameter of the zone of inhibition surrounding the discs. + + + = susceptible (16 mm to 14 mm); + + and + = intermediate (14 mm to 10 mm); − = resistant (no zone of inhibition).

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

of inhibition (Table 2) against the respective peptide were evaluated. KK-14, Dip KK-14 and dKK-14 all showed zones of inhibition at concentrations of 50 and 25 μg/mL. KK-14 (R10) had a halo around the disc containing 50 μg/mL peptide but not 25 μg/mL. As expected, all four peptides did not inhibit the yeasts at 12.5 μg/mL. Dip KK-14 (50 μg/mL) was not as prominent on the plate, even though this concentration of peptide was two-fold the MIC determined in the microdilution broth assay (Fig. 1).

3.2. Antifungal microtiter plate assay

The antifungal activity of the peptides was tested through incubation with the yeast in a microtitre plate. The growth of the yeast was observed over time to detect their antifungal properties. Each of the four peptides showed variation in the degree of their anti-yeast activity. Apart from *Z. rouxii,* each yeast strain was found to be sensitive to each of the peptides at one or more of the tested concentrations (Table 3). The most sensitive yeast was *Z. bailii*, displaying the lowest MIC, at 50 μg/mL, while the most active peptide was Dip KK-14 against all 5 yeast strains. The fungistatic or fungicidal ability of the peptides on the yeast varied and was dependant on the strain and peptide. The peptide with the highest fungicidal activity and completely inhibiting most yeast at its MIC was Dip KK-14. While KK-14 (R10) also displayed broad fungicidal activity at its MIC, this MIC was higher than that of Dip KK-14.

The Minimal Fungicidal Concentration (MFC) of the peptides which displayed only fungistatic activity at their MIC was found to be as high as 800 μg/mL. *Z. rouxii* and *S. cerevisiae* were the least susceptible to the fungistatic properties of Dip KK-14 and dKK-14, respectively (Table 4).

3.3. Salt stability of the peptides

The antifungal assay was repeated with the addition of different salt concentrations ($MgCl_2$ and KCl) to test the stability of the each of the peptides. The presence of $MgCl_2$ at both concentrations tested (1 mM and 5 mM) decreased the antifungal activity of KK-14 enabling *Z. bailii* to grow at the MIC (50 μg/mL). The higher concentration of 5 mM was more effective at disrupting the activity of the peptide, allowing growth to occur at double the MIC (i.e. 100 μg/mL). dKK-14 was more effected at the higher concentration of $MgCl_2$, with *Z. bailii* growing even at the MIC (50 μg/mL). KK-14 (R10) was least effected by $MgCl_2$, as observed by the inhibition of growth at its MIC, 50 μg/mL. Dip KK-14 were not affected by 1 mM $MgCl_2$, as it still displayed inhibitory activity at its MIC (50μg/mL); however, 5 mM $MgCl_2$ affected the peptide to some extent, as growth was observed at the MIC (Fig. 2).

The presence of KK-14 in 50 and 150 mM KCl resulted in its antifungal activity being lessened, both at its MIC and double that MIC. dKK-14 was more affected by the higher concentration of KCl in comparison to the lower concentration, with *Z. bailii* growing at both its MIC and double the MIC. The higher KCl concentration decreased the antifungal activity of KK-14 (R10), enabled the growth of *Z. bailii* at 50 and 100 μg/mL. The lower concentration of KCl (50 mM) was less effective at disrupting the antifungal activity. The MIC of Dip KK-14 was affected at both KCl concentrations, with 150 mM causing more disruption to the peptide's ability to inhibit *Z. bailii* (Fig. 3).

After performing ANOVA, significant differences were observed between the peptide concentrations that were found to inhibit *Z. bailii* in the salt solutions, as can be seen from Figs. 2, 3 and 4.

3.4. Temperature stability of the peptides

The peptides were subjected to temperature of 100 °C and tested in an antifungal assay for their inhibitory effect against the yeast. Heating of KK-14 at 100 °C for 15 min caused a reduction in the antifungal properties of the peptide, resulting in the increased growth of *Z. bailii* in the presence of this peptide. At both the MIC (50 μg/mL) and double the

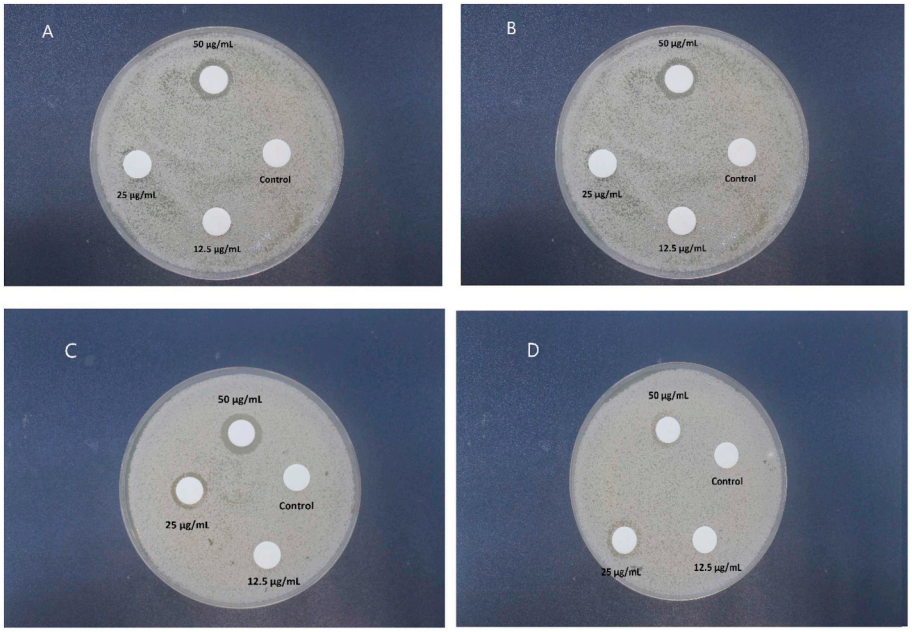

Fig. 1. Results of the disc diffusion assay against *Z. bailii* Sa1403. Each peptide was tested at 50, 25 and 12.5 μg/mL. KK-14 (A), Dip KK-14 (B), dKK-14 (C) and KK-14 (R10) (D) can be seen to show varying degrees of inhibition.

Table 3
Minimum Inhibitory Concentration values of the peptides against the yeast strains. The nature of the inhibitory activity – fungistatic or fungicidal – is also shown.

	Kluyveromyces lactis ATCC56498	*Zygosaccharomyces bailli* Sa1403	*Zygosaccharomyces rouxii* ATCC14679	*Debaromyces hansenii* CBS2334	*Saccharomyces cerevisiae* Baker's yeast
KK-14	100 µg/mL –Fungicidal	50 µg/mL –Fungistatic	No Inhibition	50 µg/mL - Fungicidal	200 µg/mL - Fungicidal
Dip KK-14	25 µg/mL –Fungicidal	25 µg/mL –Fungicidal	200 µg/mL - Fungistatic	50 µg/mL -Fungicidal	50 µg/mL - Fungicidal
dKK-14	100 µg/mL –Fungistatic	50 µg/mL –Fungicidal	No Inhibition	25 µg/mL -Fungicidal	200 µg/mL - Fungistatic
KK-14 (R10)	200 µg/mL –Fungicidal	50 µg/mL –Fungicidal	No Inhibition	100 µg/mL -Fungicidal	200 µg/mL - Fungicidal

Table 4
Minimum Fungicidal Concentration values of the peptides shown to have fungistatic properties against the different yeast strains. Maximum concentration tested was 800 µg/mL.

Yeast strain	Peptide	MFC (µg/mL)
Z. bailii Sa1403	KK-14	400
K. lactis ATCC56498	dKK-14	400
S. cerevisiae - Baker's yeast	dKK-14	800
Z. rouxii ATCC14679	Dip KK-14	800

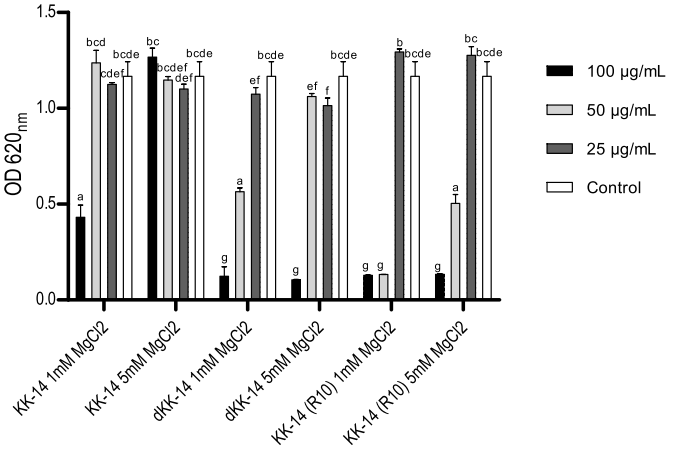

Fig. 2. Effect of $MgCl_2$ on the inhibitory activity of the peptides KK-14, dKK-14 and KK-14 (R10) against *Z. bailii* Sa1403, at concentrations of 1 and 5 mM. Means sharing the same letter do not differ significantly at the 95% confidence level based on the Tukey mean comparison method; $p < 0.05$.

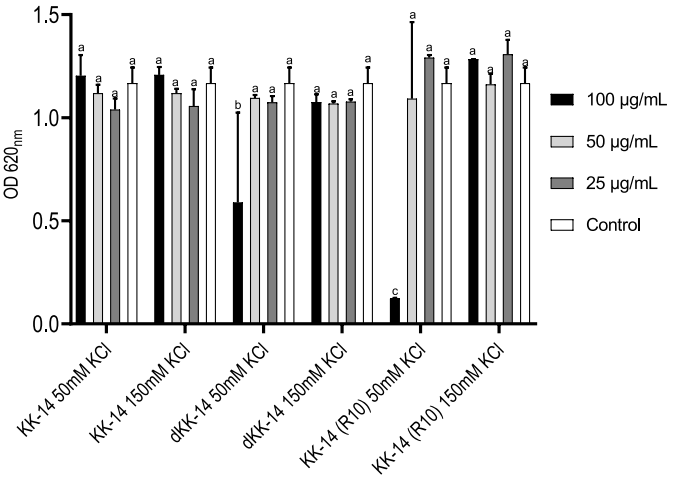

Fig. 3. Effect of KCl on the inhibitory activity of the peptides KK-14, dKK-14 and KK-14 (R10) against *Z. bailii* Sa1403, at concentrations of 50 and 150 mM. Values with different letters vary statistically. Tukey Test; $p < 0.05$.

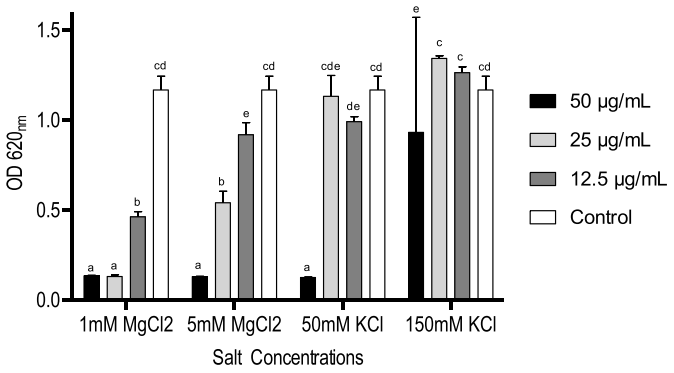

Fig. 4. Effect of KCl and MgCl2 at concentrations of 50 and 150 mM, and 1 and 5 mM, respectively, on the inhibitory activity of Dip KK-14 against *Z. bailii* Sa1403. Values with different letters vary statistically. Tukey Test; $p < 0.05$.

Table 5
Effect of heat treatment (100 °C for 15 min) on each peptide and the resulting antifungal activity against Z. bailii Sa1403 post-heat treatment.

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

n.d., concentration not tested.

MIC (100 µg/mL), the peptide caused no inhibition of *Z. bailii* as was originally observed in the antifungal assay. dKK-14, KK-14 (R10) and Dip KK-14 were not affected by this increase in temperature, evident by the inhibition caused at the MIC. The inhibition of the yeast at all 3 concentrations (double the MIC, its MIC and half the MIC) was observed (Table 5).

3.5. Effect of pH on antifungal activity of the peptides

The antifungal assay was repeated using YMM changed to different pH to observe the inhibitory effect of each peptide under such pH stress (Table 6). Changing the pH of the media to pH 3 (control pH 5.33) resulted in a decrease in *Z. bailii* inhibition caused by the four peptides. Increasing the pH to 9 and 11 resulted no yeast growth in the control comprising of *Z. bailii* in the absence of peptides. The greatest effect caused by change in pH was seen in pH 5 and 7. KK-14 was affected at pH 5 with a decrease in its antifungal activity, thus enabling growth of *Z. bailii* at the MIC and even at double that MIC. At pH 7, inhibition was only observed at the higher peptide concentration tested (100 µg/mL, double the MIC). For the dKK-14 peptide, at pH 5, inhibition was only observed in double its MIC, indicating a decrease in its antifungal activity. pH 7 did not affect the antifungal activity of dKK-14. An increase in antifungal activity was observed for Dip KK-14 at pH 5 and 7. KK-14 (R10) was more effective at pH 7, compared to the control (unadjusted) medium, where complete inhibition was observed at half the MIC. At

Table 6
The effect of different pH on the stability and antifungal activity of the four peptides against Z. bailii Sa1403. Inhibition = No growth in the MIC and double the MIC; Complete Inhibition = No growth in all three concentrations tested; No inhibition = Growth in all three concentrations.

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

pH 5, use of the MIC and double that resulted in a similar level of inhibition to that observed in the microtitre plate antifungal assay.

3.6. Membrane permeabilisation activity

Membrane permeabilisation is becoming increasingly recognised as a mechanism by which antimicrobial peptides target both yeast and both Gram positive and Gram negative bacterial cells (Dias et al., 2017; Lyu et al., 2016; Pérez-Peinado et al., 2018). *S. cerevisiae* was incubated with the different concentrations of peptide found to be inhibitory and viewed under the CLSM to observe the mode of action of the peptides. Membrane permeabilisation of *S. cerevisiae* was assessed in the presence of the peptides. The yeast showed strong fluorescence when treated with 400 µg/mL of the peptides, Dip KK-14, dKK-14 and KK-14 (R10). KK-14, however did not affect the cell membrane at 400 µg/mL (Fig. 5). The degree of permeabilisation decreased as the concentration of peptides decreased, in a dose-dependent manner. The lowest concentration for all four peptides (25 µg/mL) showed almost no permeability. The original synthetic peptide, KK-14, in general, caused less permeabilisation of *S. cerevisiae* (Figures for KK-14, Dip KK-14 and dKK-14 not shown).

3.7. Kinetics of membrane permeabilisation

Each peptide was incubated with *S. cerevisiae* and propidium iodide in a microtitre to follow the progress of permeabilisation of the yeast in the presence of the peptides. For KK-14 (R10) and dKK-14, the kinetics revealed faster permeabilisation over 6 h. As predicted, both peptides resulted in a steady increase in the measured fluorescence over the period of 6 h, indicating the occurrence of membrane permabilisation (Fig. 6). KK-14 (R10) at the concentrations of 400, 200 and 100 µg/mL all showed permeabilisation while only 400 and 200 µg/mL of dKK-14 caused observable permeabilisation (Fig. 7). Significantly lower

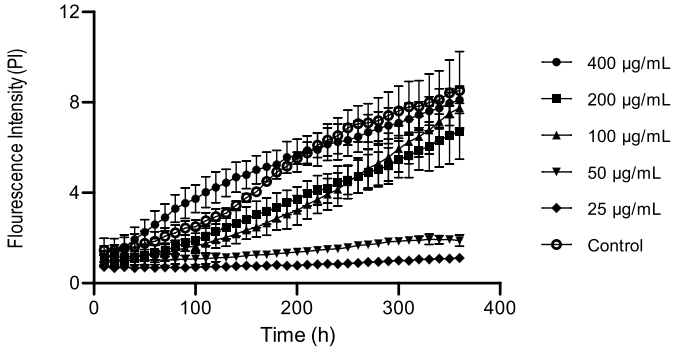

Fig. 6. Kinetics indicating the rate of membrane permeabilisation of *S. cerevisiae* – Baker's yeast in the presence of KK-14 (R10).

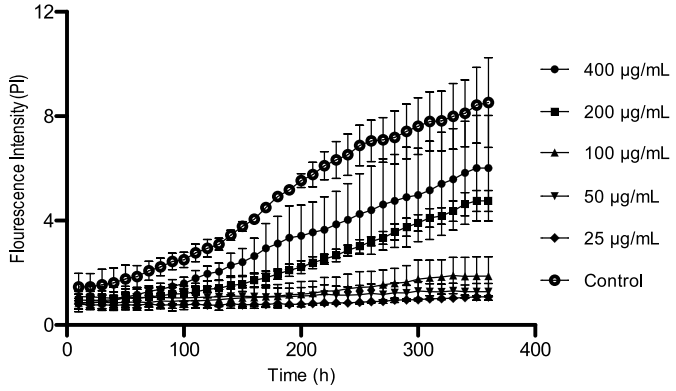

Fig. 7. Kinetics indicating the rate of membrane permeabilisation of *S. cerevisiae* – Baker's yeast in the presence of dKK-14.

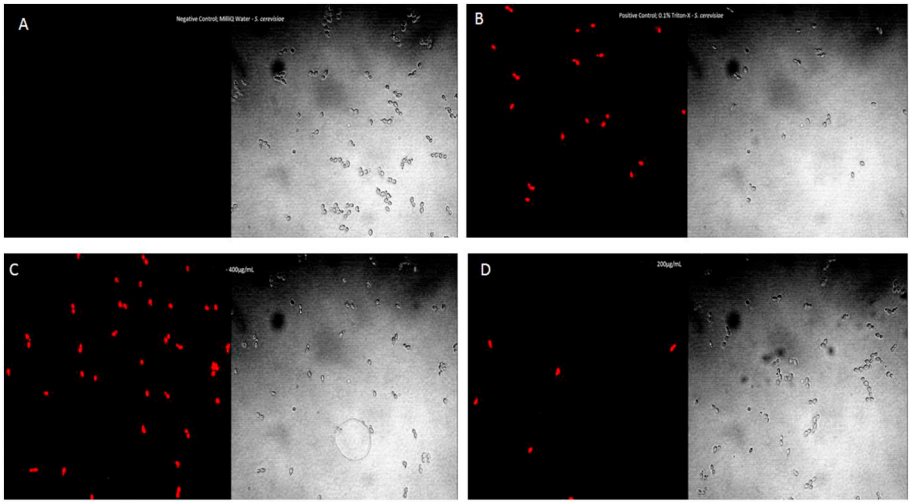

Fig. 5. Results of the permabilisation effects of KK-14 (R10) on *S. cerevisiae* – Baker's yeast. A, B, C and D show the negative control, positive control, 400 µg/mL and 200 µg/mL peptide, respectively.

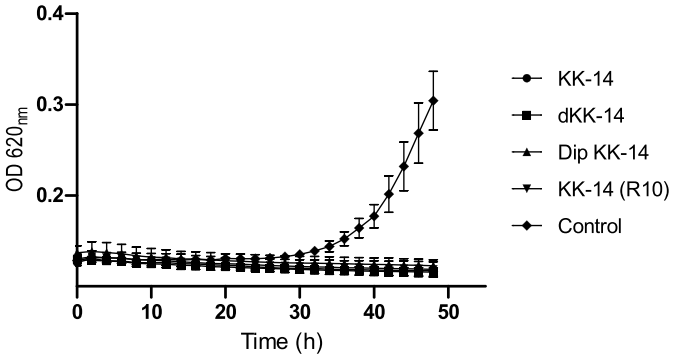

Fig. 8. Antifungal assay of peptide KK-14, Dip KK-14, dKK-14 and KK-14 (R10) against *Z. bailii* Sa1403 in Fanta Orange at concentration of 12.5 μg/mL.

permeability was observed after 6 h with KK-14 and Dip KK-14 (data not shown).

3.8. Stimulation of reactive oxygen species production

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

3.9. Application of peptides in a food matrix

The antifungal assay was repeated using Fanta Orange as the main medium for the yeast to grow. Investigation of the application and effect of the peptides in a soft drink (Fanta Orange) found that they caused complete inhibition of *Z. bailii,* inoculated at 10^2 cfu/mL, at all concentrations tested. Fig. 8 shows the extent of inhibition that each peptide caused at 12.5 μg/mL.

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

4. Discussion

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

The generation of a novel peptide sequence based on numerous antifungal peptides was modified to generate three derivative peptides. This was accomplished by altering specific residues in the original sequence. Their ability to cause inhibition of the yeast species tested was found to vary, being strain- and peptide-dependant. The original peptide, KK-14, inhibited 4 of the 5 yeasts, with *Z. rouxii* showing complete resistance even at 200 μg/mL. *K. lactis, Z. bailii, D. hansenii* and *S. cerevisiae* showed inhibition at concentrations of 100, 50, 50 and 200 μg/mL, respectively. The incorporation of Ala, Trp, Pro, Phe and Leucine (Leu) in the primary sequence served to impart hydrophobicity on the peptide. In general, an increase in hydrophobicity promotes the spontaneous insertion of such peptides into cell membranes (Engelman and Steitz, 1981). In addition, the presence of two Lys residues on the N- and C-terminal of the sequence helped to provide the peptide a position on the yeast cell membrane into which it could better infiltrate (Stark et al., 2002). It has previously been shown that peptides which displayed antimicrobial activity against Gram positive and Gram negative bacteria contained these residues which imparted a hydrophobic nature on these peptides, and likely contributed to their antimicrobial activity. Thus, the incorporation of such residues in novel peptides should promote antimicrobial activity, making them suitable for the generation of novel AMPs. Dip KK-14 consisted of the same amino acid sequence as the original peptide with the exception of a β-di-phenylalanine (Dip) residue substituted for Trp10. β-di-phenylalanine is an amino acid with a similar structure to alanine and phenylalanine and has its amino group bonded to the β carbon (Cheng et al., 1992). The antifungal activity of this peptide was found to be higher than KK-14 against all yeast may be attributed to the insertion of the Dip residue. A previous study showed that the presence of two Phe residues in a modified peptide sequence significantly improved the antimicrobial activity of the peptide by causing a greater disruption to bacterial cell membranes (Shahmiri et al., 2015). In the present study, β-di-phenylalanine replaced a non-hydrophobic Trp, thus increasing the hydrophobicity of the peptide, which could explain the observed increase in antifungal activity. The addition of the β diphenylalanine as a modification of the original KK-14 peptide produced peptide with longer side chains, which could have promoted cell membrane penetration in comparison to a peptide with the Trp residue in that position. The use of a β- instead of an α-diphenylalanine was applied to increase the proteolytic resistance of the peptide, as it is less likely to be degraded in this form (Cabrele et al., 2014).

The antimicrobial activity of the D-enantiomeric peptide showed similar inhibitory capacity to that of KK-14 against all yeasts, except *D. hansenii*. This decrease in yeast resistance of *D. hansenii* could be attributed to an increased ability of the D-enantiomer to cause membrane permeabilisation or it could potentially be due to this peptides increased resistance to proteases secreted by this yeast (de la Fuente-Núñez et al., 2015). It has been shown that the D-enantiomeric form of studied peptides not only display an increase in antifungal activity compared to their original counterpart, but also demonstrate proteolytic resistance, including to human serum proteases (Jung et al., 2007; Tugyi et al., 2005). Another reason for the increase in activity against *D. hansenii* could be due to the backbone of the D-enantiomer; having different rotations in three-dimensional space may lead to its side chains having different topologies, therefore effecting its antimicrobial activity (Mohamed et al., 2017).

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

At the concentration of 800 μg/mL, *S. cerevisiae* and *Z. rouxii* were completely inhibited by dKK-14 and Dip KK-14, respectively, in both the disc diffusion assay and microdilution broth assay. This indicates that the minimum amount of these peptides needed to cause complete killing of the yeast was as high as 800 μg/mL. For KK-14 and dKK-14 against *Z. bailii* and *K. lactis*, respectively, 400 μg/mL was the minimum fungicidal concentration.

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

The kinetics of the membrane permeabilisation demonstrated that KK-14 (R10) caused permeabilisation at a faster rate than KK-14 and Dip KK-14. The higher helicity caused by the Arg residue, as well as the Trp/Arg succession in this peptide sequence could have resulted in the increased permeabilisation of the yeast (Deslouches et al., 2016). Many well studied AMPs are recognised for their ability to induce cell death via a number of different mechanisms. One of which is through the stimulation of ROS overproduction (Bondaryk et al., 2017). This was not detected, even at the highest concentration of 400 μg/mL. This suggests that ROS production was not a factor in the antifungal activity of the peptides. Thus, membrane permeabilisation was the more likely mode of action of the peptides. Interactions between the peptides and the binding sites on the yeast's membrane known as sphingolipids, could have facilitated such membrane permeabilisation (Bondaryk et al., 2017). The hydrophobicity of the peptides also contributed to this mode of action. It has been shown that the amino acid residues Phe, Trp, Leu and Ala, present in each the peptides, contain hydrophobicities above a threshold value (a value obtained from the calculated indices of a peptide's amino acid residues and which determines the peptide's hydrophobicity and thus its ability to integrate into cell membranes) enabling them to spontaneously insert into membranes (Liu et al., 1996; Stark et al., 2002). Thevissen et al., 1999 demonstrated that the primary cause of growth inhibition by plant defensins is through binding-site mediated insertion.

The stability of the peptides was tested under high salt concentrations the concentrations of which were tested being based on previous research (Betts et al., 1999; Kandasamy and Larson, 2006; Wu et al., 2008). Both salts showed a similar negative effect on the antifungal activity, particularly at the higher concentration; however the modified peptides were effected to a lesser extent than the original KK-14 peptide. There was a statistical difference in the peptides' inability to inhibit *Z. bailii* under the influence of the different salt concentrations, in particular for $MgCl_2$. The presence of cations in the medium may be a factor that could have prevented the peptides from interacting with the cell membrane of the yeast, a significant step required for optimal effect. It has been shown that at a higher salt concentration, peptides are less likely to interact with the cell membrane. In addition, the presence of salts may have altered the peptides' overall charge causing a modification to their structure (Baldauf et al., 2013). This interference could be due to a reduction of the available head group area for the lipids, which leads to their tighter packing, making it more difficult for the peptides to penetrate and cause effect (Wu et al., 2008). The sensitivity of these peptides to the monovalent cation K^+, in the form of KCl, reduced their antifungal activity at high salt concentration. Testing the peptides against the salts enabled for the better understanding of the peptides' response to a high salt matrix, an environment commonly found in food spoiled by such yeast (Rawat, 2015).

Subjecting KK-14 to a heat treatment caused a decrease in peptide activity against *Z. bailii*. Heating could have caused irreversible changes to its secondary or tertiary structure. This loss of structure may have consequently caused a reduction in its antifungal activity (Ozkan et al., 2016). The stability of dKK-14, KK-14 (R10) and Dip KK-14 was unaffected, indicating their stability in high temperature. One potential reason for this could be the lack of tertiary structure and the flexibility of the peptide. It's been shown that the greater the flexibility of some cationic peptides, the more enhanced the antimicrobial effects (Liu et al., 2013).

Adjusting the medium to pH 3 resulted in no inhibition of *Z. bailii* growth in the presence of any peptide. This could be due to the ability of yeast to thrive in acidic environments. *Z. bailii* is a yeast notorious for its resistance to acidic, low pH, environmental stresses (Palma et al., 2017). In addition, significantly changing the pH of the medium could have modified the charge on the peptides, leading to the alteration in peptide folding. This could have then resulted in a decrease in their activity (Di Russo et al., 2012). This change in peptide structure coupled with *Z. bailii*'s resistance to the low pH media may explain this complete lack of inhibition observed (Yang and Honig, 1993). At high pH, there was complete inhibition of growth; however, this was likely due to the unfavourably high pH being unsuitable for growth, rather than peptide activity (Dang et al., 2010).

Dip KK-14 was the most favourable peptide that could be considered in the application as an antifungal agent. The combination of its low MIC, good membrane permeability, and stability against most of the salt concentrations, make for a promising antifungal agent.

Application of the peptides to two food group with which *Z. bailii* is commonly associated, enabled investigation of the peptides for real-world applications. In the soft drink (Fanta Orange) the growth of *Z. bailii* was completely inhibited by all peptides at all concentrations tested, even at the lowest concentration (12.5 μg/mL). This could indicate a potential synergistic effect between the peptides and a component of the soft drink, which may have enhanced their inhibitory effect. Indeed, growth was observed for the control, consisting of *Z. bailii* alone inoculated in the Fanta Orange, indicating that the presence of preservatives in the soft drink had no effect. Testing of the peptides against *Z. bailii* in mayonnaise resulted in no inhibition. This could be attributed to the viscosity of the mayonnaise, physically inhibiting the interaction between peptide and yeast. This highlights a limitation of use of these peptides when applied to such a viscous matrix, making them unsuitable for use in certain types of foods. Although limitations may arise, the overall ability of each peptide to cause inhibition of yeast makes them an appealing substitute for chemical preservation of certain food types. This study highlights the potential for using peptide modification as a means of creating synthetic peptides suitable for application as anti-yeast agents in food. The cost of synthesis of such synthetic peptides may, however, currently be a limitation to their incorporation as food preservatives. An alternative to this challenge associated with synthetic peptides can be the incorporation of natural peptides extracted from plants.

Acknowledgements

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

References

Baldauf, C., Pagel, K., Warnke, S., Von Helden, G., Koksch, B., Blum, V., Scheffler, M., 2013. How cations change peptide structure. Chem. Eur. J. 19, 11224–11234. https://doi.org/10.1002/chem.201204554.

Bechinger, B., Gorr, S.-U., 2017. Antimicrobial peptides: mechanisms of action and resistance. J. Dent. Res. 96, 254–260. https://doi.org/10.1177/0022034516679973.

Betts, G.D., Linton, P., Betteridge, R.J., 1999. Food spoilage yeasts: effects of pH, NaCl and temperature on growth. Food Control 10, 27–33. https://doi.org/10.1016/

S0956-7135(98)00151-0.
Bondaryk, M., Staniszewska, M., Zielińska, P., Urbańczyk-Lipkowska, Z., 2017. Natural antimicrobial peptides as inspiration for Design of a new Generation Antifungal Compounds. J. Fungi. https://doi.org/10.3390/jof3030046.
Cabrele, C., Martinek, T.A., Reiser, O., Berlicki, Ł., 2014. Peptides containing β-amino acid patterns: challenges and successes in medicinal chemistry. J. Med. Chem. 57, 9718–9739. https://doi.org/10.1021/jm5010896.
Cai, H., Hauser, M., Naider, F., Becker, J., 2016. Halo assay for toxic peptides and other compounds in microorganisms. Bio-Protocol 6. https://doi.org/10.21769/BioProtoc.2025.
Cheng, L., Goodwin, C.A., Schully, M.F., Kakkar, V.V., Claeson, G., 1992. Synthesis and biological activity of Ketomethylene Pseudopeptide analogues as thrombin inhibitors. J. Med. Chem. 35, 3364–3369. https://doi.org/10.1021/jm00096a010.
Cools, T.L., Vriens, K., Struyfs, C., Verbandt, S., Ramada, M.H.S., Brand, G.D., Bloch, C., Koch, B., Traven, A., Drijfhout, J.W., Demuyser, L., Kucharíková, S., Van Dijck, P., Spasic, D., Lammertyn, J., Cammue, B.P.A., Thevissen, K., 2017. The antifungal plant Defensin HsAFP1 is a phosphatidic acid-interacting peptide inducing membrane Permeabilization. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.02295.
Crowley, L.C., Scott, A.P., Marfell, B.J., Boughaba, J.A., Chojnowski, G., Waterhouse, N.J., 2016. Measuring cell death by Propidium iodide uptake and flow cytometry. Cold Spring Harb Protoc 2016https://doi.org/10.1101/pdb.prot087163. pdb.prot087163.
Dang, T.D.T., Mertens, L., Vermeulen, A., Geeraerd, A.H., Van Impe, J.F., Debevere, J., Devlieghere, F., 2010. Modelling the growth/no growth boundary of Zygosaccharomyces bailii in acidic conditions: a contribution to the alternative method to preserve foods without using chemical preservatives. Int. J. Food Microbiol. 137, 1–12. https://doi.org/10.1016/j.ijfoodmicro.2009.10.020.
de la Fuente-Núñez, C., Reffuveille, F., Mansour, S.C., Reckseidler-Zenteno, S.L., Hernández, D., Brackman, G., Coenye, T., Hancock, R.E.W., 2015. D-enantiomeric peptides that eradicate wild-type and multidrug-resistant biofilms and protect against lethal Pseudomonas aeruginosa infections. Chem. Biol. 22, 196–205. https://doi.org/10.1016/j.chembiol.2015.01.002.
Deslouches, B., Hasek, M.L., Craigo, J.K., Steckbeck, J.D., Montelaro, R.C., 2016. Comparative functional properties of engineered cationic antimicrobial peptides consisting exclusively of tryptophan and either lysine or arginine. J. Med. Microbiol. 65, 554–565. https://doi.org/10.1099/jmm.0.000258.
Di Russo, N.V., Estrin, D.A., Martí, M.A., Roitberg, A.E., 2012. pH-dependent conformational changes in proteins and their effect on experimental pKas: the case of Nitrophorin 4. PLoS Comput. Biol. 8. https://doi.org/10.1371/journal.pcbi.1002761.
Dias, S.A., Freire, J.M., Pérez-Peinado, C., Domingues, M.M., Gaspar, D., Vale, N., Gomes, P., Andreu, D., Henriques, S.T., Castanho, M.A.R.B., Veiga, A.S., 2017. New potent membrane-targeting antibacterial peptides from viral capsid proteins. Front. Microbiol. 8, 1–9. https://doi.org/10.3389/fmicb.2017.00775.
Diz, M.S.S., Carvalho, A.O., Rodrigues, R., Neves-Ferreira, A.G.C., Da Cunha, M., Alves, E.W., Okorokova-Façanha, A.L., Oliveira, M.A., Perales, J., Machado, O.L.T., Gomes, V.M., 2006. Antimicrobial peptides from chilli pepper seeds causes yeast plasma membrane permeabilization and inhibits the acidification of the medium by yeast cells. Biochim. Biophys. Acta Gen. Subj. 1760, 1323–1332. https://doi.org/10.1016/j.bbagen.2006.04.010.
Djiadeu, P., Azzouz, D., Khan, M.A., Kotra, L.P., Sweezey, N., Palaniyar, N., 2017. Ultraviolet irradiation increases green fluorescence of dihydrorhodamine (DHR) 123: false-positive results for reactive oxygen species generation. Pharmacol. Res. Perspect. 5, e00303. https://doi.org/10.1002/prp2.303.
Ebbensgaard, A., Mordhorst, H., Overgaard, M.T., Aarestrup, F.M., Hansen, E.B., 2018. Dissection of the antimicrobial and hemolytic activity of Cap18: generation of Cap18 derivatives with enhanced specificity. PLoS One 13, 1–25. https://doi.org/10.1371/journal.pone.0197742.
Engelman, D.M., Steitz, T.A., 1981. The spontaneous insertion of proteins into and across membranes: the helical hairpin hypothesis. Cell 23, 411–422. https://doi.org/10.1016/0092-8674(81)90136-7.
Fjell, C.D., Hiss, J.A., Hancock, R.E.W., Schneider, G., 2012. Designing antimicrobial peptides: form follows function. Nat. Rev. Drug Discov. 11, 37–51. https://doi.org/10.1038/nrd3591.
Fleet, G.H., 2006. The commercial and community significance. In: Yeast Handb, pp. 1–5.
Gil-Rodríguez, A.M., Carrascosa, A.V., Requena, T., 2015. Yeasts in foods and beverages: in vitro characterisation of probiotic traits. LWT Food Sci. Technol. 64, 1156–1162. https://doi.org/10.1016/j.lwt.2015.07.042.
Gould, G.W., 1996. Methods for preservation and extension of shelf life. Int. J. Food Microbiol. 33 Intsmotionol Journal o&odMicmbii&gy.
Hayes, B.M.E., Bleackley, M.R., Wiltshire, J.L., Anderson, M.A., Traven, A., Van Der Weerden, N.L., 2013. Identification and mechanism of action of the plant defensin nad1 as a new member of the antifungal drug arsenal against candida albicans. Antimicrob. Agents Chemother. 57, 3667–3675. https://doi.org/10.1128/AAC.00365-13.
Hittinger, C.T., Steele, J.L., Ryder, D.S., 2018. Diverse yeasts for diverse fermented beverages and foods. Curr. Opin. Biotechnol. 49, 199–206. https://doi.org/10.1016/j.copbio.2017.10.004.
Ho, V.T.T., Zhao, J., Fleet, G., 2014. Yeasts are essential for cocoa bean fermentation. Int. J. Food Microbiol. 174, 72–87. https://doi.org/10.1016/j.ijfoodmicro.2013.12.014.
Jenssen, H., Hamill, P., Hancock, R.E.W., 2006. Peptide antimicrobial agents. Clin. Microbiol. Rev. 19, 491–511. https://doi.org/10.1128/CMR.00056-05.
Jespersen, L., Jakobsen, M., 1996. Specific spoilage organisms in breweries and laboratory media for their detection. Int. J. Food Microbiol. 33, 139–155. https://doi.org/10.1016/0168-1605(96)01154-3.
Jin, L., Bai, X., Luan, N., Yao, H., Zhang, Z., Liu, W., Chen, Y., Yan, X., Rong, M., Lai, R., Lu, Q., 2016. A designed tryptophan- and lysine/arginine-rich antimicrobial peptide with therapeutic potential for clinical antibiotic-resistant Candida albicans vaginitis. J. Med. Chem. 59, 1791–1799. https://doi.org/10.1021/acs.jmedchem.5b01264.
Jung, H.J., Park, Y., Sung, W.S., Suh, B.K., Lee, J., Hahm, K.S., Lee, D.G., 2007. Fungicidal effect of pleurocidin by membrane-active mechanism and design of enantiomeric analogue for proteolytic resistance. Biochim. Biophys. Acta Biomembr. 1768, 1400–1405. https://doi.org/10.1016/j.bbamem.2007.02.024.
Kandasamy, S.K., Larson, R.G., 2006. Effect of salt on the interactions of antimicrobial peptides with zwitterionic lipid bilayers. Biochim. Biophys. Acta Biomembr. 1758, 1274–1284. https://doi.org/10.1016/j.bbamem.2006.02.030.
Kumar, P., Kizhakkedathu, J.N., Straus, S.K., 2018. Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. Biomolecules 8. https://doi.org/10.3390/biom8010004.
Kurtzman, C.P., Rogers, R., Hesseltine, C.W., 1971. Microbiological spoilage of mayonnaise and salad dressings. Appl. Microbiol. 21, 870–874.
Ledenbach, L.H., Marshall, R.T., 2009. Compendium of the Microbiological Spoilage of Foods and Beverages. https://doi.org/10.1007/978-1-4419-0826-1.
Liu, L.P., Li, S.C., Goto, N.K., Deber, C.M., 1996. Threshold hydrophobicity dictates helical conformations of peptides in membrane environments. Biopolymers 39, 465–470. https://doi.org/10.1002/(SICI)1097-0282(199609)39:3<465::AID-BIP17>3.0.CO;2-A.
Liu, L., Fang, Y., Wu, J., 2013. Flexibility is a mechanical determinant of antimicrobial activity for amphipathic cationic α-helical antimicrobial peptides. Biochim. Biophys. Acta Biomembr. 1828, 2479–2486. https://doi.org/10.1016/j.bbamem.2013.06.017.
Loureiro, V., Malfeito-Ferreira, M., 2003. Spoilage yeasts in the wine industry. Int. J. Food Microbiol. 86, 23–50. https://doi.org/10.1016/S0168-1605(03)00246-0.
Lyu, Y., Yang, Y., Lyu, X., Dong, N., Shan, A., 2016. Antimicrobial activity, improved cell selectivity and mode of action of short PMAP-36-derived peptides against bacteria and Candida. Sci. Rep. 6, 1–12. https://doi.org/10.1038/srep27258.
Mahlapuu, M., Håkansson, J., Ringstad, L., Björn, C., 2016. Antimicrobial peptides: an emerging category of therapeutic agents. Front. Cell. Infect. Microbiol. 6, 1–12. https://doi.org/10.3389/fcimb.2016.00194.
Mayan, M.D., 2010. Drug-induced Permeabilization of *S. cerevisiae*. In: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K. (Eds.), Current Protocols in Molecular Biology. John Wiley & Sons, Inc., Hoboken, NJ, USA.
Mohamed, M.F., Abdelkhalek, A., Seleem, M.N., 2016. Evaluation of short synthetic antimicrobial peptides for treatment of drug-resistant and intracellular Staphylococcus aureus. Sci. Rep. 6, 2–15. https://doi.org/10.1038/srep29707.
Mohamed, M.F., Brezden, A., Mohammad, H., Chmielewski, J., Seleem, M.N., 2017. A short D-enantiomeric antimicrobial peptide with potent immunomodulatory and antibiofilm activity against multidrug-resistant Pseudomonas aeruginosa and Acinetobacter baumannii. Sci. Rep. 7, 1–13. https://doi.org/10.1038/s41598-017-07440-0.
Muñoz, A., López-García, B., Pérez-Payá, E., Marcos, J.F., 2007. Antimicrobial properties of derivatives of the cationic tryptophan-rich hexapeptide PAF26. Biochem. Biophys. Res. Commun. 354, 172–177. https://doi.org/10.1016/j.bbrc.2006.12.173.
Ozkan, A.D., Tekinay, A.B., Guler, M.O., Tekin, E.D., 2016. Effects of temperature, pH and counterions on the stability of peptide amphiphile nanofiber structures. RSC Adv. 6, 104201–104214. https://doi.org/10.1039/c6ra21261a.
Palma, M., Münsterkötter, M., Peça, J., Güldener, U., Sá-Correia, I., 2017. Genome sequence of the highly weak-acid-tolerant Zygosaccharomyces bailii IST302, amenable to genetic manipulations and physiological studies. FEMS Yeast Res. 17, 1–15. https://doi.org/10.1093/femsyr/fox025.
Pérez-Peinado, C., Dias, S.A., Domingues, M.M., Benfield, A.H., Freire, J.M., Rádis-Baptista, G., Gaspar, D., Castanho, M.A.R.B., Craik, D.J., Henriques, S.T., Veiga, A.S., Andreu, D., 2018. Mechanisms of bacterial membrane permeabilization by crotalicidin (Ctn) and its fragment Ctn(15–34), antimicrobial peptides from rattlesnake venom. J. Biol. Chem. 293, 1536–1549. https://doi.org/10.1074/jbc.RA117.000125.
Rai, M., Pandit, R., Gaikwad, S., Kövics, G., 2016. Antimicrobial peptides as natural biopreservative to enhance the shelf-life of food. J. Food Sci. Technol. 53, 3381–3394. https://doi.org/10.1007/s13197-016-2318-5.
Rawat, S., 2015. Food spoilage: microorganisms and their prevention. Pelagia Res. Libr. Asian J. Plant Sci. Res. 5, 47–56.
Shahmiri, M., Enciso, M., Mechler, A., 2015. Controls and constrains of the membrane disrupting action of Aurein 1.2. Sci. Rep. 5, 1–14. https://doi.org/10.1038/srep16378.
Stark, M., Liu, L.-P., Deber, C.M., 2002. Cationic hydrophobic peptides with antimicrobial activity. Antimicrob. Agents Chemother. 46, 3585–3590. https://doi.org/10.1128/AAC.46.11.3585-3590.2002.
Strøm, M.B., Rekdal, Ø., Svendsen, J.S., 2002. Antimicrobial activity of short arginine- and trytophan-rich peptides. J. Pept. Sci. 8, 431–437. https://doi.org/10.1002/psc.398.
Taniguchi, M., Ochiai, A., Takahashi, K., Nakamichi, S., Nomoto, T., Saitoh, E., Kato, T., Tanaka, T., 2016. Effect of alanine, leucine, and arginine substitution on antimicrobial activity against candida albicans and action mechanism of a cationic octadecapeptide derived from α-amylase of rice: antifungal activity of AmyI-1-18 analogs against *C. albicans*. Biopolymers 106, 219–229. https://doi.org/10.1002/bip.22817.
Thevissen, K., Terras, F.R.G., Broekaert, W.F., 1999. Permeabilization of fungal membranes by plant defensins inhibits fungal growth. Appl. Environ. Microbiol. 65, 5451–5458.
Tournas, V.H., Heeres, J., Burgess, L., 2006. Moulds and yeasts in fruit salads and fruit juices. Food Microbiol. 23, 684–688. https://doi.org/10.1016/j.fm.2006.01.003.
Tugyi, R., Uray, K., Ivan, D., Fellinger, E., Perkins, A., Hudecz, F., 2005. Partial D-amino acid substitution: improved enzymatic stability and preserved ab recognition of a MUC2 epitope peptide. Proc. Natl. Acad. Sci. 102, 413–418. https://doi.org/10.1073/pnas.0407677102.

Wareing, P., Davenport, R.R., 2005. Microbiology of soft drinks and fruit juices. Chem. Technol. Soft Drink. Fruit Juices 279–297. https://doi.org/10.1002/9780470995822.ch11.

Wessolowski, A., Bienert, M., Dathe, M., 2004. Antimicrobial activity of arginine- and tryptophan-rich hexapeptides: the effects of aromatic clusters, D-amino acid substitution and cyclization. J. Pept. Res. 64, 159–169. https://doi.org/10.1111/j.1399-3011.2004.00182.x.

Wimley, W.C., 2010. Describing the mechanism of antimicrobial peptide action with the interfacial activity model. ACS Chem. Biol. 5, 905–917. https://doi.org/10.1021/cb1001558.

Wu, G., Ding, J., Li, H., Li, L., Zhao, R., Shen, Z., Fan, X., Xi, T., 2008. Effects of cations and PH on antimicrobial activity of thanatin and s-thanatin against Escherichia coli ATCC25922 and B. subtilis ATCC 21332. Curr. Microbiol. 57, 552–557. https://doi.org/10.1007/s00284-008-9241-6.

Yang, A.S., Honig, B., 1993. On the pH dependence of protein stability. J. Mol. Biol. https://doi.org/10.1006/jmbi.1993.1294.