Antimicrobial properties and death-inducing mechanisms of saccharomycin, a biocide secreted by *Saccharomyces cerevisiae*

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Abstract We recently found that *Saccharomyces cerevisiae* (strain CCMI 885) secretes antimicrobial peptides (AMPs) derived from the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) that are active against various wine-related yeast and bacteria. Here, we show that several other *S. cerevisiae* strains also secrete natural biocide fractions during alcoholic fermentation, although at different levels, which correlates with the antagonistic effect exerted against non-*Saccharomyces* yeasts. We, therefore, term this biocide saccharomycin. The native AMPs were purified by gel-filtration chromatography and its antimicrobial activity was compared to that exhibited by chemically synthesized analogues (AMP1 and AMP2/3). Results show that the antimicrobial activity of the native AMPs is significantly higher than that of the synthetic analogues (AMP1 and AMP2/3), but a conjugated action of the two synthetic peptides is observed. Moreover, while the natural AMPs are active at pH 3.5, the synthetic peptides are not, since they are anionic and cannot dissolve at this acidic pH. These findings suggest that the molecular structure of the native biocide probably involves the formation of aggregates of several peptides that render them soluble under acidic conditions. The death mechanisms induced by the AMPs were also evaluated by means of epifluorescence microscopy-based methods. Sensitive yeast cells treated with the synthetic AMPs show cell membrane disruption, apoptotic molecular markers, and internalization of the AMPs. In conclusion, our work shows that saccharomycin is a natural biocide secreted by *S. cerevisiae* whose activity depends on the conjugated action of GAPDH-derived peptides. This study also reveals that *S. cerevisiae* secretes GAPDH-derived peptides as a strategy to combat other microbial species during alcoholic fermentations.

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Introduction

The antagonism exerted by *Saccharomyces cerevisiae* against wine-related yeast and bacteria during alcoholic fermentation has been related to the secretion of antimicrobial peptides (AMPs) (Albergaria et al. 2010; Branco et al. 2014; Kemsawasd et al. 2015). In a recent work, Branco et al. (2014) isolated a peptidic fraction from *S. cerevisiae* fermentation supernatants (strain CCMI 885) containing AMPs derived from the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Two main peptides were identified in that bioactive fraction: the AMP2/3 and the AMP1, with the amino acid residues V S W Y D N E Y G Y S T R and

ISWYDNEYGYSAR, molecular masses of 1.638 and 1.622 kDa, respectively, and a theoretical isoelectric point (pI) of 4.37 (Branco et al. 2014). These anionic peptides correspond to fragments of the *S. cerevisiae* GAPDH2/3 (AMP2/3) and GAPDH1 (AMP1) isoenzymes.

AMPs are evolutionarily conserved components of the innate immune system and constitute the first line of antimicrobial defense in organisms across the eukaryotic kingdom (Sang and Blecha 2009; Wong et al. 2007). In the majority of cases, AMPs are cationic in nature and kill microbes by interacting with the anionic components of target cell membranes (Brogden 2005). Nevertheless, several anionic AMPs have also been found in animals and plants and, in recent years, it has become clear that they are also involved in the innate immune response of different organisms (Harris et al. 2009). The minimum inhibitory concentration (MIC) of anionic AMPs is usually weaker (MIC > 600 μM) than that of cationic AMPs (MIC ranging 10–100 μM) (Matsuzaki 2009). But the activity of anionic AMPs can be enhanced by several factors such as by the action of divalent metal cations (Dashper et al. 2005) or by additional peptides, as it was reported for lactococcin G (Nissen-Meyer et al. 1992). Anionic AMPs use a diverse range of antimicrobial mechanisms such as translocation across the membrane and permeabilization of cell membranes via pore formation (Harris et al. 2009).

Apoptosis in yeast was first discovered by Madeo et al. (1997) and was considered to be an unexpected finding since unicellular organisms seem to have no advantage in committing suicide. Nevertheless, apoptosis in yeast is now firmly confirmed, and several intrinsic, as well as exogenous stresses such as H_2O_2, UV irradiation, and acetic acid, have been described as apoptosis inducers in yeast cells (Madeo et al. 1999; Laun et al. 2001; Ludovico et al. 2001). Moreover, AMPs have been found to induce apoptosis in different sensitive microorganisms (Jin et al. 2010; Reiter et al. 2005). For instance, in *S. cerevisiae*, the virally encoded killer toxins K1 and K28 induce an apoptotic cell response in sensitive yeast strains (Reiter et al. 2005).

The aim of the present work was to characterize the antimicrobial properties and the death-inducing mechanisms of the GAPDH-derived AMPs, and to evaluate the role they play in the ability of *S. cerevisiae* strains to combat other microbial species during wine fermentation. With that purpose, several *S. cerevisiae* strains were screened regarding the levels of the natural biocide secreted during mixed-culture alcoholic fermentations and the antagonistic effect exerted against a sensitive non-*Saccharomyces* strain. Chemically synthesized analogues of the two main peptides (AMP2/3 and AMP1) that compose the native biocide were used to evaluate its antimicrobial activity and death-inducing mechanisms (e.g., cell membrane disruption, death by apoptosis, and internalization of AMPs).

Materials and methods

Strains and growth conditions

In this work we used the following *S. cerevisiae* strains: CCMI 885 (Culture Collection of Industrial Microorganisms of ex-INETI, Portugal); ISA 1000 (Culture collection of Instituto Superior de Agronomia, Portugal), ISA 1028, ISA 1029, ISA 1046, ISA 1063, ISA 1200; S101 (Saint Georges S101, Bio Springer, France); and ATCC 6269 (American Type Culture Collection). The non-*Saccharomyces* yeast strains used were as follows: *Dekkera bruxellensis* ISA 2211; *Hanseniaspora guilliermondii* NCYC 2380 (National Collection of Yeast Cultures, Norwich, United Kingdom); *Kluyveromyces marxianus* PYCC 2671 (Portuguese Yeast Culture Collection, FCT/UNL, Caparica, Portugal); *Lachancea thermotolerans* PYCC 2908; and *Torulaspora delbrueckii* PYCC 4478. Yeast strains were maintained on yeast extract peptone dextrose (YEPD)-agar slants (20 g/l of glucose, 20 g/l of peptone, 10 g/l yeast extract, 20 g/l agar) and stored at 4 °C. Inoculums were prepared by transferring biomass from one YEPD-agar slant (pre-grown at 30 °C for 48 h) into in 250-ml flasks with 100 ml of YEPD and incubating flasks at 30 °C and 150 rpm, for 16 h. All media were autoclaved at 120 °C for 20 min.

Mixed-culture alcoholic fermentations

Synthetic grape juice (SGJ) (110 g/l of glucose plus 110 g/l of fructose, pH 3.5, prepared as described in Pérez-Nevado et al. (2006)) fermentations were performed with mixed cultures of *H. guilliermondii* and each of the following *S. cerevisiae* strains: CCMI 885, ISA 1000, ISA 1028, ISA 1029, ISA 1046, ISA 1063, ISA 1200, S101, and ATCC 6269. One SGJ-fermentation was performed with *H. guilliermondii* in single-culture and used as negative control of the antagonism exerted by the *S. cerevisiae* strains. All fermentations were carried out in 500-ml flasks containing 300 ml of SGJ that were inoculated with 10^5 cells per milliliter of each yeast and incubated at 25 °C, under gentle agitation (80 rpm). Fermentations were carried out in duplicates and daily samples were taken to determine cell growth, sugars consumption, and ethanol production. Cell growth was assessed by colony forming units (CFU) counts. Briefly, 100 μl of culture sample were spread onto YEPD-agar plates, after appropriate dilution, and incubated at 30 °C in a vertical incubator (Infors, Anjou, Canada) for 2–6 days. In the mixed-culture fermentations, CFU counts of *H. guilliermondii* were obtained on YEPD-agar plates with 0.001 % of cycloheximide and the CFU counts of *S. cerevisiae* determined as the difference between the total number of CFU on YEPD-agar plates (both species grow) and the number of CFU on cycloheximide-YEPD-agar plates (only *H. guilliermondii* grows). Sugars (glucose and

fructose) and ethanol concentrations were determined by high-performance liquid chromatography (HPLC) using an HPLC apparatus (Merck Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany). Cell-free samples (filtration by 0.45 μm Millipore membranes) were injected into a Sugar-Pak column (Waters Hitachi, Milford, USA) and eluted with a degassed CaEDTA (50 mg/l) aqueous mobile phase at 90 °C and 0.5 ml/min.

Purification of native biocide fractions by gel-filtration chromatography

Cell-free supernatants (7-day-old) from each of the mixed-culture fermentations performed were ultrafiltrated by centrifugal filter units (Vivaspin 15R, Sartorius, Gottingen, Germany) equipped with 10 and 2 kDa cutoff membranes. Peptidic fractions (2–10 kDa) were obtained by first passing the fermentation supernatants through 10 kDa centrifugal filter units and then concentrating (10-fold) those permeates in 2 kDa centrifugal filter units. These peptidic fractions (2–10 kDa) were then fractionated by gel-filtration chromatography using a Superdex-Peptide column (10/300 GL, GE Healthcare, London, UK) coupled to an HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with a UV detector (Merck Hitachi, Darmstadt, Germany). The peptidic supernatant fractions (2–10 kDa) were eluted with 0.1 M ammonium acetate at a flow rate of 0.7 ml/min. The eluate fractions between the retention time 27–29 min were collected and lyophilized.

Spectrum of action and antimicrobial properties of the native biocide

The minimum inhibitory concentration (MIC) and half inhibitory concentration (IC50) of the native biocide (i.e., GAPDH-derived AMPs) were determined against *H. guilliermondii*, *L. thermotolerans*, *K. marxianus*, *T. delbrueckii* and *D. bruxellensis*. The gel-filtration lyophilized fraction-II obtained from the *S. cerevisiae* strain CCMI 885 fermentation supernatant was resuspended in YEPD with 30 g/l of ethanol and pH 3.5. Growth inhibitory assays were performed in 96-well microplates containing 100 μl of YEPD medium, without fraction-II (control) and with fraction-II at final protein concentrations of 125, 250, 500, and 1000 μg/ml. Media were inoculated with 10^5 cells per milliliter of each of the above-mentioned non-*Saccharomyces* yeasts, and the microplates incubated in a Thermo-Shaker (Infors HT, Bottmingen, Switzerland) at 30 °C, under strong agitation (700 rpm). Cell growth was followed by optical density measurements (at 590 nm) in a Microplate Reader (Dinex Technologies Inc., Chantilly, USA) and by CFU counts. The MIC was defined as the minimum concentration of biocide that completely inhibited the growth of the sensitive yeast, and the IC50 as the concentration of biocide that induced a growth reduction of 50 % as compared with growth in the respective control assay.

Antimicrobial activity of synthetic peptide analogues (AMP2/3 and AMP1)

Analogues of the AMP2/3 (amino acids residues: VSWYDNEYGYSTR) and AMP1 (amino acids residues: ISWYDNEYGYSAR) were chemically synthetized according to standard procedures and purchased from GenScript Inc. Company (GenScript HK Limited, Hong Kong). The synthetic peptides were obtained in lyophilized form; stock-solutions of each peptide were prepared by dissolving 2 mg of lyophilized powder in 1 ml of deionized water and the pH was adjusted to 8.0 with a sodium hydroxide solution until total solubilization was attained. The antimicrobial activity of the synthetic peptides AMP2/3 and AMP1 was determined against *H. guilliermondii* in growth inhibitory assays performed as described in the "Spectrum of action and antimicrobial properties of the native biocide" section. Briefly, a 50-μl aliquot of each AMP stock solution was mixed with 50 μl of 2 × YEPD (two-fold concentrated YEPD with 60 g/l ethanol) and the final pH was adjusted to 6.0. The AMPs solutions were used in growth assays at the following concentrations (μg/ml): 125, 250, 500, and 1000. Media were inoculated with 10^5 cells per milliliter of *H. guilliermondii* and cultures were incubated in a Thermo-Shaker (Infors HT, Bottmingen, Switzerland) at 30 °C, under strong shaking (700 rpm). The combined action of the two synthetic peptides was also tested against the same yeast strain, using mixtures of the synthetic AMP2/3 + AMP1 at the ratios of 1:1; 2:1; 4:1, and 6:1, to a final concentration of 1000 μg/ml.

Internalization of AMPs fluorescently labeled with fluorescein

Exponentially grown cells of *H. guilliermondii* and *D. bruxellensis* were separately incubated in deionized water and in YEPD medium (pH = 6.0) at room temperature (*ca.* 20–25 °C) with synthetic peptides (AMP2/3 and AMP1) fluorescently labeled with fluorescein (FITC). The AMPs were chemically synthesized and fluorescently labeled with FITC according to standard procedures and purchased from GenScript Inc. Company (GenScript HK Limited, Hong Kong). The AMPs-FITC were added to deionized water and to YEPD medium using a mixture of AMP2/3 + AMP1 in a ratio of (4:1) to a final concentration of 1000 μg/ml. The initial cell density in the assays was 10^6 cells per milliliter, and four different media were used as follows: deionized water and YEPD, without and with ethanol (30 g/l). Each assay was performed in duplicates. After 1 h of incubation with the

AMPs-FITC, cells were harvested by centrifugation ($7000 \times g$, for 5 min), stained with 10 μl of propidium iodide (PI) solution (1 mg/ml) and incubated for 30 min in the dark. Finally, cells were visualized in an epifluorescent microscope (Zeiss Axiovert 50, Oberkochen, Germany) equipped with a Zeiss Neofluor ×40 objective (numerical aperture 0.75) and the number of cells emitting green fluorescence (internalization of the AMPs-FITC) and red fluorescence (PI-stained cells) was quantified to determine the percentage of cells that were able to internalize the AMPs-FITC and those with permeabilized membranes (PI-stained cells).

Analyses of apoptotic and necrotic markers

Apoptotic and/or necrotic markers induced by the synthetic AMPs in sensitive yeast cells were assessed in *H. guilliermondii* incubated (10^5 cells/ml) for 2 h in YEPD (with 30 g/l of ethanol at pH 6.0) with the synthetic AMPs. The AMPs were added to the YEPD medium using mixtures of the two synthetic peptides (i.e., AMP2/3 + AMP1) in a ratio of 4:1, to final concentration of 100 μg/ml. *H. guilliermondii* cells incubated in YEPD without the AMPs were used as negative control, and *H. guilliermondii* cells incubated in YEPD with 5 mM of peroxide of hydrogen (H_2O_2) were used as positive control. Since cycloheximide inhibits the protein synthesis in yeast, this antibiotic is typically used to validate the apoptosis-inducing ability of a given stress (e.g., H_2O_2). Thus, to confirm the ability of these AMPs to induce apoptosis, *H. guilliermondii* was incubated in YEPD with 0.01 % of cycloheximide and 100 μg/ml of the synthetic AMPs. Apoptotic cellular markers (i.e., DNA strand breaks, phosphatidylserine exposure at the surface of the cytoplasmatic membrane, and chromatin condensation) were detected in the AMP-treated cells by the epifluorescent microscopic methods described in the following section.

TUNEL method

DNA strand breaks were confirmed by the incorporation of modified dUTPs at the 3′-OH ends of fragmented DNA using the enzyme terminal deoxynucleotidyl transferase (TdT). Modifications were directly detected by epifluorescent microscopy using a fluorescently modified nucleotide (i.e., fluorescein-dUTP) (Click-iT TUNEL Alexa Fluor imaging Assay, Invitrogen, USA) and the following procedure: firstly, yeast cells were fixed during 1 h with 4 % paraformaldehyde, digested with zymolyase and β-glucuronidase during 1 h and 30 min at 37 °C under agitation (150 rpm) and permeabilized with sodium citrate 0.1 M for 30 min at 70 °C. Then, cells were washed with PBS buffer and incubated with 20 μl of TUNEL reaction mixture (60 U/ml of terminal deoxynucleotidyl transferase, 1 μl of EdUTP nucleotide mixture, and 47 μl of reaction buffer) for 1 h at 37 °C in the dark;

finally, cells were washed with PBS and spotted onto a Neubauer chamber to enumerate the cells exhibiting green fluorescence by epifluorescence microscopy (Olympus BX-60 microscope, Tokyo, Japan).

ANNEXIN V/PI staining

The exposure of phosphatidylserine at the surface of the cytoplasmatic membrane in apoptotic cells was detected by applying fluorescein conjugated with Annexin V (Alexa fluor 488, Invitrogen, Paisley, UK) together with PI. Briefly, cells were washed in sorbitol buffer (2 M sorbitol, 0.5 mM MgCl$_2$, 35 mM potassium phosphate, pH 6.8), digested with zymolyase and β-glucuronidase during 1 h and 30 min at 37 °C under agitation (150 rpm) and after this washed with biding buffer/sorbitol (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl$_2$). Then, 5 μl of Annexin V and 2 μl of PI (0.5 μg/ml) were added and cells were incubated for 20 min at room temperature in the dark. These cells were harvested by centrifugation, resuspended in binding buffer/sorbitol and spotted onto a Neubauer chamber to enumerate cells stained with PI (necrotic cells) and cells emitting green fluorescence at the surface of the cytoplasmatic membrane (apoptotic cells) by epifluorescence microscopy (Olympus BX-60 microscope, Tokyo, Japan).

DAPI staining

Chromatin condensation was accessed by microscopic observations of cells stained with the fluorescent dye 4,6 diamidino-2-phenylindole (DAPI). Briefly, AMP-treated cells were incubated for 20 min with 1 mg/ml of DAPI (Invitrogen, Paisley, UK) in the dark, at room temperature. Cells were harvested by centrifugation and resuspended in PBS and spotted onto a Neubauer chamber to enumerate cells exhibiting blue fluorescence (DAPI-stained cells) by epifluorescence microscopy (Olympus BX-60 microscope, Tokyo, Japan).

Results

Antagonism of *S. cerevisiae* strains and secretion of native biocide fractions

The antagonism exerted by different *S. cerevisiae* strains against non-*Saccharomyces* yeast was assessed by performing synthetic grape juice (SGJ) fermentations with mixed-cultures of *H. guilliermondii* and several *S. cerevisiae* strains. SGJ-fermentation performed with *H. guilliermondii* in single culture was used as negative control. Comparing the growth profiles of *H. guilliermondii* during the mixed-culture fermentations (Fig. 1a–i) with the single-culture fermentation (Fig. 1j), it is clear that all *S. cerevisiae* strains induced death of

Fig. 1 Cell density profiles (CFU/ml) of *S. cerevisiae* (*diamonds*) and *H. guilliermondii* (*squares*) during mixed-culture fermentations performed with *S. cerevisiae* strains CCMI 885 (**a**), ISA 1000 (**b**), ISA 1028 (**c**), ISA1029 (**d**), ISA 1046 (**e**), ISA 1063 (**f**), ISA 1200 (**g**), S101 (**h**), ATCC 6269 (**i**), and during the single-culture fermentation of *H. guilliermondii* (**j**). Values represented are means of triplicate measurements ± SD (*error bars*) of two independent biological experiments
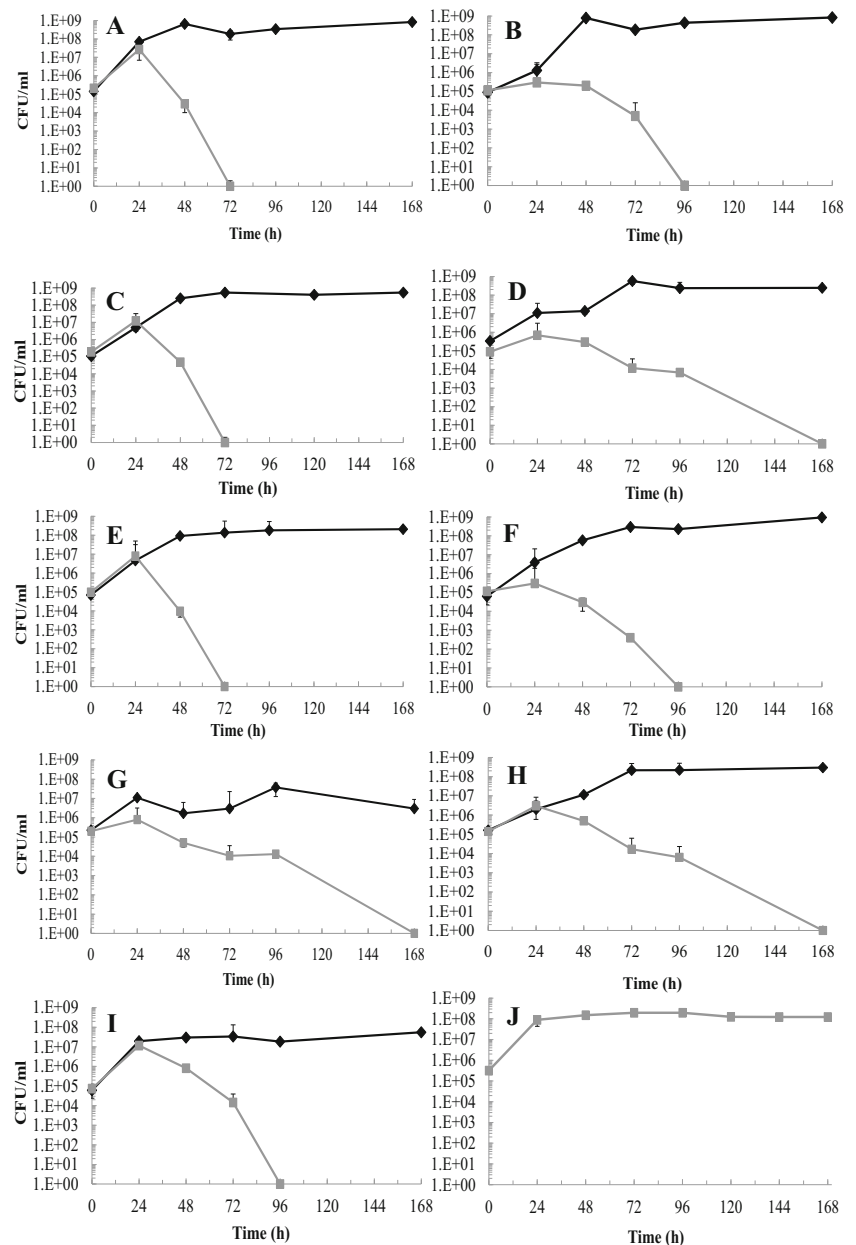
H. guilliermondii, although at different rates. While the cell viability of *H. guilliermondii* was entirely lost within the first 72 h in the mixed-culture fermentations performed with the *S. cerevisiae* strains CCMI 885, ISA 1028, and ISA 1046, a similar effect occurred only after 96 h with the strains ISA 1000, ISA 1063, and S101 and only after 168 h with the strains ISA 1029, ISA 1200, and ATCC 6269. In all mixed-culture fermentations, the initial sugars (220 g/l of glucose + fructose) were entirely consumed within 3–7 days, whereas in the single-culture fermentation *H. guilliermondii* consumed only 72 % of the initial sugars, leaving 62 g/l of residual sugars (glucose + fructose) after 7 days and producing 66 g/l of ethanol (Fig. S1 in the Electronic Supplementary Material (ESM)).

The peptidic fractions (2–10 kDa) of cell-free supernatants (7-day-old) obtained from every fermentation were fractionated by gel-filtration chromatography to isolate the natural biocide fraction in which the GAPDH-derived AMPs were previously identified by Branco et al. (2014) (i.e., peak-II indicated in Fig. 2). Death rates of *H. guilliermondii* during the mixed-culture fermentations show a positive correlation with the relative amount of the natural biocide fraction present in each supernatant (Table 1). Indeed, the supernatants from the fermentations where *H. guilliermondii* died within 72 h (strains CCMI 885, ISA 1028, and ISA 1046) showed the largest peak II areas, while the supernatants from the fermentations where *H. guilliermondii* took 168 h to die off (strains ISA 1029, ISA 1200. and ATCC 6269) showed the smallest
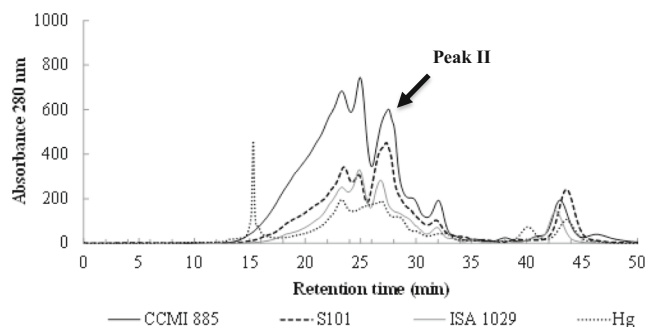
Fig. 2 Gel-filtration chromatographic profiles of the peptidic fractions (2–10 kDa) of supernatants obtained from the mixed-culture fermentations performed with *H. guilliermondii* and different *S. cerevisiae* strains (CCMI 885, S101, and ISA 1029), and from the single-culture fermentation of *H. guilliermondii* (Hg). Fractions indicated as peak-II correspond to the bioactive fraction in which the GAPDH-derived AMPs were previously identified by Branco et al. (2014)

peak-II areas (Table 1). The area of peak-II in these supernatants varied by two-fold, with the supernatants from the *S. cerevisiae* strains CCMI 885 and ISA 1029 exhibiting the largest area and the smallest area, respectively.

Sequence alignments of GAPDH isoenzymes for wine-related yeasts

In *S. cerevisiae*, three related but not identical GAPDH isoenzymes (GAPDH1, GAPDH2, and GAPDH3) are encoded by unlinked genes designated *TDH1*, *TDH2*, and *TDH3* (McAlister and Holland 1985). The GAPDH-derived peptides (AMP2/3 and AMP1) identified in *S. cerevisiae* fermentation supernatants match the C-terminal (309–321) sequence of the isoenzymes GAPDH2/3 (amino acids residues: VSWYDNEYGYSTR) and GAPDH1 (amino acids residues: ISWYDNEYGYSAR), respectively. To investigate the species-specificity of the amino acid sequences of these AMPs, we performed the sequence alignment of GAPDH isoenzymes of several non-*Saccharomyces* yeasts, in the region containing the AMP2/3 and the AMP1 fragments

(Fig. S2 in the ESM). Results show a high homology among the GAPDH sequences in the AMP2/3's region. However, the amino acid sequence of the AMP1 fragment seems to be quite unique, since it does not fully match any of the GAPDH sequences of the non-*Saccharomyces* yeasts analyzed (at least one amino acid is always different). It should be mentioned that we did not use *H. guilliermondii* in the sequence alignments because the genome of this species has not been sequenced yet.

Antimicrobial properties of the native and synthetic AMPs

Minimum inhibitory concentrations (MICs) of the native biocide (GAPDH-derived AMPs) against *H. guilliermondii*, *K. marxianus*, and *L. thermotolerans* were 250 μg/ml, while against *T. delbrueckii* and *D. bruxellensis* higher values were observed (500 and 1000 μg/ml, respectively) (Table 2). Half inhibitory concentrations (IC50) agree well with MICs for the same non-*Saccharomyces* yeasts (Table 2). The fungicidal effect of the native AMPs against these non-*Saccharomyces* yeasts was quantified as the number of LOGs of [CFU/ml] reduction (Table 3). The native AMPs show a strong fungicidal effect against *H. guilliermondii*, reducing its cell density by 4.2 and 5.2 orders of magnitude at 250 and 500 μg/ml, respectively (Table 3). Against *T. delbrueckii* and *D. bruxellensis*, the fungicidal effect of the native AMPs was lower, with the cell density of these yeasts being reduced by 3.6 and 2.9 orders of magnitude, respectively, at 500 and 2000 μg/ml (Table 3).

To further investigate the mode of action of the GAPDH-derived AMPs, synthetic analogues of the two main peptides that were isolated from the native biocide fraction (i.e., AMP1 and AMP2/3) were used to assess their antimicrobial effect against the sensitive yeast *H. guilliermondii*. However, due to the anionic nature of these synthetic peptides ($pI = 4.35$) it was not possible to test their inhibitory effect at the same acidic conditions used for the native biocide (i.e., YEPD at pH = 3.5), since they did not dissolve at this acidic pH. In fact, the

Table 1 Death rates of *H. guilliermondii* (Hg) during the mixed-culture fermentations performed with different *S. cerevisiae* strains, and the relative amount of the native biocide fraction (i.e., area of peak-II indicated in Fig. 2) in the respective supernatants

S. cerevisiae strains	Time (h) of initial death (T_{id}) of Hg	[Eth] (g/l) at T_{id}	Time (h) till total death (T_{td}) of Hg	[Eth] (g/l) at T_{td}	Area of peak-II indicated in Fig. 2
CCMI 885	24	13.5	72	68.4	107646681
ISA 1000	48	19.5	96	64.7	73742789
ISA 1028	24	7.3	72	58.3	98014390
ISA 1029	48	16.8	168	60.1	52280855
ISA 1046	24	7.3	72	52.7	93746340
ISA 1063	24	7.7	96	87.8	92708870
ISA 1200	48	2.1	168	15.7	64881770
S101	24	18.8	96	95.0	88723244
ATCC 6269	24	7.3	168	56.7	58831257

Table 2 Minimum inhibitory concentration (MIC) and half inhibitory concentration (IC50) of the native biocide determined against several wine-related non-*Saccharomyces* yeasts

Yeast	MIC (µg/ml)	IC50 (µg/ml)
H. guilliermondii	250	80
L. thermotolerans	250	65
K. marxianus	250	80
T. delbrueckii	500	135
D. bruxellensis	1000	260

MIC was defined as the lowest concentration of the native biocide fraction that prevents any visible growth (measured by absorbance) of yeast culture; IC50 was defined as the lowest concentration of the native biocide fraction that induces a 50 % reduction of yeast growth as compared with the control assay (measured by absorbance)

synthetic AMPs contain a majority of acidic amino acids in their primary structure, which prevents its solubilization at pH = 3.5. Therefore, the antimicrobial activity of the synthetic peptides was assessed in YEPD at pH 6.0, using increasing concentrations (0, 125, 250, 500, and 1000 µg/ml) of AMP2/3 and AMP1, either alone or mixed at different ratios. Results showed (Fig. 3) that both AMPs inhibited the growth of *H. guilliermondii*, although the AMP1 exhibited a much stronger effect than the AMP2/3 (76 % of inhibition for the AMP1 and only 30 % for AMP2/3, both at 1000 µg/ml). Besides, none of the synthetic AMPs (used either alone or mixed in a 1:1 ratio) was able to kill *H. guilliermondii* with the same efficiency of the natural AMPs (Table 3). Nevertheless, an increased antimicrobial effect was observed when the two peptides were used together (Fig. 3). Given these observations, we evaluated the effect of mixing the AMP2/3 with the AMP1 at different proportions, namely at the ratios of 2:1, 4:1, and 6:1 (final concentrations of 1000 µg/ml), on the growth inhibition of *H. guilliermondii*. These ratios were chosen based on the fact that the GAPDH2/3 isoenzymes from which the AMP2/3 derives are produced by *S. cerevisiae* cells at much higher proportions than the GAPDH1 isoenzyme, from which the AMP1 derives. In fact, McAlister and Holland (1985) found that the contribution of the *TDH1*, *TDH2*, and *TDH3* gene products to the total GAPDH activity in *S. cerevisiae* cells is 10–15, 25–30, and 50–60 %, respectively. Results revealed that, under such conditions, the AMPs were able to kill *H. guilliermondii* (cell viability was reduced by about 1–2 orders of magnitude), with the strongest fungicidal effect being achieved at the ratio of 4:1 (Fig. 4). The conjugated action of the two synthetic peptides can also be confirmed by comparing the inhibitory capacity of the AMP1 used alone or mixed with the AMP2/3 at a ratio of 4:1 (AMP2/3:AMP1), for the same concentration of AMP1 in each situation (Fig. S3). However, regardless of the way the synthetic AMPs were used (alone or mixed at any ratio) its antimicrobial activity was always lower than that of the natural biocide. Indeed, the synthetic AMPs were only able to reduce the cell viability of *H. guilliermondii* by *ca.* two orders of magnitude (using mixtures of AMP2/3 + AMP1 in a ratio of 4:1) at 1000 µg/ml (Fig. 4), while the natural AMPs reduced the cell viability of the same yeast strain by *ca* four orders of magnitude (Table 3) at a much lower concentration (at 250 µg/ml).


Fig. 3 Growth inhibition of *H. guilliermondii* (relative to control) in the assays performed in YEPD (pH = 6.0) with increasing concentrations of the synthetic AMP2/3 alone (*triangles*), of the synthetic AMP1 alone (*circles*) and of a mixture of AMP2/3 and AMP1 (*squares*) at a ratio of 1:1. Values represented are means of triplicate measurements ± SD (*error bars*) of two independent biological experiments

Table 3 Fungicidal effect of the native biocide at different concentrations, determined as LOG of [CFU/ml] reduction, against several wine-related non-*Saccharomyces* yeasts

Yeast	LOG of [CFU/ml] reduction			
	[native biocide fraction]			
	250 µg/ml	500 µg/ml	1000 µg/ml	2000 µg/ml
H. guilliermondii	4.2	5.2	–	–
L. thermotolerans	2.7	3.7	–	–
K. marxianus	2.6	3.2	–	–
T. delbrueckii	3.3	3.6	–	–
D. bruxellensis	–	–	0.4	2.9

LOG of [CFU/ml] reduction corresponds to the number of logarithms (LOGs) that cell density [CFU/ml] decreased in the biocide-assay, from an initial value of 10^5 CFU per milliliter to a final value determined when the respective control assay reached the stationary growth phase

Internalization of the synthetic AMPs by sensitive yeast cells

To investigate the internalization ability of the AMPs, exponentially grown cells of *H. guilliermondii* and *D. bruxellensis* were separately incubated in deionized water and in YEPD (both media with and without ethanol) in the presence of the AMPs fluorescently labeled with FITC (AMPs-FITC). Results showed that the synthetic AMPs were able to enter

Fig. 4 Cell viability (CFU/ml) of *H. guilliermondii* after 18 h of incubation in YEPD (pH = 6.0) without AMPs (control) and with mixtures of the synthetic AMP2/3 and AMP1 (final concentrations of 1000 μg/ml) at ratios of 6:1, 4:1, and 2:1. The initial cell density was 10^5 CFU/ml in all the assays. Values are means of triplicate measurements ± SD (*error bars*) of two biological independent assays
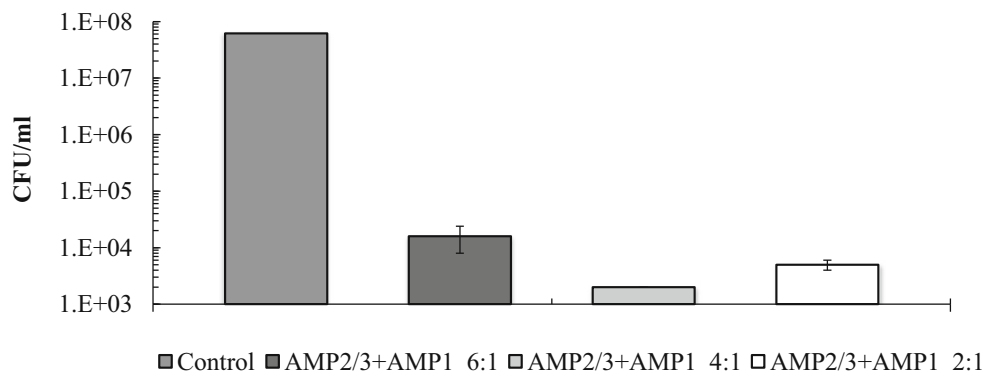
in cells of both yeasts (Figs. 5 and 6). However, the percentage of cells that internalized the AMPs significantly increased when cells were incubated in YEPD (ca. 25–30 %) instead of water (less than 10 %) (Fig. 6a, b). On the other hand, ethanol had no impact on the ability of the AMPs to penetrate *H. guilliermondii* cells (Fig. 6a), whereas an increased internalization was observed in *D. bruxellensis* cells (Fig. 6b). The membrane integrity of *H. guilliermondii* and *D. bruxellensis* cells was assessed by staining cells with PI, revealing that all cells that internalized the AMPs (AMPs-FITC) also showed compromised cell membranes (PI-stained) (Fig. 6a, b).

Apoptotic/necrotic molecular markers in AMPs-treated cells

Apoptotic cell death induced by AMPs has been reported by several authors (Jin et al. 2010) and (Reiter et al. 2005). This led us to investigate whether an apoptosis-like process occurs in sensitive yeast cells exposed to the synthetic AMPs. Cells dying by apoptosis display typical molecular markers such as the following: DNA strand breaks, detectable by the TUNEL-assay; chromatin condensation, detectable by DAPI-staining; and exposure of phosphatidylserine at the outer cell membrane, detectable by Annexin V-FITC staining. In the latter assay, apoptotic and necrotic cells can be distinguished by double staining cells with Annexin V (green fluorescence) and PI (red fluorescence), which is a membrane-impermeant fluorescent dye. These cellular markers were assessed by epifluorescent microscopy in *H. guilliermondii* cells incubated in YEPD without the AMPs (control) and with 100 μg/ml of synthetic AMPs. Cells were also incubated in YEPD with 5 mM of H_2O_2 (positive control) and in YEPD with 100 μg/ml of synthetic AMPs plus cycloheximide (negative control). Results (Fig. 7) showed that *H. guilliermondii* cells treated with 100 μg/ml of AMPs exhibited 28 % of cells with DNA strand breaks (TUNEL-positive), 4 % of cells with phosphatidylserine exposure at the membrane surface (Annexin+/PI−), 1 % of necrotic cells (Annexin+/PI+), and no cells with chromatin condensation (DAPI-positive). Prior to these assays, *H. guilliermondii* cells were incubated in

YEPD medium in the presence of 3.0, 5.0, and 180 mM of H_2O_2 and the above-mentioned apoptotic molecular markers were assessed, following the procedure described by Madeo et al. (1999). Results revealed that *H. guilliermondii* cells exposed to 5 mM of H_2O_2 exhibited a higher percentage of apoptotic cells than when exposed to 3 mM of a H_2O_2, while 180 mM of H_2O_2 induced necrosis (PI-stained cells) in 95 % of cells (data not shown). Thus, H_2O_2 at 5.0 mM was used as positive control of death by apoptosis in *H. guilliermondii* cells (Fig. 7). Conversely, cycloheximide inhibits apoptosis in yeast since it blocks the protein synthesis machinery required to execute the programmed cell death mechanism. By comparing the apoptosis molecular markers exhibited by *H. guilliermondii* cells treated with the synthetic AMPs in the absence and in the presence of cycloheximide (Fig. 7), our results suggest that the AMPs indeed induce apoptosis in the sensitive yeast *H. guilliermondii*.

Discussion

We had previously reported that *S. cerevisiae* (strain CCMI 885) secretes AMPs derived from the glycolytic enzyme GAPDH (Albergaria et al. 2010; Branco et al. 2014), active against several wine-related non-*Saccharomyces* yeasts (e.g., *D. bruxellensis*, *K. marxianus*, *L. thermotolerans*, and *T. delbrueckii*) and bacteria (e.g., *Oenococcus oeni*). In the present work, we show that several other *S. cerevisiae* strains also secrete these AMPs during alcoholic fermentation and, therefore, we term the native biocide saccharomycin. In addition, we demonstrate that there is a positive correlation between the death rates of *H. guilliermondii* during mixed-culture fermentations performed with different *S. cerevisiae* strains and the levels of saccharomycin excreted to the extracellular medium. Since saccharomycin exhibited a fungicidal effect against several wine-related non-*Saccharomyces* yeasts, our results strongly suggest that secretion of GAPDH-derived peptides is a defensive strategy used by *S. cerevisiae* strains to combat other microbial species during wine fermentation.

Fig. 5 Internalization of the synthetic AMPs fluorescently labeled with FITC by *D. bruxellensis* (**a**, **b**) and *H. guilliermondii* (**c**, **d**) cells. Microscopic observation in bright field (**a**, **c**) and in fluorescent filter (470 nm) (**b**, **d**) using a ×100 objective amplification
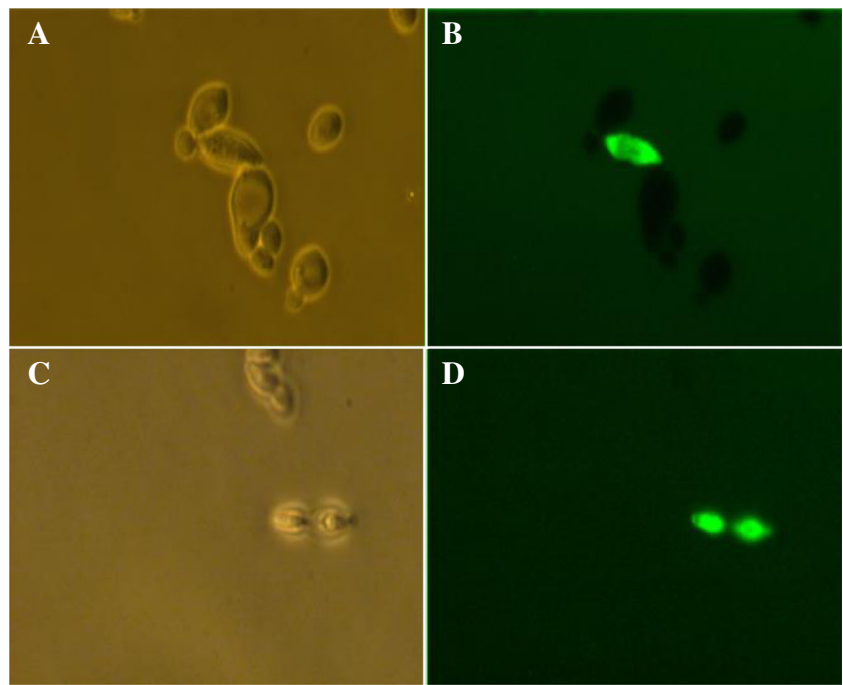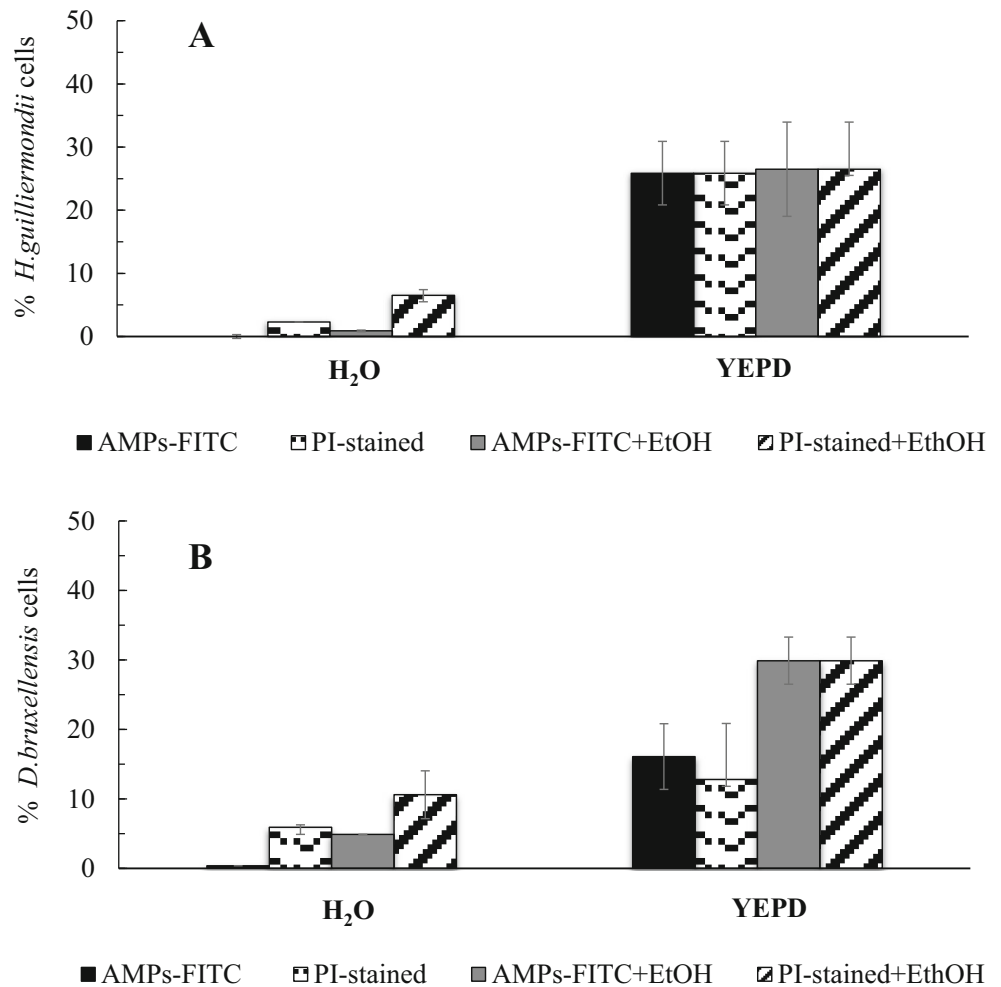

Fig. 6 Percentage of *H. guilliermondii* (**a**) and *D. bruxellensis* (**b**) cells that internalized the synthetic AMPs fluorescently labeled with FITC (AMPs-FITC) and that lost membrane integrity (PI-stained) after incubation in deionized water and in YEPD, without ethanol (AMPs-FITC/PI-stained) and with 30 g/l of ethanol (AMPs-FITC + EtOH/ PI-stained + EtOH). Values correspond to means of triplicate measurements ± SD (*error bars*) from two biological independent assays
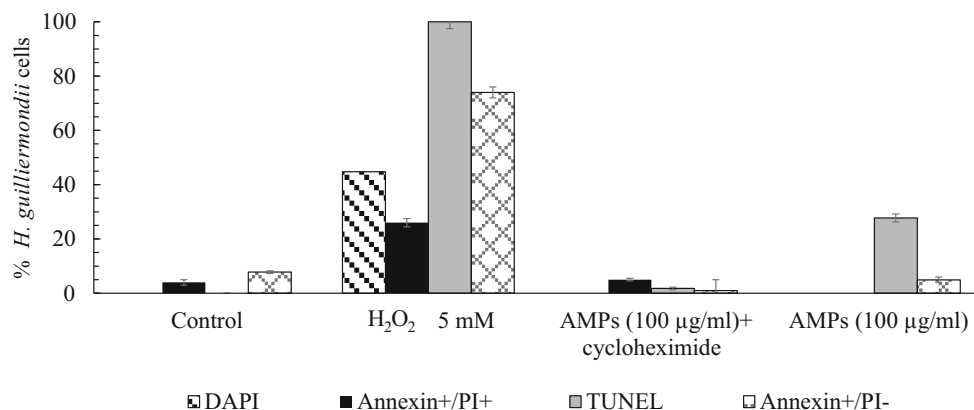
Fig. 7 Percentage of *H. guilliermondii* cells exhibiting apoptotic/necrotic molecular markers after incubation in YEPD medium, for 2 h, at different conditions: without AMPs (control); with 5 mM of H_2O_2 (positive control); with 100 µg/ml of AMPs plus 0.01 % cycloheximide (negative control); with 100 µg/ml of AMPs. *DAPI* apoptotic cells with chromatin condensation; *Annexin+/PI+* necrotic cells with compromised membranes; *TUNEL* apoptotic cells with DNA-strand breaks; *Annexin+/PI−* apoptotic cells with phosphatidylserine exposed at the surface of cytoplasmatic membrane. Values correspond to means of triplicate measurements ± SD (*error bars*) from two biological independent assays

The involvement of GAPDH in the defense system of *S. cerevisiae* seems surprising, since this protein is mainly associated with its glycolytic role. However, recent studies have shown that GAPDH also displays several other activities in different subcellular locations (Nakajima et al. 2009; Silva et al. 2011; Sirover 2005, 2011). For example, GAPDH is a cell-wall-associated protein with adhesion properties in bacteria (Izquierdo et al. 2009) and in the yeast *Candida albicans* where it plays a role in virulence (Gil et al. 1999). GAPDH has also been found on the cell surface of different yeasts such as in *K. marxianus*, involved in cell flocculation (Fernandes et al. 1992), and in *S. cerevisiae*, with unknown functions (Delgado et al. 2001, 2003). In mammalian cells, GAPDH is overexpressed in neuronal apoptotic cells and involved in Alzheimer's disease (Sunaga et al. 1995), while, in parasite, GAPDH is an immuno-suppressor (Sahoo et al 2013). Due to its diverse activities, GAPDH has been called a "moonlighting protein" (Sirover 2011). Besides, two different GAPDH-derived peptides with antifungal activity were recently isolated: one from the human placental tissue (Wagener et al. 2013) and the other from the skin of yellowfin tuna (Seo et al. 2012). On the other hand, GAPDH is a highly conserved protein, which means that its amino acid sequence should not vary significantly among close-related species. Indeed, our sequence alignments of the GAPDH isoenzymes for *S. cerevisiae* and some wine-related non-*Saccharomyces* yeasts show huge homology within the region that contains the AMP2/3. Nevertheless, the amino acid sequence of the AMP1 varies for, at least, one amino acid within the GAPDH sequences of those non-*Saccharomyces* yeasts. Interestingly, our results show that the antimicrobial activity of the AMP1 is much higher than that of the AMP2/3. The AMP1 originates from the GAPDH1 isoenzyme, which is only synthesized when *S. cerevisiae* cells enter the stationary growth phase (Boucherie 1995). Taken together, these findings could explain why the non-*Saccharomyces* yeasts invariable die off more intensely after *S. cerevisiae* attains the stationary growth phase during alcoholic fermentations (Albergaria et al. 2010; Nissen and Arneborg 2003; Pérez-Nevado et al. 2006).

Using synthetic analogues of the main peptides that compose the natural biocide (i.e., AMP2/3 and AMP1) we found that the antimicrobial activity of the native AMPs depends on the conjugated action of these GAPDH-derived peptides. Besides, a maximal antimicrobial effect was found when the AMP2/3 was mixed with the AMP1 at a ratio of 4:1. It is worth noting that, if the naturally secreted GAPDH-derived peptides are able to form aggregates of five molecules, the global molecular weight (MW) of those aggregates would be of about 8.0 kDa (MW of each peptide is ca. 1.6 kDa), which agrees with the apparent MW of the bioactive fractions isolated from the gel-filtration chromatography (data not shown). Moreover, while saccharomycin is active at acidic conditions (YEPD at pH = 3.5), the synthetic peptides are not. These findings prompt us to propose that the natural biocide may adopt a molecular structure involving the formation of aggregates of several peptide molecules (probably, five peptides) which render them soluble and bioactive at acidic conditions. In fact, different studies have shown that the activity of some AMPs depends on the conjugated action of several molecules (Nissen-Meyer et al. 1992; Straus and Hancock 2006). That is the case of lactococcin G, a bacteriocin whose activity depends on the complementary action of two peptides at approximately equal proportions (Nissen-Meyer et al. 1992). Also, daptomycin is an anionic AMP that was first isolated from *Streptomyces roseosporus* (Debono et al. 1987) and whose activity depends on the formation of aggregates of 14–16 daptomycin molecules that form a

micelle-like structure by the action of calcium cations (Ca^{2+}) (Straus and Hancock 2006).

Most AMPs induce death of sensitive cells by interacting with cell membranes and permeabilizing them (Pandey et al. 2011). However, some AMPs have developed unique mechanisms to translocate across membranes and to act on cytoplasmic targets without disrupting cell membranes (Powers and Hancock 2003). Indeed, translocation across membranes by a micellar aggregate mechanism was first proposed for the frog-derived antimicrobial peptide buforin II (Park et al. 2000), which rather than causing large membrane perturbations induces a transient disruption without permanent permeabilization (Powers and Hancock 2003). Once present in the cytoplasm, AMPs are thought to interact with DNA, RNA, and/or cellular proteins and to inhibit synthesis of these compounds (Brown and Hancock 2006). In the present work, we investigated the death mechanisms induced by the *S. cerevisiae* AMPs, by treating sensitive yeast cells with synthetic AMPs fluorescently labeled with FITC (internalization of peptides) and by staining those cells with PI (loss of cell membrane integrity). Results showed that all sensitive cells that internalized the AMPs, also exhibited cell membrane permeabilization. In a previous work (Branco et al. 2015), we had already found that the natural *S. cerevisiae* AMPs induce membrane permeabilization of *H. guilliermondii* cells. Here, we show that the synthetic AMPs are also able to cross the cell membrane and enter in the cytoplasm of sensitive yeast cells (both *H. guilliermondii* and *D. bruxellensis*), which means these AMPs are cell-penetrating peptides. Besides, both of these cellular effects were much more pronounced when sensitive cells were treated with the AMPs in YEPD (25–30 % of cells with disrupted membranes and AMPs internalization) than in deionized water (less than 10 % of cells with disrupted membranes and AMPs internalization). These results suggest that some component of the YEPD medium, probably a metal cation (e.g., Fe^{2+}, Mn^{2+}, Mg^{2+}, etc.), may enhance the activity of these AMPs. Although the effect of metal cations on the internalization of AMPs has never been reported, several studies (Dashper et al. 2005, 2007) have shown that the antimicrobial activity of anionic AMPs can be enhanced by the action of metal cations, since they promote the biding with the negatively charged cell wall. That is the case of kappacins, isolated from bovine milk and the first anionic AMPs to be investigated (Malkoski et al. 2001). In fact, Dashper et al. (2005) demonstrated that the antibacterial effect of kappacins is enhanced by the presence of divalent metal cations (both Zn^{2+} and Ca^{2+}). In addition, those authors found that under acidic conditions the membranolytic ability of kappacins in the presence of Zn^{2+} was higher, which could promote the influx of hydrogen ions, lower the intracellular pH, and thus increase the antibacterial activity (Dashper et al. 2005, 2007).

AMPs are known to trigger cell death by inducing molecular markers typical of death by apoptosis (Jin et al. 2010; Reiter et al. 2005). Here, we show that sensitive yeast cells treated with sub-lethal concentrations (i.e., 100 μg/ml) of synthetic analogues of the GAPDH-derived AMPs exhibit cellular markers characteristic of death by apoptosis such as DNA fragmentation, a typical late apoptosis phenomenon.

In conclusion, our work shows that saccharomycin is a natural biocide secreted by different *S. cerevisiae* strains that induces the death of several wine-related non-*Saccharomyces* yeasts, and whose activity depends on the conjugated action of GAPDH-derived peptides. The death mechanisms induced by these AMPs on sensitive yeasts involve cell membrane permeabilization, internalization of peptides and induction of apoptotic molecular markers.

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