**Antimicrobial peptides (AMPs) produced by *Saccharomyces cerevisiae*
induce alterations in the intracellular pH, membrane permeability and
culturability of *Hanseniaspora guilliermondii* cells**

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Abstract

Saccharomyces cerevisiae produces antimicrobial peptides (AMPs) during alcoholic fermentation that are active against several wine-related yeasts (e.g. *Hanseniaspora guilliermondii*) and bacteria (e.g. *Oenococcus oeni*). In the present study, the physiological changes induced by those AMPs on sensitive *H. guilliermondii* cells were evaluated in terms of intracellular pH (pHi), membrane permeability and culturability. Membrane permeability was evaluated by staining cells with propidium iodide (PI), pHi was determined by the fluorescence ratio imaging microscopy (FRIM) technique and culturability by the classical plating method. Results showed that the average pHi of *H. guilliermondii* cells dropped from 6.5 (healthy cells) to 5.4 (damaged cells) after 20 min of exposure to inhibitory concentrations of AMPs, and after 24 h 77.0% of the cells completely lost their pH gradient ($\Delta\text{pH}=\text{pHi}-\text{pH}_{\text{ext}}$). After 24 h of exposure to AMPs, PI-stained (dead) cells increased from 0% to 77.7% and the number of viable cells fell from 1×10^5 to 10 CFU/ml. This means that virtually all cells (99.99%) became unculturable but that a sub-population of 22.3% of the cells remained viable (as determined by PI staining). Besides, pHi results showed that after 24 h, 23% of the AMP-treated cells were sub-lethally injured (with $0<\Delta\text{pH}<3$). Taken together, these results indicated that this subpopulation was under a viable but non-culturable (VBNC) state, which was further confirmed by recuperation assays. In summary, our study reveals that these AMPs compromise the plasma membrane integrity (and possibly also the vacuole membrane) of *H. guilliermondii* cells, disturbing the pHi homeostasis and inducing a loss of culturability.

Keywords: antimicrobial peptides; membrane integrity; fluorescence ratio imaging microscopy (FRIM); intracellular pH (pHi); wine yeasts.

1. Introduction

In the early stages of wine fermentation (4-5% of ethanol) non-*Saccharomyces* yeast species such as *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum* and *Candida stellata* are predominant. However, as the fermentation progresses the highly fermentative and ethanol-tolerant strains of *S. cerevisiae* take over and complete the fermentation process (Pretorius, 2000; Sabate *et al.*, 2002). Several studies have shown that the early death of some non-*Saccharomyces* yeast during wine fermentation is due to microbial interactions induced by *S. cerevisiae* through different mechanisms (Arneborg *et al.*, 2005, Nissen *et al.*, 2003, Pérez-Nevado *et al.*, 2006). Moreover, in a work carried out by Albergaria *et al.* (2010) it was shown that *S. cerevisiae* produces antimicrobial peptides (AMPs) that are active against several wine-related microbial species. However, the physiological changes that these AMPs induce in sensitive yeast cells (i.e., cells that are inhibited and/or killed off by the AMPs) have not, as yet, been investigated.

AMPs are low molecular weight proteins (typically 20–40 amino acids in length) that constitute a diverse class of naturally occurring molecules with broad antimicrobial spectrum of action against bacteria, viruses, and fungi (Bradshaw, 2003, Izadpanah and Gallo, 2005). Many different types of organisms use AMPs for defence against infection and membrane interaction appears to be the key to the antimicrobial function of AMPs. AMPs generally present amphiphilic structures what facilitate these interactions. The peptides action may implicate membrane permeabilization, depolarization, leakage or lysis, resulting in cell death (Bradshaw, 2003; Izadpanah and Gallo, 2005; Matsuzaki, 2009).

Most AMPs are cationic in nature and kill microbes by interacting with anionic components of target cell membranes. Nevertheless, several anionic AMPs (AAMPs) have been found in animals and plants and it has become clear that they play an important role in their innate immune system (Harris *et al.*, 2009). Also, the previously found *S. cerevisiae* AMPs (Albergaria *et al.*, 2010) were recently characterised and sequenced, and they were shown to be anionic in nature (Branco *et al.*, 2014).

Although the mode of action of most AAMPs remains unclear, it has been reported that they use a diverse range of antimicrobial mechanisms such as translocation across the membrane and in other cases, such as with cyclotides, the membrane itself is the major site of action for AAMPs, which permeabilize membranes via pore formation (Harris et al., 2009). AAMPs generally exhibit weaker antimicrobial activity than cationic AMPs with minimal inhibitory concentrations (MIC) usually higher than 600 μ M such as in kappacins, the first AAMPs isolated from bovine milk (Malkoski et al., 2001), while cationic AMPs typically exhibit MIC ranging from 10-100 μ M (Matsuzaki, 2009). Investigation on the mode of action of kappacins showed that these peptides are strongly membranolytic at acidic pH, suggesting that divalent cations may facilitate the interaction of kappacins with the bacterial membrane and/or its ability to aggregate in the membrane to form anionic pores, thus increasing its permeability to cations. Under acidic conditions, this action could facilitate the influx of hydrogen ions thereby lowering intracellular pH and contributing to the antibacterial activity of the peptide (Dashper et al., 2005).

Fluorescence ratio imaging microscopy (FRIM) is a technique that measures intracellular pH (pHi) by using fluorescent pH sensitive probes (Arneborg et al, 2000; Siegmund et al, 1999). This technique, based on the linear response between the ratiometric intensity of fluorescence emitted by the probe and the pHi of the cells, gives information at the single-cell level, which allows determining the pHi of different sub-populations of cells simultaneously. Several authors (Hornbæk et al, 2002; Mortensen et al, 2006; Mortensen et al, 2008; Smigic et al., 2009; Vindeløv and Arneborg, 2002) used the fluorescent probe 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) to measure the pHi of bacteria and yeast cells at a pH ranging from 5.5 to 8. However, CFDA-SE is not adequate for pHi measurements of cells under acidic conditions, like in the case of wine fermentations where the pH of the medium ranges from 3 to 3.5, since they are not sensitive to pH values lower than 5.5. As an alternative, Shabala et al. (2006)

successfully used the fluorescent probe 5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate succinimidyl ester (CDCFDA-SE) to determine the pHi changes of *Listeria innocua* and *Lactobacillus delbrueckii* cells, when exposed to acidic stress.

However, in yeast CDCFDA-SE seems to stain preferentially the vacuole of cells and thus this probe has been used to estimate the vacuolar pH of *S. cerevisiae* cells (Mira et al., 2009). Carmelo et al. (1997) proposed that the yeast vacuole plays an important role in maintaining the pH homeostasis within the yeast cell. In *S. cerevisiae* cells the vacuolar pH is maintained at mildly acidic values (i.e. around 6.0) and the cytoplasmic pH close to neutral values in non-stressed cells, while in stressed cells the vacuolar pH decreases (Carmelo et al., 1997). To the best of our knowledge, the pHi of *H. guilliermondii* cells was never determined, neither under non-stressful nor under stressful conditions.

Thus, the aim of the present work was to determine the physiological alterations induced by the *S. cerevisiae* AMPs on the membrane permeability and pHi of sensitive *H. guilliermondii* cells at enological growth conditions. We also investigated if CDCFDA-SE stains the vacuole or the cytoplasm of *H. guilliermondii* cells, under stressful (in the presence of AMPs) and non-stressful (in the absence of AMPs) growth conditions, and which probe (CFDA-SE or CDCFDA-SE) was most adequate to measure the pHi of cells in the presence of the AMPs.

2. Materials and methods

2.1 Strains and growth conditions

Two yeast strains were used in this study: *Hanseniaspora guilliermondii*, NCYC 2380 (National Collection of Yeast Cultures, Norwich, United Kingdom) and *S. cerevisiae* CCMI 885 (Culture Collection of Industrial Microorganisms, ex-INETI, Lisbon, Portugal). *S. cerevisiae* and *H. guilliermondii* were maintained on YEPD-agar slants (20 g/l of glucose, 20 g/l of peptone, 10 g/l of yeast extract, 20 g/l agar, pH 6) and stored at 4°C.

Inocula of both yeasts were prepared by transferring biomass from one YEPD-agar slant (pre-grown at 30 °C for 48 h) into 100 ml of YEPD medium in 250 ml flasks that were incubated at 30 °C and 150 rpm for 16 h. All media were autoclaved at 120°C for 20 min.

2.2. AMPs production and purification

A 7 day-old supernatant of *S. cerevisiae* CCMI 885 was obtained from an alcoholic fermentation performed at 25 °C without agitation in synthetic grape juice (SGJ). The SGJ (D-glucose 110 g/l, D-fructose, 110 g/l, L-(1)-tartaric acid, 6.0 g/l, L-(2)-malic acid, 3.0 g/l, citric acid, 0.5 g/l, YNB, 1.7 g/l, CAA, 2.0 g/l, CaCl₂, 0.2 g/l, arginine-HCl, 0.8 g/l, L-(2)-proline, 1.0 g/l, L-(2)-tryptophan, 0.1 g/l, pH 3.5) was prepared as described in Pérez-Nevado et al. (2006). The 7 day-old supernatant was first filtrated by 0.22 µ Millipore membranes (**Merck Millipore, Algés, Portugal**) and then ultrafiltrated through centrifugal filter units (Vivaspin 15R, Sartorius, Germany) equipped with 10 and 2 kDa cut-off membranes. A concentrated peptidic fraction (2-10 kDa) was obtained by first passing the supernatant through the 10 kDa (10-fold) centrifugal unit and then concentrating this permeate (40-fold) in the 2 kDa centrifugal unit.

2.3. Buffers and solutions

Citrate phosphate buffer solutions with adjusted pH values within the range of 3.5 to 8.0 were prepared by mixing appropriate volumes of 200 mM Na₂HPO₄ (Merck, Darmstadt, Germany) and 100 mM citric acid (Merck, Darmstadt, Germany).

The fluorescent probes 5(6)-carboxy-fluorescein diacetate succinimidyl ester (CFDA-SE) (Molecular Probe Inc., OR, USA) and 5(6)-Carboxy-2',7'-dichlorofluorescein diacetate succinimidyl ester (CDCFDA-SE) (Molecular Probes Inc., OR, USA) were dissolved in water-free dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) to a final concentration of 4.48 mM and 9.16 mM, respectively. Propidium iodide (PI) (Molecular Probes Inc., OR, USA) was dissolved in sterile Milli-Q water (18.2 mΩ) to a final concentration of 1 mg/ml.

2.4. AMP-assays to determine culturability, pHi and membrane permeability

To determine physiological changes induced by the *S. cerevisiae* AMPs on *H. guilliermondii* cells, two growth assays were performed in 25 ml of YEPD medium (at pH 3.5) in the absence (control assay) and in the presence of the AMPs (AMP-assay). Native AMPs, obtained by purification of peptides from *S. cerevisiae* supernatants (as described in subsection 2.2), were added to the medium to a final protein concentration of 1 mg/ml, which corresponds to the maximum concentration of AMPs found in *S. cerevisiae* fermentation supernatants after 7 days of fermentation (data not shown). Both cultures were inoculated at an initial cell density of 10^5 cells/ml of *H. guilliermondii* and incubated at 30 °C, under strong agitation (150 rpm), for 24 h. Each culture was performed in duplicate and samples were taken at 0 h, 0.34 h, 8 h and 24 h, respectively, to determine the culturability (CFU/ml), intracellular pH (pHi) and cell viability (membrane permeability) of cells.

Recuperation assays were performed with *H. guilliermondii* cells after they had been incubated with the AMPs for 24 h. Cells from the AMP-assay after 24 h of incubation were centrifuged and then resuspended in fresh YEPD medium (pH=3.5, without the AMPs) and incubated under the same conditions as those used for AMP assays (i.e. 10^5 cells/ml, 30 °C, 150 rpm). Cells were allowed to recover for 24 h and then samples (from duplicates assays) were taken for culturability and pHi determination.

Culturability was determined by the classical plating method. Briefly, samples were plated onto YEPD-agar plates, after appropriate dilution, and plates were incubated at 25°C. The number of colony forming units (CFU) was counted after 2-6 days.

Intracellular pH (pHi) of *H. guilliermondii* cells was determined by fluorescence ratio imaging microscopy (FRIM) using fluorescent pH-sensitive probes (CDCFDA-SE and CFDA-SE) and membrane permeability (viability) was evaluated using the membrane impermeant fluorescent dye, propidium iodide (PI). Culture samples were first centrifuged

at 7000 rpm for 5 min and the respective pellets resuspended in 990 μ l of YEPD medium to obtain *ca* 10^7 cells/ml. Cells were double staining with pH-sensitive probes (CDCFDA-SE or CFDA-SE) by adding 10 μ l of a 9.16 mM CDCFDA-SE solution or 4.48 mM CFDA-SE to 990 μ l of cell suspension that was incubated at 25 °C for 3 h. After 2 h and 30 min of incubation with the probes, 10 μ l of PI solution (1 mg/ml) was added and cells incubated for more 30 min at 25 °C. After 3 h of incubation the cells were centrifuged at 7000 rpm for 5 min at 4 °C and then resuspended in 100 μ l of YEPD.

2.5. Fluorescence Ratio Imaging Microscopy (FRIM) and data analysis

The pHi of single cells was determined by the FRIM method, as described in Mortensen et al. (2006). The set-up used consisted of a fluorescent microscope (Zeiss Axioscop 50, Germany) equipped with a Zeiss Neofluar 40 $\times$ objective (numerical aperture 0.75) and a HBO 50 W halogen lamp to provide excitation of the probes (CFDA-SE and CDCFDA-SE). Stained cells were excited for 3 seconds at 470 nm and 440 nm, respectively and fluorescence emission (above 520 nm) was recorded on a cooled CCD-camera (CoolSnapfx, Photometrics, Birkerød, Denmark). Images were analysed using the RS Image software (Roper Scientific, version 1.9.2). For PI staining measurements, cells were excited for 3 seconds at 540 nm and fluorescence emission (above 610 nm) was recorded. To minimize photo bleaching of CDCFDA-SE or CFDA-SE stained cells, a 2.5% neutral-density filter was used in the excitation path. Data analysis was performed using the Image J 1.37v software program (<http://rsb.info.nih.gov/ij>). The pHi of single cells was determined by calculating the ratio of the fluorescence intensity emitted by cells stained with the pH-sensitive probes excited at 470 and 440 nm (R470/440) and the respective calibration curves (R470/440 vs pHi). These ratio values were obtained by dividing the fluorescence intensity of individual pixels from an image taken at 470 nm and the corresponding image taken at 440 nm. The background fluorescence intensity (regions

without cells) was subtracted from the fluorescence intensity of the stained cells. In each experiment 50 cells were analysed. Each experiment was repeated twice.

2.6. Calibration curves

To establish the relationship between the fluorescence emitted by cells stained with each probe (i.e. with CFDA-SE and CDCFDA-SE) and the respective pH_i, calibration curves were constructed for each pH-sensitive probe (**Fig. 1**). Briefly, after the step of fluorescence staining with the pH-sensitive probes, cells were harvested by centrifugation at 7000 rpm for 5 min at 4°C and incubated with 500 µl of ethanol (70% v/v) at 25°C for 30 min to permeabilize the membrane. Those cells (dead cells) were then harvested by centrifugation (7000 rpm for 5 min at 4 °C) and resuspended in 150 µl of supernatant. Aliquots of 10 µl of this cell suspension were added to 500 µl of several citrate phosphate buffers with pH values ranging from 3.0 up to 8.0 for CDCFDA-SE stained cells and from 5.0 up to 8.0 for CFDA-SE stained cells. Citrate phosphate buffers with adjusted pH values (3.0-8.0) were prepared through the mixture of appropriate volumes of 200 mM Na₂HPO₄ and 100 mM citric acid (Merck, Darmstadt, Germany). Those cells were then incubated for 10 min at 25°C in order to equilibrate the external pH (pH of the buffer solution) with the internal pH of cells (pH_i), and used as standards to construct the respective calibration curves. The fluorescence intensity emitted by the above-mentioned standards after excitation at 470 nm and 440 nm was measured and the ratio R_{470/440} calculated as described above and plotted against the respective pH_i value. Calibration data was fitted by a third degree polynomial curve.

The PI fluorescence intensity emitted by double-stained cells (PI+CDCFDA-SE or PI+CFDA-SE) was measured at 610 nm. In order to determine the minimal PI fluorescence value emitted by dead cells, a suspension of ethanol-treated cells (i.e. cells incubated with 70% (v/v) ethanol for 30 min at 25°C) was stained with 10 µl of PI solution (1 mg/ml), incubated for 30 min at 25°C, and then analysed by epifluorescence microscopy.

2.7. Accumulation of CDCFDA-SE probe on the vacuole of *H. guilliermondii* cells

To determine if CDCFDA-SE probe would stain preferentially the vacuole of *H. guilliermondii* cells, two growth assays were performed in the presence (AMP-assay) and in the absence of the AMPs (Control), as described in section 2.4. Each culture was performed in duplicate and samples were taken at 0 h, 8 h and 24 h, respectively. CDCFDA-SE-stained cells were analysed along the incubation time (from 0 to 24 h) by epifluorescence microscopy (Olympus BX-60, Tokyo, Japan). The fluorescence emitted by *H. guilliermondii* cells stained with CDCFDA-SE was quantified using a U-MWB filter (Excitation 450-480 nm) and total cells were quantified on the bright field with U-MNU filter (excitation 360-370 nm). 100 cells were analysed to determine the percentage of cells exhibiting fluorescence exclusively in the vacuole.

3. Results and Discussion

3.1. Selection of the best probe to measure the pHi of *H. guilliermondii* cells

In order to choose the most adequate probe to use in the FRIM method, we first constructed calibration curves with each pH-sensitive probes, i.e. with CFDA-SE and with CDCFDA-SE. Results (**Fig. 1-A,B**) clearly showed that CDCFDA-SE was the adequate probe to use under acidic conditions of our assays (external pH 3.5) since CFDA-SE did not show any fluorescence sensitivity at low pH values (<5.5-6.0).

Since several authors have reported (Fernandes et al., 2003; Preston et al., 1997; Roberts et al., 1991) that CDCFDA-SE stains preferentially the vacuole of *S. cerevisiae* cells, we checked if this was also true for *H. guilliermondii* cells. With that purpose, we analysed *H. guilliermondii* cells stained with CDCFDA-SE along 24 h of incubation in the presence and in the absence of the AMPs (**Fig. 2**). Microscopic observation data showed that for cells incubated in the presence of the AMPs (stress conditions), only a small percentage of them exhibited CDCFDA-SE fluorescence in the vacuoles during the whole

incubation period (9.8% of cells at 0 h and 0% of cells at 24 h), whereas all the remaining cells (90-100%) showed CDCFDA-SE fluorescence in the cytoplasm (**Fig. 3-B**). These results indicate that for *H. guilliermondii* cells under this stress conditions, CDCFDA-SE does not measure the vacuolar pH (pHvac) but rather the cytoplasmatic pH. Given these results, we assumed that in the AMP-assay (pH 3.5) this probe measures the cytoplasmatic pH of *H. guilliermondii* cells.

Nevertheless, when *H. guilliermondii* cells were incubated under non-stressful growth conditions (control assay) with CDCFDA-SE, fluorescent vacuoles were detected in 88% of the cells after 24 h (**Fig. 3-A**). Hence, in the control assay the information obtained after 24 h by using the CDCFDA-SE probe refers mainly to pHvac. While, by using the CFDA-SE probe, mainly the cytoplasmatic pH was determined after 24 h both in the control and AMP assay (**Table 2**).

3.2. Alterations induced by the AMPs on the membrane permeability, internal pH (pHi) and culturability of *H. guilliermondii* cells

Since it has been reported that most AMPs disturb the membrane integrity of sensitive cells (Harris et al., 2009), it is expected that under acidic conditions the intracellular pH (pHi) of those cells will fall. These cell physiological alterations, i.e. membrane integrity and pHi, can be directly assessed by using the appropriate fluorescent probes, i.e. propidium iodide (PI) to detect membrane permeability and pH-sensitive probes (CDCFDA-SE or CFDA-SE) to measure pHi.

In order to evaluate the physiological alterations induced by the *S. cerevisiae* AMPs on sensitive yeast cells, we assessed culturability, membrane permeability and pHi of *H. guilliermondii* cells during 24 h of incubation in the absence (control assay) and in the presence of AMPs (AMP-assay) (**Fig. 4**). Culturability was determined by the classical plating method, while membrane permeability and pHi were assessed by epifluorescent microscopy in a single-cell approach.

The pHi values of 50 individual cells (as well as their mean value) were determined by the FRIM method both in the control (**Fig. 4-A**) and in the AMP-assay (**Fig. 4-C**). Results showed that after 20 min of incubation with the AMPs the average pHi of *H. guilliermondii* cells dropped from an initial value of 6.5 to an average value of 5.4, further decreasing in the next hours up to a final value of 3.5 (equal to the external pH) after 24 h (**Fig. 4-C**). This means that *H. guilliermondii* cells were not able to maintain their pH homeostasis in the presence of the AMPs. Conversely, during the control assay the average pHi of *H. guilliermondii* cells showed no significant decrease within the first 8 h of growth, although a drop from 6.5 to 5.7 was observed after 24 h (**Fig. 4-A**).

The single-cell approach of the FRIM method allowed to detect a high heterogeneity within the cell population regarding pHi of individual cells, both in the control and in the AMP-assay (**Fig. 4-A,C**). For this reason, we grouped cells into three subpopulations according with the pH gradient ($\Delta\text{pH}=\text{pHi}-\text{pH}_{\text{ext}}$) of cells. Since the pHi of *H. guilliermondii* healthy cells was found to be 6.5 (**Fig. 4-A**), close to the value reported by several authors (Cimprich, et al., 1995; Guldeldt and Arneborg, 1998; Imai et al., 1994; Imai et al., 1995; Rowe et al., 1994) for healthy cells of *S. cerevisiae* (7.0), the following subpopulations were defined: healthy cells for $\Delta\text{pH}\geq 3.0$ ($=6.5-3.5$); sub-lethally injured cells for $0<\Delta\text{pH}<3.0$; severely injured cells for $\Delta\text{pH}=0$. The evolution of these cell subpopulations, as well as the culturability of the whole population, during the control and AMP assays are represented in **Fig. 4-B,D**. Results show that in the AMP-assay the percentage of healthy cells ($\Delta\text{pH}\geq 3.0$) and their culturability rapidly decreased (**Fig. 4-D**), with the subpopulation of severely injured cells ($\Delta\text{pH}=0$) reaching 77% after 24 h and culturability dropping from an initial value of 10^5 CFU/ml to a final value of 10 CFU/ml. Conversely, in the control assay (**Fig. 4-B**) culturability increased from 10^5 CFU/ml to 2×10^8 CFU/ml in 24 h, with the subpopulation of severely injured cells ($\Delta\text{pH}=0$) attaining just 3.3% of the whole population.

Membrane permeability was evaluated by staining cells with propidium iodide (PI) both in the control and in the AMP assay (**Table 1**). A sharp increase on the percentage of PI-stained cells (from 0% to 77.7%) was observed within 24 h in the AMP-assay, while in the control-assay only 28.3% of cells lost their membrane permeability. These results show that AMPs severely affect the membrane permeability of *H. guilliermondii* cells. They also show that 77.7% of *H. guilliermondii* cells became dead after 24 h of incubation in the presence of the AMPs. Indeed, PI is commonly used to assess cell viability (Davey and Winson, 2003), although some authors (Davey and Hexley, 2011) reported that *S. cerevisiae* cells can be reversibly permeable to PI. However, in a previous work (Branco et al., 2012) we showed that cell viability of *H. guilliermondii* cells is reliably assessed by PI staining. After 24 h of incubation with the AMPs, virtually all (99.99%) cells became unculturable, but 22.3% of the cells were viable (**Table 1**) and 23% sub-lethally injured (with $0 < \Delta \text{pH} < 3$) (**Fig. 4D**). Taken together, these data suggest that the subpopulation of viable cells (22.3%) entered into a viable but non-culturable (VBNC) state. To investigate if these cells were actually in a VBNC state, we checked whether they could recover its culturability and pH_i homeostasis after transferring these AMP-treated cells into fresh YEPD medium (without AMPs) and incubating them for 24 h. Results showed that the AMP-treated cells were able to recover their culturability and pH_i (**Fig. 5**), confirming that the subpopulation of viable cells were, in fact, in a VBNC state and, consequently, just sub-lethally injured. It should be noticed that, although there was initially 10 CFU/ml in the recuperation assay, this residual subpopulation of cultivable cells (0.01%) could never have been able to grow up to 10⁷ CFU/ml within 24 h once the specific growth rate under the same growth conditions (control assay) is just 0.28 h⁻¹.

Results showed that after 24 h in the control assay CDCFDA-SE stains mainly the vacuole of *H. guilliermondii* cells (**Figs. 2 and 3-A**), which means that pH_i values measured for cells in the control after 24 h (**Fig. 4-A**) refers mainly to the vacuolar pH.

This might explain why pH_i in the control assay dropped from 6.5 to 5.7 at 24 h in the control assay (**Fig. 4-A**), also suggesting that, likewise in *S. cerevisiae* cells (Carmelo et al., 2007), vacuolar pH of *H. guilliermondii* cells is maintained at mildly acidic pH (at 5.7) in non-stressful conditions, while cytoplasm is close to neutral pH (around 6.5). Conversely, CFDA-SE stained the cytoplasm of all cells both in the control and in the AMP-assay (**Table 2**). Therefore, we also determined the pH_i of 24 h-incubated cells by using CFDA-SE, thereby measuring the cytoplasmatic pH both in the control assay and in the AMP assay (**Table 2**). Considering the range at which CFDA-SE gave a good estimation of single cell pH_i (i.e., between 5.5 and 8.0) (**Fig. 1-A**), the following subpopulations were defined: healthy cells for $\Delta pH \geq 1.5$ ($=7.0-5.5$); sub-lethally injured cells for $0 < \Delta pH < 1.5$; severely injured cells for $\Delta pH = 0$. Results showed that the subpopulation of severely injured cells found in the control assay was almost irrelevant (i.e. 2%) and very similar to that found when using the CDCFDA-SE probe for 24 h-incubated cells (3.3%), while in the AMP-assay it represented 84.3% of the total population. Thus, also in the AMP-assay results obtained with the CFDA-SE probe were in agreement with those found with the CDCFDA-SE probe, namely for the percentage of severely injured cells (77 %).

The present paper also confirms that there is a good correlation between the pH_i and the culturability exhibited by cells, as previously reported by several other authors (Fang et al., 2006; Gaggia et al., 2010; Imai and Ohno, 1995; Rechinger and Siegmund, 2002). Even if some compounds can have a pronounced antimicrobial effect without targeting membrane integrity (Powers and Hancock, 2003) our results show that these *S. cerevisiae* AMPs actually disturb the membrane integrity of cells. They also demonstrate that pH_i is a good physiological parameter to evaluate the physiological state of cells subjected to stress conditions. These findings, together with the observation that during the AMP-assays CDCFDA-SE stained indistinctly the cytoplasm and the vacuole of *H.*

guilliermondii cells (**Fig. 3B**), strongly suggest that the AMPs negatively affect the vacuole membrane (tonoplast). Since it has been proposed that vacuoles play an important role in maintaining the homeostasis of pHi in yeast (Carmelo et al., 1997; Mira et al., 2009), the loss of tonoplast integrity might explain why *H. guilliermondii* cells lose their ability to maintain pHi homeostasis in the presence of the AMPs.

In conclusion, the present study demonstrates that the AMPs secreted by *S. cerevisiae* (Albergaria et al., 2010; Branco et al., 2014) compromise the plasma membrane integrity of *H. guilliermondii* cells, and possibly also that of the vacuole membrane, thereby disturbing the pHi homeostasis and inducing a loss of culturability. It should be noted, however, that these physiological alterations cannot be specifically related with the anionic nature of the AMPs investigated in this work, since similar alterations have also been reported for cationic AMPs (Harris et al., 2009). Thus, future work is needed to fully understand the mode of action of these AMPs.

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Table 1: Percentage of *H. guilliermondii* cells with compromised membrane integrity (PI-stained cells) during the control and AMP assays.

Time (h)	% PI-stained cells	
	Control-assay	AMP-assay
0	0.0	0.0
8	7.22	38.3
24	28.3	77.7

Table 2: Subpopulations of *H. guilliermondii* cells present in the control assay (without AMPs) and in the AMP-assay (with AMPs) after 24 h of incubation and percentage of cells exhibiting fluorescence in the cytoplasm when stained with the CFDA-SE probe. Cells were grouped according with their ΔpH ($=\text{pHi}-\text{pH}_{\text{ext}}$) range and pHi values determined by the FRIM method using the CFDA-SE probe.

	% Healthy cells ($\Delta\text{pH} \geq 1.5$)	% Sub-lethally injured cells ($0 < \Delta\text{pH} < 1.5$)	% Severely injured cells ($\Delta\text{pH} = 0$)	%cytoplasm-stained cells
Control-assay	2.0	96.0	2.0	100
AMP-assay	5.9	9.8	84.3	100

Legends:

Fig. 1: Calibration curves of the fluorescence ratio (R470/440 nm) emitted by *H. guilliermondii* cells stained with the pH-sensitive probes CDCFDA-SE (**A**) and CFDA-SE (**B**) at different pH_i values. Values represented correspond to means of 50 single cells measured in each of two independent assays and error bars to standard deviation ($\pm$ SD). Calibration points were fitted by a third degree polynomial curve.

Fig. 2: Percentage of *H. guilliermondii* cells exhibiting CDCFDA-SE fluorescence exclusively in the vacuole after 0 h, 8 h and 24 h of incubation in the absence (Control assay) and in the presence of AMPs (AMP-assay). Percentages were calculated based on the microscopic observation of 100 individual cells.

Fig. 3: Microscope images (WB-filter) of *H. guilliermondii* cells stained with CDCFDA-SE after 24 h of incubation in the absence of AMPs (control assay) (**A**) and in the presence of the AMPs (AMP-assay) (**B**). Objective amplification of 100 x. Microscope observation of the AMP-assay sample showed the same cells both in the WB-filter (fluorescent cells) and in the bright-field (data not shown).

Fig. 4: Changes in the culturability (CFU/ml) and pH_i, as determined by the FRIM method with the CDCFDA-SE probe, of *H. guilliermondii* cells during the control (**A,B**) and AMP-assay (**C,D**). Evolution of pH_i of individual cells (50 cells analysed *per* sample) and of the pH_i average value (**A,C**), as well as of subpopulations exhibiting Δ pH $\geq$ 3 (healthy cells), $0 < \Delta$ pH < 3 (sub-lethally injured cells) and Δ pH=0 (severely injured cells) and of culturability (**B,D**). Each variable represented correspond to mean values of duplicate experiments $\pm$ SD.

Fig. 5: Culturability (CFU/ml) and sub-populations of *H. guilliermondii* cells with $\Delta\text{pH} \geq 3$, healthy; $0 < \Delta\text{pH} < 3$, sub-lethally-injured; $\Delta\text{pH} = 0$, severely injured after being exposed to AMPs for 24 h (with the AMPs 24 h) and after being allowed to recover for 24 h in YEPD medium (without the AMPs 24 h). Values represented are means of duplicate experiments $\pm$ SD (error bars).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5