PURIFICATION AND BIOCHEMICAL/MOLECULAR CHARACTERISATION OF ANTIMICROBIAL PEPTIDES PRODUCED BY *SACCHAROMYCES CEREVISIAE* AND EVALUATION OF THEIR MODE OF ACTION

PATRÍCIA ALEXANDRA BATISTA BRANCO

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Doutora Maria Helena Whytton da Terra Soares de Albergaria; Investigadora Auxiliar; Laboratório Nacional de Energia e Geologia, I.P.;

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TESE APRESENTADA PARA OBTENÇÃO DO GRAU DE DOUTOR EM ENGENHARIA ALIMENTAR

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3. Branco P, Kemsawasd V, Santos L, Diniz M, Caldeira J, Almeida MG, Arneborg N, Albergaria H (2017) *Saccharomyces cerevisiae* accumulates GAPDH-derived peptides on its cell surface that induce death of non-*Saccharomyces* yeasts by cell-to-cell contact. FEMS Microbiol Ecol 93:1-10. doi: 10.1093/femsec/fix055.
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5. Branco P, Viana T, Albergaria H, Arneborg N (2015) Antimicrobial peptides (AMPs) produced by *Saccharomyces cerevisiae* induce alterations in the intracellular pH, membrane permeability and culturability of *Hanseniaspora guilliermondii* cells. Int J Food Microbiol 205:112-118. doi:10.1016/j.ijfoodmicro.2015.04.015.
6. Branco P, Francisco D, Chambon C, Hébraud M, Arneborg N, Almeida MG, Caldeira J, Albergaria H (2014). Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions. Appl Microbiol Biotechnol 98:843-853. doi: 10.1007/s00253-013-5411-y.

"Science must be used to improve the lives of others"

Louis Pasteur

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ABSTRACT

The antagonistic effect exerted by *Saccharomyces cerevisiae* against other microbial species during wine fermentations was recently ascribed to its capacity to secrete antimicrobial peptides (AMPs). The main goal of the present work was to purify, identify and characterize those AMPs. Firstly, the AMPs were purified by means of chromatographic techniques (size-exclusion and ion-exchange) and then characterized regarding their amino acid sequence, codifying genes and antimicrobial/biochemical properties. Analysis of the purified AMPs by mass spectrometry revealed that the natural biocide is mainly composed by two peptides (AMP1 and AMP2/3) derived from the isoenzymes of the glycolytic protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The spectrum of action of the naturally-excreted AMPs, which we named saccharomycin, is wide and includes several wine-related non-*Saccharomyces* yeasts, such as *Hanseniaspora guilliermondii*, *Torulaspora delbrueckii*, *Kluyveromyces marxianus*, *Lachancea thermotolerans* and *Dekkera bruxellensis*, as well as bacteria such as *Oenococcus oeni*. The antimicrobial effect of saccharomycin is significantly higher than that of synthetic analogues (AMP1 and AMP2/3) and depends on their complementary action and relative proportion. The mode of action of the AMPs was evaluated against sensitive yeast cells. The AMPs induce cell membrane permeabilization, loss of pH homeostasis and increase/decrease of H^+-influx/-efflux. They also induce cell molecular markers typical of death by apoptosis in *H. guilliermondii*. Our work also revealed the accumulation of these GAPDH-derived peptides on the surface of stationary-grown (48 h) cells of *S. cerevisiae*, which induce death of non-*Saccharomyces* yeasts (*H. guilliermondii* and *L. thermotolerans*) by direct cell-cell contact. Finally, *S. cerevisiae* strains over-expressing these AMPs prevented growth of *D. bruxellensis* in co-fermentations, decreasing the levels of sulphur dioxide needed to control wine spoilage. Thus, the potential of these AMPs to be used as biopreservative in wine seems promising.

Keywords: Wine microbiology, *Saccharomyces cerevisiae*; Antimicrobial peptides; yeast-yeast interactions; glyceraldehyde-3-phosphate dehydrogenase

RESUMO

O efeito antagónico de *Saccharomyces cerevisiae* contra outras espécies microbianas durante fermentações vínicas foi recentemente atribuído à sua capacidade de excretar péptidos antimicrobianos (PAMs). O principal objetivo do presente estudo foi purificar, identificar e caracterizar estes PAMs. Primeiramente, os PAMs foram purificados por técnicas cromatográficas (exclusão molecular e permuta iónica) e posteriormente caracterizados quanto à sua sequência de aminoácidos, aos genes que os codificam e às suas propriedades antimicrobianas/bioquímicas. Os PAMs purificados foram, em seguida, analisados por espectrometria de massa, revelando que o biocida natural é composto maioritariamente por dois PAMs (PAM1 e PAM2/3) originários das três isoenzimas da proteína glicolítica gliceraldeído-3-fosfato desidrogenase. O espectro de ação dos PAMs naturais, os quais designámos por saccharomycin, é amplo e inclui várias leveduras vínicas, tais como *Hanseniaspora guilliermondii*, *Torulaspora delbrueckii*, *Kluyveromyces marxianus*, *Lachancea thermotolerans* e *Dekkera bruxellensis*, assim como a bactéria vínica *Oenococcus oeni*. O efeito antimicrobiano de saccharomycin é bastante mais acentuado do que o efeito dos análogos quimicamente sintetizados (PAM1 e PAM2/3) e depende da sua ação complementar, assim como da sua proporção relativa. O modo de ação dos PAMs foi analisado em leveduras sensíveis, verificando-se que estes induzem permeabilização da membrana celular, perda da homeostase do pH e aumento/decréscimo do influxo/efluxo de H^+. Verificou-se igualmente, que os PAMs induzem morte por apoptose em *H. guilliermondii*. Descobrimos, ainda, que estes PAMs se acumulam na superfície de células estacionárias (48 h) de *S. cerevisiae*, as quais são capazes de induzir a morte de leveduras não-*Saccharomyces* (*H. guilliermondii* e *L. thermotolerans*) por contacto celular direto. Por fim, uma estirpe laboratorial de *S. cerevisiae* foi manipulada geneticamente de forma a sobre-expressar cada um dos PAMs, verificando-se que as estirpes manipuladas apresentaram um elevado efeito antimicrobiano contra *D. bruxellensis*, o que permitiu reduzir os níveis de dióxido de enxofre normalmente aplicados em vinhos. Assim, a utilização destes PAMs como um bioconservante alternativo no vinho parece promissora.

Palavras-chave: Microbiologia enológica; péptidos antimicrobianos; interações levedura-levedura, gliceraldeído-3-fosfato desidrogenase.

RESUMO ALARGADO

Nas fermentações vínicas espontâneas, as leveduras não-*Saccharomyces* pertencentes à microflora natural dos mostos de uva, tais como *Hanseniaspora guilliermondii*, *Lachancea thermotolerans (Klyuveromyces thermotolerans*), *Kluyveromyces marxianus* e *Torulaspora delbrueckii,* são responsáveis pelo arranque da fermentação, crescendo até se atingirem concentrações de etanol à volta de 4-5% (v/v). Após esta primeira fase da fermentação, as condições do meio tornam-se gradualmente mais hostis e as leveduras não-*Saccharomyces* começam a morrer. Apesar disto, a maioria das estirpes *S. cerevisiae* é capaz de sobreviver e concluir a fermentação.

A morte prematura das leveduras não-*Saccharomyces* durante as fermentações vínicas tem sido tradicionalmente atribuída à sua baixa capacidade para suportar as condições adversas do meio de crescimento. No entanto, durante a última década, vários estudos têm questionado a influência destes fatores na morte prematura destas leveduras e outras causas têm sido propostas por diferentes autores. Um estudo pioneiro demonstrou que durante fermentações vínicas *S. cerevisiae* excreta péptidos (peso molecular <10 kDa) que induzem a morte de leveduras não-*Saccharomyces*. A presente tese teve como principal objetivo purificar, sequenciar e caracterizar esses péptidos antimicrobianos (PAMs). Para tal, realizaram-se fermentações alcoólicas em mosto sintético com *S. cerevisiae* e os sobrenadantes resultantes da fermentação foram ultrafiltrados e concentrados, utilizando membranas de 10 kDa e 2 kDa. A fração peptídica (2-10 kDa) foi, em seguida, purificada utilizando técnicas cromatográficas de exclusão molecular e troca iónica. As diferentes frações obtidas em cada passo do processo de purificação foram testadas quanto à sua atividade antimicrobiana contra leveduras sensíveis (e.g. *H. guilliermondii*). A fração purificada que apresentou um maior efeito antimicrobiano foi analisada por espectrometria de massa (LC-ESI-MS/MS), e revelou conter dois péptidos maioritários com as seguintes sequências de aminoácidos: ISWYDNEYGYSAR (PAM1) e VSWYDNEYGYSTR (PAM2/3). Estas sequências correspondem a fragmentos das três isoenzimas da proteína glicolítica gliceraldeido-3-fosfato desidrogenase (GAPDH).

O biocida natural contendo os PAMs excretados por *S. cerevisiae* durante a fermentação alcoólica foi designado por saccharomycin e o seu espectro de ação, bem como as concentrações inibitórias mínimas (CIMs) e as concentrações que induzem 50 % de redução do crescimento (CI50), foram determinados para as leveduras vínicas: *H. guilliermondii*, *T.*

delbrueckii, K. marxianus, L. thermotolerans e *Dekkera bruxellensis*. Os resultados mostraram que *H. guilliermondii*, *L. thermotolerans* e *K. marxianus* são mais sensíveis aos PAMs, exibindo CIMs de 250 µg/ml e CI50 de 80, 65 e 80 µg/ml, respetivamente; enquanto *T. delbrueckii* e *D. bruxellensis* são mais resistentes apresentando CIMs de 500 e 1000 µg/ml e CI50 de 135 e 260 µg/ml, respetivamente. Posteriormente, a atividade antimicrobiana da saccharomycin foi comparada com aquela exibida pelos análogos químicos PAM1 (derivado da isoenzima GAPDH1) e PAM2/3 (derivado das isoenzimas GAPDH2/3). A ação dos péptidos sintéticos foi testada contra *H. guilliermondii*, utilizando os péptidos em separado e em misturas de PAM2/3+PAM1 nas proporções de 1:1, 2:1, 4:1 e 6:1. Contudo, devido à natureza aniónica destes PAMs (p*I*=4.35) não foi possível solubilizá-los em meio YEPD a pH 3.5 (condição utilizada nos testes da saccharomycin) mas apenas a pH 6.0. Os resultados mostraram que o PAM1 tem um maior efeito antimicrobiano (76% de inibição) do que o PAM2/3 (30% de inibição), à mesma concentração (1000 µg/ml). Além disso, verificou-se que a ação conjugada dos dois péptidos resulta num maior efeito fungicida (100% de inibição), que é máximo na proporção 4:1. Estes resultados sugerem que o biocida natural (i.e., a saccharomycin) deverá ser composto por agregados destes PAMs e que algum metal catiónico (e.g. Fe^{2+} ou Mg^{2+}) presente nos mostos de uvas poderá aumentar o seu efeito antimicrobiano e promover a sua solubilização a pH acídico (i.e., a pH 3.5).

O modo de ação dos PAMs foi avaliado em células de *H. guilliermondii* em termos de alterações na permeabilidade da membrana celular (analisado por marcação das células com iodeto de propídeo), no pH intracelular (determinado pela técnica *Fluorescence Ratio Imaging Microscopy*) e nos fluxos de protões H^+ através da membrana (quantificado pela medição da velocidade de acidificação/alcalinização do meio externo). Os resultados mostraram que os PAMs induziram a perda da integridade membranar em 77,7% das células incubadas com 1000 µg/ml de saccharomycin, reduzindo o pHi de 6,5 para 3,5 em 77% das células e o efluxo de protões em 75,6 %.

Avaliou-se ainda o tipo de morte celular (apoptose/ necrose) induzido pelos PAMs. Para isso, incubou-se *H. guilliermondii* na presença dos péptidos sintéticos (PAM2/3+PAM1 numa proporção de 4:1) em concentrações sub-letais (i.e. 25, 50 e 100 µg/ml). Verificou-se que os PAMs induziram apoptose em 28% das células de *H. guilliermondii*, nomeadamente através de indução da fragmentação do ADN, uma característica típica de apoptose tardia.

A capacidade de internalização dos PAMs foi também avaliada em *H. guilliermondii* e *D. bruxellensis*, incubando estas células (pré-cultivadas e recolhidas em fase exponencial) em água e em meio YEPD na presença/ausência de etanol e dos péptidos sintéticos (PAM1 e PAM2/3), marcados com um fluorocromo (isotiocianato de fluoresceína). Os resultados mostraram que a percentagem de células que internalizaram os péptidos sintéticos foi significativamente maior em YEPD (ca 25-30%) do que em água (menos de 10%). A internalização dos péptidos não foi afetada pelo etanol, mas antes pelo próprio meio (i.e., água ou YEPD). Uma vez que está descrito que a capacidade antimicrobiana de certos PAMs aniónicos pode aumentar pela ação de metais catiónicos (e.g. Fe^{2+} e Mg^{2+}), estes resultados sugerem que algum metal catiónico presente no meio YEPD possa ter contribuído para este efeito.

A morte prematura de algumas leveduras não-*Saccharomyces* durante fermentações realizadas com *S. cerevisiae* foi atribuída por alguns autores a um mecanismo de morte mediado pelo contacto direto célula-a-célula com *S. cerevisiae*. Uma vez que o GAPDH foi encontrado na parede celular de *S. cerevisiae*, colocamos a hipótese de que estes PAMs se poderiam acumular na superfície celular de *S. cerevisiae* e assim induzir a morte das leveduras não-*Saccharomyces* por contacto celular direto. Para testar esta hipótese realizaram-se ensaios onde se colocaram células de *S. cerevisiae*, recolhidas respetivamente na fase exponencial (12 h) e na fase estacionária (48 h) de crescimento, em contacto direto com células de *H. guilliermondii* e de *L. thermotolerans* num meio isento de fontes carbonadas (para evitar a produção de quaisquer metabolitos) e a elevadas densidades celulares (para promover o contacto celular). Os resultados mostraram que as leveduras não-*Saccharomyces* em contacto direto com células exponenciais de *S. cerevisiae* mantiveram a sua viabilidade celular inalterada durante 30 h, enquanto as mesmas leveduras em contacto direto com células estacionárias de *S. cerevisiae* perderam 40-80% da sua viabilidade celular após apenas 5 h. Para confirmar a presença dos PAMs na superfície celular de *S. cerevisiae*, as proteínas de membrana das células exponenciais e estacionárias foram extraídas e separadas por cromatografia de exclusão molecular. Uma fração peptídica proveniente da superfície celular de células estacionárias de *S. cerevisiae* mostrou bioatividade e foi analisada quer por espectrometria de massa, quer por ensaios imunológicos usando um anticorpo policlonal específico (anti-PAM1 e anti-PAM2/3). Os resultados mostraram a presença dos PAMs na parede de células estacionárias de *S. cerevisiae*,

mas não de células exponenciais, o que demonstra que uma das razões da morte de leveduras não-*Saccharomyces* por contacto celular direto com *S. cerevisiae* é induzida pela presença destes PAMs na parede celular de *S. cerevisiae*.

Na indústria vínica ocorrem grandes perdas económicas devido a contaminações com microrganismos indesejáveis. Entre os vários contaminantes, *D. bruxellensis* é sem dúvida um dos mais perigosos. O dióxido de enxofre (SO_2) é o conservante químico mais utilizado para evitar o crescimento de contaminantes microbiológicos no vinho, nomeadamente de *D. bruxellensis*. Contudo, a adição deste composto em elevadas concentrações deve ser evitada devido ao impacto que tem na saúde. Com o propósito de utilizar estes PAMs como conservantes alternativos no vinho, ou como coadjuvantes dos conservantes químicos, construímos estirpes *S. cerevisiae* geneticamente modificadas para sobre-expressarem cada um dos PAMs (i.e., PAM2/3 e PAM1) em concentrações mais elevadas do que as estirpes selvagens. Os níveis de expressão dos PAMs foram analisados por *PCR* em tempo-real ao longo de uma fermentação alcoólica e a sua concentração no meio foi determinada pelo método ELISA (*Enzyme-linked immunosorbent assay*) utilizando um anticorpo policlonal específico. As estirpes modificadas mostraram níveis mais elevados de expressão e de produção dos PAMs (PAM2/3 e PAM1) do que a estirpe selvagem. De forma a confirmar o efeito antagonístico exercido pelas estirpes modificadas, realizaram-se fermentações alcoólicas com culturas mistas de *S. cerevisiae* e *D. bruxellensis*, utilizando quer as estirpes modificadas, quer a estirpe selvagem de *S. cerevisiae*. Os resultados mostraram que na presença das estirpes *S. cerevisiae* geneticamente modificadas a *D. bruxellensis* perdeu a sua viabilidade celular, enquanto na presença da estirpe selvagem essa levedura foi capaz de crescer. Verificou-se ainda que a adição de 1000 µg/ml dos PAMs a vinhos simulados (13% e 14% (v/v) de etanol), artificialmente contaminados com *D. bruxellensis*, permitiu induzir a perda de viabilidade de *D. bruxellensis* em apenas 48 h, permitindo reduzir os níveis de SO_2 utilizados até 14,25 mg/l (limite legal 150 mg/l). Estes resultados abrem interessantes perspetivas relativamente à possibilidade de utilização destes PAMs como bioconservantes alternativos, ou como coadjuvantes do SO_2, no vinho.

Palavras-chave: Microbiologia enológica; péptidos antimicrobianos; interações levedura-levedura, gliceraldeido-3-fosfato desidrogenase.

LIST OF ABBREVIATIONS

Abbreviations	Full meaning
ΔpH	Proton gradient
AAB	Acetic acid bacteria
AMPs	Antimicrobial peptides
AAMPs	Anionic antimicrobial peptides
ATP	Adenosine-5'-triphosphate
DNA	Deoxyribonucleic acid
CI50	Metade da concentração máxima de inibição
CIMs	Concentrações inibitórias mínimas
CFU	Colony forming units
CO_2	Carbon dioxide
EDTA	Ethylenediamine tetraacetic acid
ESI	Electrospray ionization
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GF	Gel filtration
GPD	Glycerol-3-phosphate dehydrogenase
HPLC	High-performance liquid chromatography
IC50	Half maximal inhibitory concentration
IEXC	Ion-exchange chromatography
LAB	Lactic acid bacteria
MALDI	Matrix assisted laser desorption/ionization
MIC	Minimal inhibitory concentration

MLF	Malolactic fermentation
MS	Mass espectrometry
NADH	Nicotinamide adenine dinucleotide-hydrogen
PAMs	Péptidos antimicrobianos
PCR	Polymerase Chain Reaction
pHi	Intracellular pH
RNA	Ribonucleic acid
RP-HPLC	Reversed-phase high-performance liquid chromatography
ROS	Reactive oxygen species
SO_2	Sulphur dioxide
YEPD	Yeast extract peptone dextrose
TUNEL	dUTP nick-end labeling

AIMS AND ORGANIZATION OF THE THESIS

Framework

During spontaneous wine fermentations, the indigenous non-*Saccharomyces* yeasts naturally present in grape musts (e.g. *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum*, *Candida stellata*, *Lachancea thermotolerans*, *Kluyveromyces marxianus*, *Torulaspora delbrueckii*) grow during the first days of fermentation, but when ethanol reaches concentrations of 4-5% (v/v) they begin to die-off. Although *S. cerevisiae* is present in the grape musts microflora in lower numbers than non-*Saccharomyces* yeasts, strains of this species are able to ferment grape sugars until exaustion and complete the fermentation process (Du Toit & Pretorius 2000).

Until recently, the selection and use of starter yeast cultures for wine fermentations has been limited to strains of *S. cerevisiae*. However, some non-*Saccharomyces* yeasts (e.g. *T. delbrueckii*, *L. thermotolerans*, *H. uvarum*) produce higher amounts of metabolites that can enhance the wine flavour (i.e. succinic acid, esters, higher alcohols) (Jolly et al. 2003; Rodríguez et al. 2010; Sadoudi et al. 2012). Therefore, nowadays, there is an increasing demand for new wine yeast starters composed of non-*Saccharomyces* and *S. cerevisiae* strain pairs that can improve oenological properties of wines. With that purpose, several research groups have studied the microbial interactions that occur between non-*Saccharomyces* and *S. cerevisiae* strains during wine fermentations.

S. cerevisiae dominance over other microbial competitors during wine fermentation has been always attributed to its higher capacity to withstand the increasingly adverse conditions occurring as the fermentation progresses: i.e. high levels of ethanol and organic acids, low pH values, oxygen scarcity and nutrients depletion (Bisson 1999; Hansen et al. 2001). Nevertheless, the weight of these factors on the microbial succession during wine fermentations has been lately questioned, and other yeast-yeast and yeast-bacteria interactions have been proposed by different authors. First, Nissen & Arneborg (2003) and Nissen et al. (2003) reported that direct physical contact between *S. cerevisiae* and non-*Saccharomyces* yeasts (*L. thermotolerans* and *T. delbrueckii*) is responsible for their early death. Then, a study carry out by Pérez-Nevado et al. (2006) proposed that the early death of some non-*Saccharomyces* yeasts

(i.e. *H. guilliermondii* and *H. uvarum*) during wine fermentations was due to killer toxins secreted by *S. cerevisiae* strains. Later, Albergaria et al. (2010) discovered that those toxins correspond to antimicrobial peptides (AMPs) (molecular weight lower than 10 kDa) secreted by *S. cerevisiae* (strain CCMI 885) which inhibit the growth of several non-*Saccharomyces* wine-related yeasts (*H. guilliermondii, K. marxianus, K. thermotolerans, T. delbrueckii*).

General and Specific Aims

Having in mind the previous issues, the main goal of the present thesis was to purify, identify and characterize the AMPs previously-found, as well as to investigate their mode of action and death-inducing mechanisms (apoptosis/necrosis) on sensitive yeast cells. The role of these AMPs in microbial interactions established during wine fermentation was also evaluated, as well as the possibility of using these AMPs as an alternative biopreservative in wine.

The specific aims were:

- Purification of the AMPs by chromatographic techniques;
- Identification and sequenciation of the purified AMPs by mass spectrometry;
- Determination of the spectrum of action and of antimicrobial properties (MIC, IC50) of the AMPs;
- Study the mode of action of the AMPs, namely by evaluating alterations in the membrane permeability, in intracellular pH (pHi) and in the culturability of sensitive yeasts cells; assessment of the death mechanisms (apoptosis/necrosis) involved;
- Investigate the involvement of these AMPs in the early death of non-*Saccharomyces* yeasts mediated by cell-cell contact;
- Construction of genetically engineered strains that over-express the AMPs and evaluation of their biopreservative potential against the most dangerous wine contaminant *D. bruxellensis*;
- Evaluation of the conjugated effect of these AMPs with sulphur dioxide (SO_2) against *D. bruxellensis* growth.

Organization of the thesis

The present thesis includes a general introduction (**Chapter I**) with the state-of-art regarding the main subjects relevant for the thesis, followed by six chapters (**Chapters II-VII**) containing the experimental work, presented in the form of full-length manuscripts, some of them already published in peer-reviewed journals (**Chapters II-V**) and other two to be submitted (**Chapters VI and VII**), and a final chapter (**Chapter VIII**) with the main concluding remarks and future perspectives. The chapters are presented as follows:

- **Chapter I:** General Introduction
- **Chapter II**: Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions;
- **Chapter III:** Antimicrobial peptides (AMPs) produced by *Saccharomyces cerevisiae* induce alterations in the intracellular pH, membrane permeability and culturability of *Hanseniaspora guilliermondii* cells
- **Chapter IV:** Antimicrobial properties and death-inducing mechanisms of saccharomycin, a biocide secreted by *Saccharomyces cerevisiae*
- **Chapter V:** *Saccharomyces cerevisiae* accumulates GAPDH-derived peptides on its cell surface that induce death of non-*Saccharomyces* yeasts by cell-to-cell contact
- **Chapter VI:** Effect of GAPDH-derived antimicrobial peptides on sensitive yeast cells: plasma membrane permeability, intracellular pH and H^+-influx/efflux
- **Chapter VII**: Expression of the GAPDH-derived AMPs on *S. cerevisiae* and evaluation of their biopreservative potential on wine fermentations
- **Chapter VIII:** Concluding remarks and future perspectives

Chapter I

GENERAL INTRODUCTION

1. Winemaking process

1.1. Brief history of wine production

The production of wine dates back to the origin of civilization and therefore is one of the oldest world's biotechnological processes. Historical archives show that the earliest winemaking activities were documented in Mesopotamia and Caucasus by 6000 BC. Around 2000 BC, wine was produced in the ancient Greece and due to the Roman Empire expansion, the process was spread out through the Mediterranean. In 500 BC wine was already produced in the current regions of Italy, Sicily, France, Portugal, Spain, and North Africa. Later, in the sixteenth century, European explorers introduced vine into the New World, and in 1530 Spanish conquerors planted *Vitis vinifera* in Argentina, Chile, Mexico and Peru. Afterwards, in 1655, Dutch colonizers planted vineyards in South Africa and shortly after that, *V. vinifera* was introduced in California and Australia (Pretorius 2000).

Even though the production of wine is one of the world's oldest biotechnological processes, the basic principles of the winemaking process have not changed considerably along history. The wine production process starts with grapes being harvested and then crushed. Subsequently, the grape juice is fermented in wood, stainless steel or concrete vats with or without temperature control. The alcoholic fermentation process which then occurs varies according to the type of wine to be produced: white, rosé or red wine. The production process of white wine (**Fig. 1A**) diverges in some steps from the production process of red wine (**Fig. 1B**). In the case of white wines, the grape juice is separated from the lees before fermentation, while red wine fermentation occurs in the presence of grape solids. Fermentation in the presence of anthocyanins, which are the pigments of the red grape berries located in the skin of grapes, introduces a major difference in the composition and taste of white and red wines. In red wine, after alcoholic fermentation, other type of metabolic process called the malolactic fermentation (MLF), may eventually occur. MLF transforms malic acid into lactic acid, lowering the acidic flavour of wine and thus altering its organoleptic characteristics (Bisson 2004).

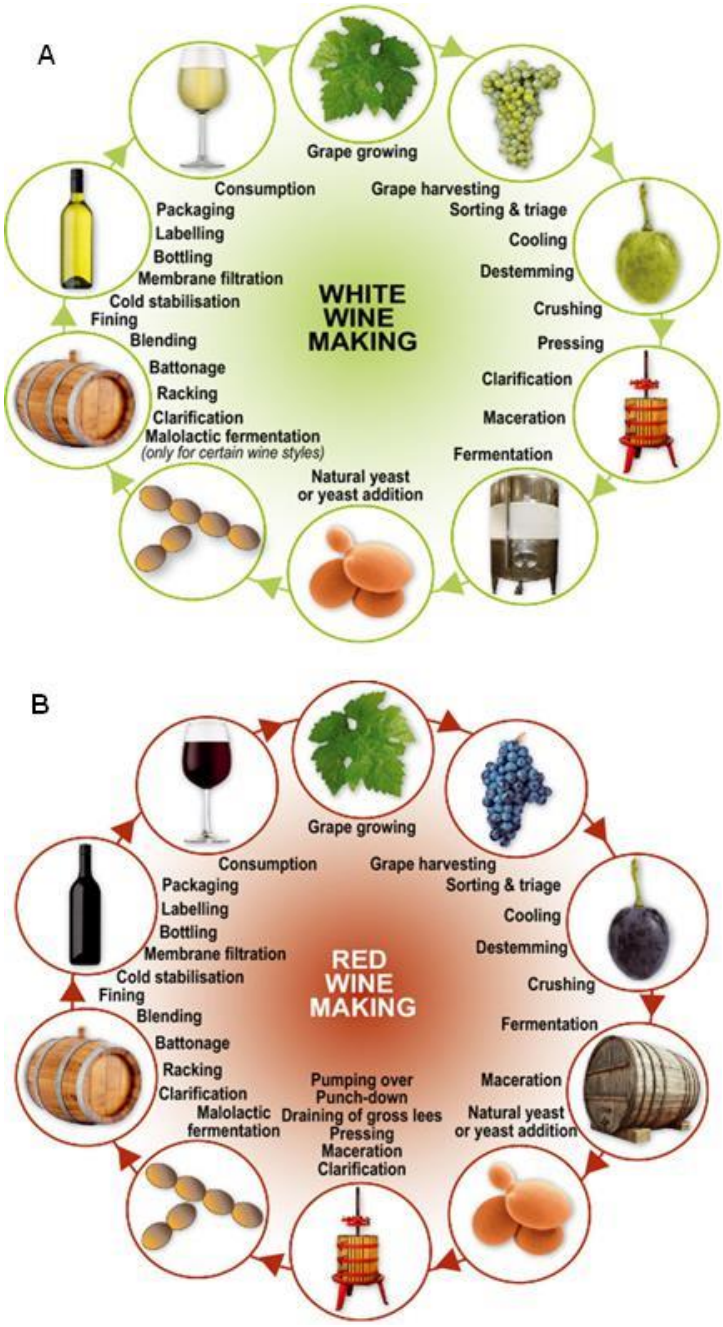

Fig. 1: Main steps of the white wine (A) and red wine (B) production processes (withdrawn from Jolly et al. (2013)).

Antoine Lavoisier was one of the founders of modern chemistry and was also the first to identify alcoholic fermentation. In 1789, Lavoisier described the main chemical reaction of alcoholic fermentation as: "grape must = carbonic acid + alcohol". He characterized this process as being one of the most amazing phenomena of chemistry. The word alcohol derives from the Arabic word *Al-kuhl* which means spirit. Later, it was used to replace what Lavoisier originally designated as the spirit of wine. Alcoholic fermentation is the most important biotransformation that occurs during the winemaking process; this biotransformation is conducted by yeast species that belong or not to the natural microbiota of the grape musts (Esteve-Zarzoso & Manzanares 1998; Romano et al. 2003). During alcoholic fermentation, yeasts metabolize carbohydrates of the grape juice obtaining energy that support their growth and producing ethanol and carbon dioxide (CO_2), (Barnett 1998; Barnett & Entian 2005).

1.2. Microorganisms associated with wine fermentation

The natural microflora associated with wine fermentation is highly complex due to the huge variety of indigenous microbial species present in the grape musts. This includes several species of yeasts, bacteria, and moulds. The microbial composition of grape musts varies according to the stage of grape ripening, physical damage of grape berries (caused by mould, insects, and birds), viticultural practicesand the presence of fungicides applied to vineyards (Pretorius et al. 1999) Damages on the surface of grapes increase the availability of nutrients for microbial growth, thus promoting population and diversity of yeasts that co-exist with various filamentous *fungi*, lactic acid bacteria (LAB) and acetic acid bacteria (AAB) (Fleet & Heard 1993). Although grape musts are relatively complete in nutrients, their low pH values (ranging 3.0-3.5) and high sugar contents (160-240 g/l) lead to a selective growth environment in which only a few yeast species can grow (Henschke 1997).

1.2.1. Yeasts

Of the 100 yeast genera representing over 700 species, 23 are associated with winemaking: *Aureobasidium, Auriculibuller, Brettanomyces, Bulleromyces, Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Issatchenkia, Kluyveromyces, Lachancea, Lipomyces, Metschnikowia, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Sporidiobolus,*

Sporobolomyces, *Torulaspora*, *Yarrowia*, *Zygoascus*, and *Zygosaccharomyces* (Barnett et al. 2000; Grangeteau 2016).

1.2.1.1. *S. cerevisiae*

Surprisingly, the population of *S. cerevisiae*, the main wine yeast species, is very low on the surface of healthy, undamaged grapes or even in vineyard soils (Barata et al. 2012; Frezier & Dubourdieu 1992; Martini et al. 1996). In fact, the origin of *S. cerevisiae* is quite controversial. While some researchers believe that damaged grapes are an important source of *S. cerevisiae* (Mortimer & Polsinelli 1999), other investigators have even argued that *S. cerevisiae* is not present in the vineyard habitat at all, and can only be found in the winery equipment's such as stemmer-crushers, pumps, pipes or fermentation vessels (Martini et al. 1996; Sabate et al. 2002). More recently in Portugal the isolation of *S. cerevisiae* from oak bark was reported (Sampaio & Gonçalves 2008). Nevertheless, due to its outstanding capacity to produce ethanol and CO_2 from sugars with high productivity, *S. cerevisiae* plays a primary role in the winemaking process (Fleet & Heard 1993; Pretorius et al. 1999; Pretorius 2003;). Therefore, *S. cerevisiae* is universally preferred for initiating alcoholic fermentation, and has earned itself the title of "the wine yeast". Interestingly, in 2003, ribosomal DNA from *S. cerevisiae* was found in a residue inside one of the earliest known wine jars from Egypt, demonstrating that this yeast species was probably responsible for wine fermentation since at least 3150 BC (Cavalieri et al. 2003).

Even if *S. cerevisiae* is referred as "the wine yeast", grape musts naturally contain a mixture of yeast species and wine fermentation is not a 'single-species' process (Fleet & Heard 1993).

1.2.1.2. Non-*Saccharomyces*

Amongst non-*Saccharomyces* yeasts associated with the natural microflora of grape musts, apiculate yeasts belonging to the genus *Hanseniaspora* are predominant on the surface of grape berries and in freshly processed grape musts, accounting for 50–75% of the total yeast population (Fleet & Heard 1993, Romano et al. 2003).

Although present in lower number, other yeasts species such as *Rhodotorula*, *Pichia*, *Candida*, *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, , *Zygosaccharomyces*, *Metschnikowia and Cryptococcus* are found in fresh must (Fleet 2003; 2008).

Non-*Saccharomyces* yeasts are often isolated from wines with anomalous analytical and sensorial profiles or from stuck fermentations. Therefore, they are frequently referred as 'wild' yeasts or 'spoilage' yeasts due to the production of large amounts of some detrimental metabolites such as ethyl acetate and acetic acid (Ciani and Picciotti 1995). Nevertheless, most non-*Saccharomyces* yeasts can also improve the organoleptic quality and complexity of wine, since they produce large amounts of various metabolites that can contribute to the sensorial profile of wine, namely: esters, terpenoids, glycerol, acetaldehyde, and succinic acid (Bisson & Kunkee 1991; Clemente-Jimenez et al. 2005; Fleet 2008; Jolly et al. 2013; Lonvaud-Funel 1996; King & Dickinson 2000; Zohre & Erten 2002). For instance, some non-*Saccharomyces* yeast such as *Lachancea thermotolerans* and *Starmerella bacillaris*, produce high amounts of glycerol which contributes to the smoothness, fullness, sweetness and complexity of wine. Although these characteristics are usually considered as positive, excessive production of glycerol can be detrimental to the quality of wine since it is often associated with increased acetic acid production (Comitini et al. 2011; Henick-Kling et al. 1998, Prior et al. 2000; Romano et al. 1997).

In most cases, the impact of non-*Saccharomyces* yeasts on the wine flavour is limited by the common practice of inoculating grape musts with pure *S. cerevisiae* cultures (wine starters), since this species can assure fast, complete and safe wine fermentations (Jolly et al. 2013). In spite of this, these yeasts can contribute to enhance several desirable organoleptic characteristics of wine. Consequently, wine starters composed of mixed *S. cerevisiae* and non-*Saccharomyces* strains have been recently developed and commercialised in order to take advantage of the positive features of non-*Saccharomyces* yeasts and, at the same time, reduce their negative effects (Jolly et al. 2013).

1.2.2. Lactic acid bacteria (LAB)

Different species of LAB have been associated with the winemaking process (Fleet 2001). In grapes, the main species of LAB that can be found are: *Lactobacillus plantarum*,

Lactobacillus hilgardii and *Lactobacillus casei*. The species *Oenococcus oeni*, which dominates the MLF, is rarely detected at the beginning of the alcoholic fermentation. Growth of LAB depends on the pH of the medium and on the concentration of sulphur dioxide (SO_2) added to the grape must. After the end of alcoholic fermentation, LAB may remain in a latent phase for quite some time. Then, the MLF may occur, depending on the temperature, pH, as well as the ethanol and SO_2 concentrations present in the medium. As soon as the malic acid is completely transformed into lactic acid (MLF), the bacterial population begins to decline. In wines without added sulphites after the MLF, LAB may remain in latent state during months (Renouf et al. 2007). LAB can drive metabolic modifications which include metabolism of citrate, amino acids, polysaccharides, synthesis and hydrolysis of esters, and degradation of phenolic acids, lipolysis, proteolysis and peptidolysis. These metabolic alterations promote microbial stability of wine and soften its taste by decreasing its total acidity and improving the flavour (Bartowsky 2005; Cappello et al. 2016). Therefore, the MLF is desirable in some white wines containing excessive total acidity and in most red wines. Yet, this process is still difficult to control as several factors affect LAB growth and activity such as nutritional deficiencies, low pH values, as well as high ethanol and SO_2 levels (Comitini et al. 2005; Eglinton & Henschke 1996; Tracey & Britz 1989).

1.2.3. Acetic acid bacteria (AAB)

AAB are obligately aerobic bacteria within the family of *Acetobacteraceae*. The AAB are normally present in acidic and alcoholic niches such as grape must (Mamlouk & Gullo 2013; Sievers & Swings 2005). AAB, such as *Acetobacter aceti*, *Acetobacter pasteurianus* and *Gluconobacter oxydans* are commonly isolated from grape musts (Drysdale & Fleet 1988). *G. oxydans* is present on grapes and disappears to give way to species of the genus *Acetobacter*, which subsists in wine (Joyeux et al. 1984). AAB usually do not develop during the winemaking process since the conditions in which the process occurs (e.g. semi-anaerobiose, high ethanol and SO_2 levels, low pH values) are not favourable for their growth. AAB are classified as obligately aerobic organism. However, momentary aeration in wine (agitation or racking of wine from one barrel into another) is sufficient to encourage significant growth of resident AAB populations (Bartowsky and Henschke 2008; Joyeux et al. 1984). AAB are oxidative microorganisms that are able to oxidize ethanol into acetic acid (Bartowsky and

Henschke 2008; Du Toit & Pretorius 2002; Joyeux et al. 1984). This unique characteristic allows AAB to transform wine into vinegar, whenever oxygen is available. For this reason, AAB are considered one of the main wine spoilage agents.

1.2.4. Contaminants and preservatives in the wine industry

Wine spoilage microorganisms include LAB, AAB, and some yeasts species (Du toit and Pretorius 2000). Many secondary metabolites produced by bacteria are volatile compounds that can affect wine sensory qualities. *O. oeni*, the most important LAB of wine, reduces the volatile acidity of wines thus positively contributing to its flavour (Bartowsky 2005). However, other species of LAB such as *Lactobacillus* sp. and *Pediococcus* sp. can produce undesirable volatile compounds such as 2,3-butandione (diacetyl), 2-ethoxy-3,5-hexadiene and acrolein which gives a flavour of buttery, nutty, caramel and bitterness to the wine (Bartowsky 2009). The other main wine spoilage bacteria are AAB, which are known for their ability to transform ethanol into acetic acid, i.e. wine into vinegar. Besides, they also produce acetaldehyde, ethyl acetate and ethyl ester that are the main detrimental compounds produced by wine-associated AAB. In prolonged barrel maturation, if wine is not topped up and monitored regularly, there is a high risk of spoilage by AAB. Likewise, reduced management during bottling and storage of red wine can promote the proliferation of spoilage by *A. pasteurianus* (Bartowsky & Henschke 2008).

Even though yeasts are responsible for alcoholic fermentation, some species such as yeasts of the genera *Dekkera*/*Brettanomyces, Candida, Hanseniaspora, Pichia, Metschnikowia, Saccharomycodes, Schizosaccharomyces and Zygosaccharomyces* can also contribute to wine spoilage (Enrique et al. 2007; Fleet 2003). Amongst those contaminants, *Dekkera bruxellensis* is considered the major cause of wine spoilage worldwide (Fugelsang 1997; Loureiro & Malfeito-Ferreira 2003). The metabolic products of *D. bruxellensis* in wines are tetrahydropyridines, acetic acid and volatile phenols (Heresztyn 1986;). The most undesirable off-flavors and off-odours are provoked by volatile phenols, namely by the 4-ethylphenol that confers phenolic off-odours described as ‘‘barnyard-like’’ or ‘‘horsey’ (Fugelsang 1997; Loureiro & Malfeito-Ferreira 2003).

In wines with low acidity (above pH 3.7) and ethanol concentrations lower than 16% (v/v) it can be challenging to stop spoilage microorganisms’ growth. The maintenance of high hygienic

standards during the various steps of wine production is essential to prevent the contamination of wine. Nowadays, control of wine spoilage is typically carried out by the filtration of wine and proper sanitization of barrels. Unfortunately, these procedures have limited efficiency against some contaminants and do not prevent subsequent recontamination (Bartowsky 2009; Millet & Lonvaud-Funel 2000). Thus, chemical preservatives such as SO_2, benzoic acid, sorbic acid and dimethyl dicarbonate (DMDC) are used to control unwanted microorganisms during winemaking (Ribéreau-Gayon et al. 2006). SO_2, the most commonly used wine preservative, acts as an antimicrobial agent and also as an antioxidant, and is usually added before fermentation to machine-harvested grapes and immediately after MLF (Romano & Suzzi 1993). However, the addition of SO_2 in excessive doses should be avoided since it can have negative impacts on the wine aroma and, above all, in human health. Nowadays consumer preferences have changed to products that are less heavily preserved more natural and healthier. This tendency has led to the possible exploitation of natural antimicrobial compounds. Consequently, numerous chemical and naturally occurring preservatives such as, sorbic acid, fumaric acid, benzoic acid, lysozyme, killer toxins as well as antimicrobial peptides such as nisin were tested as alternatives to SO_2 (du Toit & Pretorius 2000; Fugelsang & Edwards 2007). However, no new compound has been identified which could be a real substitute for SO_2 with all its oenological properties including the antioxidative and antiseptic action in winemaking (García-Ruiz et al. 2008;).

2. Yeast population dynamics during wine fermentation

Spontaneous fermentation of grape musts occurs under non-sterile conditions and several yeast species and strains are involved in the process. Therefore, spontaneous wine fermentation is a complex biochemical process characterized by the sequential growth of different yeasts. Yeast genera and species substitute each other along the fermentation process according to their optimal fitness to the changing environment (Pretorius 2000), originating a typical growth pattern (**Fig. 2**).

In the early stages of wine fermentation non-*Saccharomyces* yeasts, such as species from the genera *Hanseniaspora* and *Candida*, are predominant. These non-*Saccharomyces* yeasts species are characterized by their low fermentative capacity, low ethanol tolerance, and a limited growth phase. The grape must is a selective growth medium, in which the non-

Saccharomyces initially present at high density levels (10^4-10^5 cells/ml) grow during the first 1-3 days of fermentations, reaching populations of about 10^7-10^8 cells/ml, while the ethanol concentrations are still low (30-40 g/l). Subsequently, the strongly fermentative and highly ethanol-tolerant strains of *S. cerevisiae* take over the fermentation process until completion (Fleet & Heard 1993; Pretorius 2000).

The yeast population dynamics and succession of species throughout the fermentation process varies according to fermentation fitness and survival ability of each yeast species that in turn depends on their intrinsic physiological features.

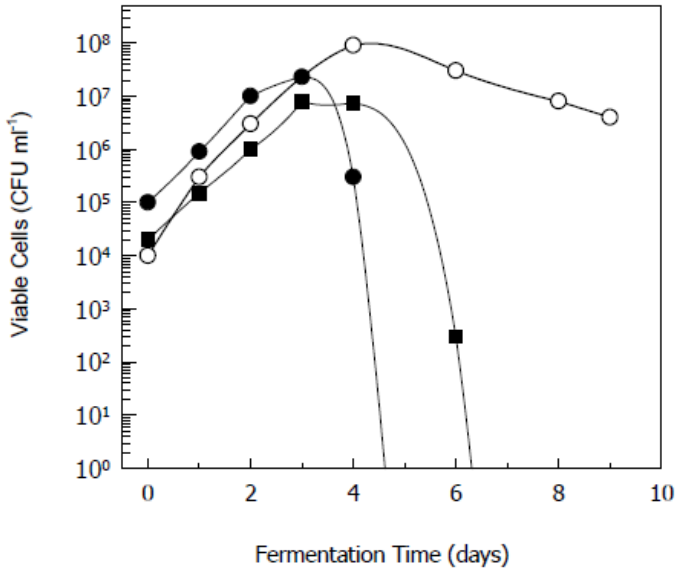

Fig. 2: General picture of the growth profiles of the most representative yeast species during wine fermentations. *Saccharomyces cerevisiae* (○); *Hanseniaspora* species (●); *Candida* species (■) (withdrawn from Fleet & Heard 1993).

2.1. Fermentation fitness of wine yeasts

The ability of wine yeasts to effectively ferment grape sugars depends on their fitness to grow and survive under the severe environment conditions of grape must. There is a direct correlation between the fermentation efficiency and stress resistance which refers to the ability of yeasts to adapt to the harsh conditions of the environment. This adaptation is determined by

different regulatory mechanisms, commonly referred as a physiological stress response (Bauer & Pretorius 2000)

Grape musts usually contain 140 to 260 g/l of an equimolar mixture of glucose and fructose. The majority of grape sugars are consumed by yeasts, producing around 10–16% (v/v) of ethanol and other metabolites and leaving approximately 2 g/l of residual sugars (Boulton et al. 1996). Several studies (Albergaria 2007; Ciani & Maccarelli 1998; Ciani & Picciotti 1995) have shown that most non-*Saccharomyce*s yeasts (e.g. *H. uvarum*, *H. guilliermondii and Starmerella bacillaris*) under oenological conditions have low limited fermentation capacity, exhibiting slow fermentation rates, producing lowest ethanol levels (5–8 % v/v) and leaving high residual sugars (30–100 g/l). Among the non-*Saccharomyces* yeasts the one that shows the slowest growth rates (0.027 h^{-1}) and low ethanol production (4-6% v/v) is *S. bacillaris*. Besides, most of those yeasts have also low SO_2 resistance. Conversely, *S. cerevisiae* is highly fermentative, exhibiting the fastest growth rates (0.085 h^{-1}) amongst the wine-related yeasts and producing the highest ethanol levels (12-16%) during wine fermentations. (Ciani & Maccarelli 1998; Ciani & Picciotti 1995)

Beyond its capacity to ferment sugars in a fast and complete way, yeasts's aptitude for wine fermentation also relies on their ability to grow and survive under the harsh wine environment conditions such as osmotic stress caused by high sugar concentrations, low pH values (3–3.5) low oxygen availability, low nitrogen concentrations (150–200 mg/l), high levels of ethanol (10–16% v/v) and organic acids (Pretorius 2000; Albergaria & Arnerborg 2016)

2.1.1. Osmotic stress

Osmotic stress can be defined as any situation where there is an imbalance between the intracellular and extracellular osmolality, sufficient to cause a harmful change in the cell physiology, such as loss of water and subsequently turgor (Blomberg & Adler 1992; Csonka & Hanson 1991; Klipp et al. 2005; Wood 1999). Grape must is a high-density substrate medium that contains a high concentration of osmotically active substances, specifically fructose and glucose.

Osmoregulation in yeast depends on the capacity of cells to sense external stimuli and to respond with changes in their physiology and biochemistry. After exposure to an osmotic

challenge cells can react to that stress by osmotolerance and osmoadaptation. Osmotolerance is an inherited feature rather than the result of adaptation and may be defined as the ability to grow in an environment with a high osmotic pressure. Osmoadaptation is a highly refined sensing and response system that may also be activated as a form of severe or persistent response (Gibson et al. 2007). The high sugar wine environment induces a hyperosmotic shock on yeast cells. As a response to this stress, yeasts developed a number of mechanisms, including alterations of the cell wall composition and of the cytoskeleton (Slaninová et al. 2000). For instance, *S. cerevisiae* under osmotic stresss induces the expression of the genes GPD1 and GPP2 that encode for NADH-dependent glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase, respectively, in order to increase the production of glycerol, a chemically inert osmolyte which re-establish the osmotic equilibrium (Albertyn et al. 1994; Hohmann 2002; Norbeck et al. 1996). Osmotolerant yeasts are usually able to synthesize glycerol as a compatible solute that acts as an osmoregulator (Hohmann 2002). Apparently, all yeast species use glycerol to compensate the increased external osmotic pressure and to prevent cellular water loss (Blomberg & Adler 1992). In summary, in order to survive in such hostile osmotic conditions, wine yeasts developed osmotic adaptation which involves the accumulation of glycerol.

2.1.2. Low pH

Depending on the yeast strain and on the environment conditions (e.g. temperature and oxygen availability), the optimal pH for yeasts growth ranges from pH 4.0 to 6.0 (Narendranath & Power 2005). The low initial pH of wine (2.8 -3.5) affects the growth and fermentation rate of yeast and influences the production of fermentation products (Nielsen & Arneborg 2007; Yalcin & Ozbas 2008). During wine fermentations, specifically during alcoholic fermentation, several other compounds are produced besides ethanol, namely glycerol, acetic acid and succinic acid (Zamora 2009). The weak organic acids, such as those above-mentioned, can affect the cell wall structure, by changing the conformation of proteins from the plasma membrane and destabilize their lipid organization (Booth & Statford, 2003). Under the low pH values of grape musts and wine (pH=2.8-4.2) (Heard & Fleet 1988), weak organic acids occur predominantly in their undissociated form and thus can cross the cell membrane. Once inside

the cell, these acids dissociate due to the higher intracellular pH (ca 6.5-7.0), liberating H^+ protons and promoting intracellular acidification (Orij et al. 2011; Piper et al. 2001; Ullah et al. 2013). Intracellular acidification leads to inhibition of essential metabolic functions (Krebs et al. 1983; Bracey et al. 1998) such as inhibition of glycolysis (Pearce et al. 2001) and consequently to a reduced ability to generate ATP. To respond to this effect, yeasts use mechanisms to regulate the pH homeostasis and maintain the intracellular pH. In *S. cerevisiae*, cells regulate pH homeostasis by pumping out the excess of protons generated through increased H^+-efflux rates via H^+-ATPase (Bracey et al. 1998). The plasma membrane H^+-ATPase enzyme is energy depend and pumps out H^+-protons using ATP hydrolysis at a 1:1 ratio (De Kok et al. 2012). At normal conditions, H^+-ATPase activity consumes about 20% of ATP (Morsomme & Boutry 2000). However, under weak organic acid stress this activity is increased consuming up to 60% of ATP (Holyoak et al. 1996).

A study performed by Nielsen & Arneborg (2007) to evaluate the effect of a weak acid (citric acid) at pH values of 3.0, 4.0, and 4.5 on growth and metabolism of *S. cerevisiae* and *Z. bailii* cultures showed that *S. cerevisiae* in the presence of citric acid at higher pH low the ethanol production as well as increases the glycerol production. However, *Z. bailii* is not so affected by citric acid since the production of glycerol is less enhanced and its ethanol production is narrowly affected as in *S. cerevisiae*.

Therefore, depending on the yeast species the primary energy metabolism is differently affected by the weak organic acids present in wine fermentations.

2.1.3. Oxygen availability

In yeast, oxygen is a crucial factor in the regulation of the sugar metabolism. In the presence of oxygen, nearly all yeasts can metabolise sugars via respiration producing CO_2 and water. Most of the yeasts species described until now are also capable of fermenting sugars to ethanol and CO_2 (Barnett et al. 1998; van Dijken & Scheffers 1986). Yet, the ability to ferment sugars to ethanol does not imply the aptitude to grow under strick anaerobic conditions and most facultative fermentative yeasts do not grow well in the complete absence of oxygen. In fact, *S. cerevisiae* is one of the few yeasts species that can grow under strictly anaerobic conditions (Visser et al. 1990). Conversely, most non-*Saccharomyces* yeasts require molecular oxygen to grow (van Dijken et al. 1993).

During wine fermentation yeasts grow under semi-anaerobic conditions and accessibility to oxygen is crucial for growth and survival of many yeasts species (Hanl et al. 2005; Hansen et al. 2001). Wine-related yeasts of the genera *Hanseniaspora, Kloeckera* and *Torulaspora*, grow poorly under these conditions, while *S. cerevisiae* exhibits fast rates of sugar consumption and ethanol production. Besides, *S. cerevisiae* is also able to ferment sugars and produce ethanol, even under fully aerobic conditions (Visser et al. 1990). This metabolic behaviour is known as the Crabtree effect and consists in the repression of the respiratory pathway whenever sugars are present in high amounts (Crabtree 1928). Since aeration of grape musts before fermentation is a common practise in winemaking to promote yeasts growth, Crabtree-positive yeasts such as *S. cerevisiae* are more likely to perform alcoholic fermentation at any growth conditions (van Dijken et al. 1993).

2.1.4. Nitrogen limitation

Numerous nitrogen sources are available in grape musts, including ammonium ions, amino acids, and peptides which are necessary for protein synthesis and yeast growth. However, many factors such as environmental features (climatic conditions and soil fertility), grape variety, maturity and viticultural practices (grape harvesting techniques) have considerable effects on the quantitative and qualitative nitrogen content of musts (Bell and Henschke 2005). Ammonium is present in grape must and is one of the first nitrogen sources to disappear from the medium during yeast cellular growth, since it is the main intermediate between catabolic and anabolic pathways (ter Schure et al. 2000). Glutamine, glutamate, and asparagine, are also described as good nitrogen sources for yeasts growth, allowing high specific growth rates. On the contrary proline, allantoin, or urea are described as non-preferred nitrogen sources (Crépin et al. 2012).

Nitrogen availability in grape musts can affect the production of many volatile compounds by yeasts, which contribute to the wine flavour, since some amino acids are direct metabolic precursors for the synthesis of higher alcohols, short to medium-chain fatty acids, and their ethyl ester or acetate ester derivatives (Hazelwood et al. 2008; Barbosa et al. 2012). Besides, nitrogen regulates the formation of yeast biomass and therefore if the concentration of nitrogen is limited it will be rapidly assimilated by the initial microflora (non-*Saccharomyces*) of wine fermentations (Constantí et al.1998; Henick-Kling et al. 1998). Consequently, will

inhibit the succeeding growth of *S. cerevisiae* strains which can lead to sluggish fermentation (Bisson 1999; Fleet 2003). Therefore, nutrient limitation is one of the factors that influence the yeast ecology of fermentation since one yeast species or strain produces or utilises a nutrient relevant to another species or strain.

2.1.5. Tolerance to high levels of ethanol

Ethanol is a highly toxic compound that inhibits the yeast metabolism and growth and the main environment stress of wine fermentations (Ingram & Buttke 1984; Viana et al. 2012). A number of factors such as media composition, plasma membrane composition, osmotic pressure, temperature and intracellular ethanol accumulation have been shown to influence the ethanol tolerance of yeast (D'amore et al. 1990). When exposed to ethanol the fluidity of yeasts cell membranes increases, which enhances the passive proton flux through the membrane, resulting in its depolarization (Leão & Van Uden 1984). All these physiological alterations can prevent the uptake of nutrients such as amino acids and ammonium (Casey & Ingledew 1986; Jones & Greenfield 1987). Yeasts have developed diverse strategies to respond to the different types of injuries induced by ethanol (Ding et al. 2009; Stanley et al. 2010) such as the ability to maintain the fluidity of their cell membranes intact in a high-ethanol environment (Alexandre et al. 1994; Ding et al. 2009; Huffer et al. 2011). Ethanol tolerance can also be influenced by sterols (Jones & Greenfield 1987; Walker-Caprioglio et al. 1990). The loss of cell viability in the presence of ethanol has been related to the content decrease of a specific sterol, ergosterol, which increases membrane rigidity (Hossack & Rose 1976; Larue et al. 1980). Indeed, ethanol-tolerant yeast strains, exhibit elevated ergosterol levels in their membrane, with higher ratios of phosphatidylinositol-to-phosphatidylcholine and larger amounts of octadecanoic fatty acids relative to the amounts of hexadecanoic fatty acids (Arneborg et al.1995). Aguilera et al. (2006) determined that oleic acid, palmitoleic acid, and ergosterol were highly correlated with H^+-ATPase activity and ethanol tolerance. Besides ergosterol, it was shown that genes involved in the intracellular pH homeostasis are also crucial for resistance to ethanol. Alexandre et al. (2001) demonstrated that genes involved in trehalose synthesis are up-regulated during ethanol stress and in addition, proved that a whole set of heat shock protein genes are induced as an ethanol stress response. In order to develop strategies for improving yeast tolerance towards ethanol, it is highly important to elucidate the molecular mechanisms involved in the stress

response (Viana 2014), since the genomic characteristics of wine yeast has been selected over billions of generations.

Ethanol is considered the major factor that rules the yeast population dynamics in wine fermentations and it has long been accepted that non-*Saccharomyces* die-off earlier because they are more sensitive to ethanol than *S. cerevisiae* (Fleet & Heard 1993; Fleet 2003; Cocolin et al. 2000; Jolly et al. 2003). However, recent studies found that some non-*Saccharomyces* yeast such as *H. uvarum*, *H. guilliermondii*, *T. delbrueckii* and *C. zemplinina* have ethanol tolerances similar to those of *S. cerevisiae* (Pérez-Nevado et al. 2006; Pina et al. 2004).

2.2. Yeast-yeast interactions

Over the last years, many studies have contributed to a better understanding of the yeast ecology and population dynamics during wine fermentations (Albergaria et al. 2010, Kemsawasd et al. 2015a; 2015b; Nissen and Arneborg 2003; Nissen et al. 2003; Pérez-Nevado et al. 2006; Renault et al. 2013; Taillandier et al. 2014; Wang et al. 2015). Those studies have shown that *S. cerevisiae* is the dominant species during wine fermentations, displacing non-*Saccharomyces* yeasts after the first days. The reasons underlying *S. cerevisiae* dominance during wine fermentations rely on its competitive advantage in the changing environment (Bauer & Pretorius 2000; Bisson 1999; Hansen et al. 2001). Beyond the classical factors discussed above (e.g. composition of grape juice, addition of SO_2, environmental conditions such as temperature, pH, oxygen availability, etc., and use of starter cultures), microbial interactions can deeply affect the growth and metabolic activity of wine yeasts (Bisson 1999; Fleet 1990).

Microbial interactions can be classified as positive (+), negative (-) and neutral (0) and can be subdivide into: mutualism (+/+ interaction), commensalism (+/0 interaction), amensalism (−/0 interaction) and competition (−/− interaction). Positive microbial interactions include mutualism, in which both sides benefit from the interaction, and commensalism in which one strain or specie has its growth enhanced by the presence of the other. Negative microbial interactions include amensalism and competition. Competition for nutrients (amino acids, nitrogen, vitamins) is a negative interaction that can occurs within a community of microbial species. Amensalism corresponds to a microbial interaction in which the growth of one organism is repressed by the secretion of other organism metabolites, but the producer

organisms are not adversely affected (Liu et al 2017). Microbial interactions in wine fermentations involve all those cases (Ciani & Comitini 2015; Liu et al 2017; Strehaiano et al. 2010). Different types of microbial interactions have been identified in mixed culture wine fermentations, and can be classified as indirect and direct interactions (Ivey et al. 2013).

2.2.1. Indirect interactions

The main indirect interactions that occur during wine fermentations are those mediated by metabolites produced during alcoholic fermentation. The most important of those metabolites is ethanol which is one of the factors that confers an ecological advantage to *S. cerevisiae* over other non-*Saccharomyces* yeast competitors due to its higher ethanol tolerance (Fleet & Heard 1993; Fleet 2003). Other compounds produced by different yeast species have been also shown to play antagonistic roles against each other such as short fatty acids, medium-chain fatty acids, acetic acid, hexanoic acid, octanoic acid, decanoic acids and acetaldehyde (Bisson 1999; Fleet 2003; Ivey et al. 2013)

Beyond ethanol and other fermentative metabolites such as organic acids, more recent studies demonstrated that antagonistic interactions are mediated by other toxic compounds such as killer-like toxins (Albergaria et al. 2010; Pérez-Nevado et al. 2006). The involvement of the killer phenomenon in wine fermentations was first reported by Bevan and Makover 1963 who discovered that certain *S. cerevisiae* strains (killer strains) produce specific extracellular proteins and glycoproteins that kill other sensitive strains of the same species. Since the classical killer toxins (K1, K2 and K28) produced by *S. cerevisiae* are only active against strains of the same species they cannot explain the early death of non-*Saccharomyces* during wine fermentations. Nevertheless, several studies have demonstrated that certain *S. cerevisiae* strains produce killer-like toxins that exert an antagonistic effect against some wine-related bacteria (Comitini et al. 2005; Osborne et al. 2007; Nehme et al. 2010) and yeasts (Pérez-Nevado et al. 2006; Albergaria et al. 2010; Branco et al. 2014). First, Comitini et al. (2005) revealed that a proteinaceous factor produced by a certain *S. cerevisiae* wine strain was able to inhibit *O. oeni* growth and MLF. Then, Pérez-Nevado et al. (2006) discovered that toxic compounds produced by *S. cerevisiae* triggered the early death of *H. guilliermondii* cells in mixed cultures with *S. cerevisiae* and (Albergaria et al. 2010) found that those toxins correspond to antimicrobial peptides (AMPs) secreted by *S. cerevisiae* (strain CCMI 885).

Finally, Branco et al. (2014) showed that those AMPs are derived from the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). More recently, Branco et al. (2017a) demonstrated that several *S. cerevisiae* strains secrete these AMPs and induce the death of other microbial competitors within the wine environment. Therefore, microbial interactions mediated by the secretion of AMPs have been shown to regulate the microbial population dynamics during wine fermentations.

During wine fermentations, there are also other interactions that promote the growth of certain species, thus modulating the yeast population dynamics. During the early stages of alcoholic fermentation, a large amount of non-*Saccharomyces* yeasts die by autolyse, releasing amino acids and vitamins into the medium that may provide nutrients for *S. cerevisiae* growth during the later stages of alcoholic fermentation (Fleet 2001). On the other hand, *S. cerevisiae* autolysis after alcoholic fermentation may well be a significant source of micronutrients for the growth of spoilage species, particularly of *Dekkera*/*Brettanomyces* spp. (Guilloux-Benatier et al. 2001). Indeed, *D. bruxellensis* due to its high ethanol tolerance is the most well-adapted non-*Saccharomyces* yeast species to the wine environment, what explains its persistence in wine (Renouf et al. 2007).

2.2.2. Direct interactions

Physical contact and quorum sensing are the direct interactions that have been considered for wine yeasts until now (Bisson 1999, Nissen & Arneborg 2003).

Physical contact

Cell growth inhibition in consequence of direct physical contact between wine yeast was firstly reported by Nissen & Arneborg (2003) and Nissen et al (2003). In those works, the authors showed that viable *S. cerevisiae* cells at high concentrations were able to induce growth arrest of two non-*Saccharomyces* yeasts, *K. thermotolerans* and *T. delbrueckii*, by a cell–cell contact-mediated mechanism. The authors performed mixed-culture fermentations using a dialysis tube set-up system in which the yeasts were physically separated in two compartments but sensing the same metabolites in the medium since the system allowed exchange of molecules between compartments. They verified that in those conditions the non-*Saccharomyces* yeasts were able to grow and ferment sugars, while when in direct physical

contact with *S. cerevisiae* they stop growing. Later, Renault et al. (2013) confirmed that *S. cerevisiae* induces death of *T. delbrueckii* by direct cell-to-cell contact (physical contact). Those authors observed a much higher cell viability of *T. delbrueckii* when physically separated from *S. cerevisiae* than in mixed-cultures with *S. cerevisiae*. Afterwards, Kemsawasd et al. (2015a) demonstrated that the early death of *L. thermotolerans* during mixed-culture fermentation with *S. cerevisiae* was due to a combination of cell-to-cell contact and AMPs secreted by *S. cerevisiae*. More recently, Branco et al. (2017b) showed that the death of wine related non-*Saccharomyces* by direct contact with cells of *S. cerevisiae* is partly mediated by the accumulation of AMPs on their surface.

Cell-cell communication, quorum-sensing

Communicative behavior can be found in every living system, even in unicellular organisms and even in the wine itself. For successful interactions, and occasionally for essential survival, microorganisms need to communicate. The main mechanism of microbial communication is quorum sensing. Quorum sensing was first identified in bacteria (Nealson & Hastings. 1979) and consists in a mechanism by which microbial cells communicate with each other through the production of low-molecular-mass signaling molecules in response to high cell density (Bassler & Losick 2006; Wuster & Babu 2007). These molecules, called quorum sensing signals, are produced during growth, but when their concentrations reach a certain threshold level, which is a non-toxic concentration for themselves, they activate or inhibit gene expression to modify the behavior of the whole population (Avbelj et al. 2016; Wuster & Babu 2007). Recently, farnesol was identified as a quorum-sensing molecule which is excreted continuously during growth of C*andida albicans* cultures at levels that are proportional to the cell density (Hornby et al. 2001). Accumulation of farnesol in the medium induces the formation of germ tubes when cultures reach high cell density, thus controlling the yeast cell-to-filamentous growth transition (Hogan 2006; Lindsay et al. 2012). Besides farnesol, another quorum-sensing molecule was detected in *C. albicans*, tyrosol; this molecule promotes cell growth and the formation of germ tubes at low cell density levels (Alem et al. 2006; Kruppa 2009).

The aromatic alcohols, 2-phenylethanol, tyrosol and tryptophol, also act as quorum-sensing molecules in *S. cerevisiae* under low-nitrogen conditions. *S. cerevisiae* starts to produce

them at a specific cell density (Avbelj et al. 2015; Hazelwood et al. 2008; Wuster & Babu 2010). These quorum-sensing molecules induce the formation of pseudohyphae at high population density levels (Wuster & Babu 2010). The release of tryptophol, 2-phenyletanol and tyrosol was likewise shown in *Debaryomyces hansenii* (Gori et al. 2011).

These quorum-sensing molecules, 2-phenylethanol, tryptophol and tyrosol have already numerous biotechnological applications especially in wine quality assessment (González-Marco et al. 2010) but also in aroma production of food and drinks (Etschmann et al. 2003; Wang et al. 2011), and they can likewise act as antioxidants and antimicrobials (Gañan et al. 2009).

3. Antimicrobial peptides: their nature and mode of action

AMPs are oligopeptides with a variable number (12-40) of amino acids (aa) that have a wide spectrum of antimicrobial activity against bacteria, fungi, viruses and eukaryotic parasites (Hancock & Chapple 1999; Hancock & Lehrer 1998). AMPs are produced by many cell types in a diversity of microorganisms, invertebrate, plant and animal species. In order to adapt to a changing environment, all organisms have developed defence mechanisms to respond to the diversity of environmental factors. One of the mechanisms is the production of AMPs which is the first line of antimicrobial defence for organisms in the entire eukaryotic kingdom. Therefore AMPs are one of the most important response elements of the innate immune system (Hirsch et al. 2008; Sang & Blecha 2009)

Alexander Fleming in the late 1920s identified the first monomeric peptide, named lysozyme, with antimicrobial activity (Flemming 1922). Lysozyme is a relatively large (148 aa) protein that kills bacteria by destroing bacterial cell wall via enzymatic activity. Although, enzymatic mechanisms are not the main mechanism of action of most AMPs (Phoenix et al. 2013), lysozyme is considered the first AMP to be identified. The discovery of AMPs dates back to 1939, when an antimicrobial substance isolated from prokaryotic cells, *Bacillus brevis*, was documented. These AMPs, gramicidins, exhibit activity both *in vitro* and *in vivo* against a wide range of gram-positive bacteria (Dubos 1939). Indeed, infected wounds on the guinea-pig skin were successfully treated with gramicidins, showing their therapeutic potential for clinical use (Gause & Brazhnikova 1944). Consequently, gramicidins were the first AMPs to be commercially manufactured as antibiotics (Van Epps 2006).

In general, AMPs are cationic in nature, possessing a net positive charge of +2 to +7 owing to an excess of basic amino acids (arginine, lysine, and histidine) over acidic amino acids (Hancock & Chapple 1999). Still, 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).

Due to their broad-spectrum activities AMPs have been considered as potential therapeutic sources of future antibiotics. However, their clinical and commercial development still have some limitations, such as potential toxicity, susceptibility to proteases, and a high cost of peptide production (Seo et al. 2012).

3.1. Purification and characterization methods

3.1.1. Purification

The goal for any purification process is to obtain a preparation that meets the quality requirements set for the compound to be purified. There is no single or simple way to purify all kinds of peptides. However, the process of peptides purification should be as simple as possible and should contain a minimum of steps, to avoid sample losses. The aim of a purification process is not only the removal of unwanted contaminants but also the concentration of the desired protein or peptide. Besides that, is also desired the transferring of the sample to an environment where it is stable and in a form ready for the intended application (Hedhammar et al. 2006).

Depending on the properties of the peptide and the type of impurities, commonly used techniques for purification are chromatographic techniques, namely, gel filtration chromatography, ion-exchange chromatography and reverse-phase high-performance liquid chromatography (Andersson et al. 2000; Kiyama et al. 1984). Chromatography denotes to a group of separation techniques which includes a retardation of molecules with respect to the solvent front that progresses through the material. Chromatography means “colour drawing” and was originally used to describe the separation of natural pigments on filter papers by differential retardation (Hedhammar et al. 2006).

3.1.1.1 Gel filtration or size exclusion chromatography

Gel filtration (GF) chromatography is widely used because it is easy to use, it has relatively high throughput and the equipment and columns are readily available (Arakawa et al. 2010).

Generally, GF matrices consist of porous beads composed of crosslinked polyacrylamide, agarose, dextran or combinations of these forming a gel with different pore sizes (Hedhammar et al. 2006). GF, allows separation of substances with differences in molecular size, under mild conditions. Sample components are eluted isocratically (single buffer, no gradient). Separation can be performed within a broad pH, ionic strength, and temperature range. GF is a non-binding method which means that no concentration of the sample components takes place. In fact, the sample zone is broadened during the passage through the column, resulting in dilution of the sample. To avoid the dilution of the peptides, the sample can be concentrated before GF (Hedhammar et al. 2006). One of the most important limitations of GF analysis is protein adsorption to the resin, particularly when using new columns and often column preconditioning protocols are required (Arakawa et al. 2010).

AMPs are small compared to most proteins. Therefore, GF chromatography can be used as the first purification step for AMPs since they are suitable for the separation of molecules with a MW lower than 10 kDa and by definition, antimicrobial peptides have less than 10 kDa (Cole & Ganz 2000)

3.1.1.2 Ion-exchange chromatography

Ionic interactions are the basis for purification of proteins by ion-exchange chromatography (IEXC). The separation is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. Typically, IEXC is used to bind the target molecule and then wash away non-bound contaminants. However, the technique can also be used to bind the impurities if required. In that case, the protein of interest should be found in the flow through. Ion exchangers are usually classified as weak or strong, which refers to pKa values of their charged groups, by analogy with weak and strong acids or bases (Hedhammar et al. 2006). The number of charges on a strong ion exchanger remains constant regardless of the buffer pH, conversely weak ion exchanger shows a pH-dependent

function and so provide optimal performance over only a small pH range (Cummins et al. 2011). In cation exchange chromatography positively charged molecules are attracted to a negatively charged solid support. Conversely, in anion exchange chromatography, negatively charged molecules are attracted to a positively charged solid support. Since all molecules with ionizable groups can be titrated, their net surface charge is highly pH dependent. In the case of proteins, which are built up of many different amino acids containing weak acidic and basic groups, the net surface charge will change gradually as the pH of the environment changes (Hedhammar et al. 2006, Karlsson & Hirsh 2011). The elution of proteins is usually performed by increases in salt concentration or changes in pH. Changes are made stepwise or with a continuous gradient. Most commonly with salt (NaCl) (Cumminis et al. 2011).

3.1.1.3 Reverse-phase high-performance liquid chromatography

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules based on hydrophobicity. The separation of molecules hangs on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase. RP-HPLC is able to separate small peptides such as those obtained by trypsin digestion and also larger proteins, even polypeptides that differ by a single amino acid residue can often be separated RP-HPLC (Rivier & McClintock 1983). RP-HPLC is a very powerful technique for the analysis of peptides and proteins, owed to the experimental simplicity with which chromatographic selectivity can be manipulated through changes in mobile phase characteristics. In addition, is possible to accomplish high recoveries of proteins and peptides and, therefore, high productivity. However, RP-HPLC can cause the irreversible denaturation of protein samples thereby reducing the recovery in a biologically active form (Aguilar & Hearn 1996; Aguilar 2004). RP-HPLC is used for the separation of peptide fragments from enzymatic digests and for purification of natural and synthetic peptides (Slemmon et al. 1994). RP-HPLC is also an extremely useful tool for final polishing and for isolation of proteins and peptides prior to mass spectrometry analysis.

3.1.2. Characterization

Characterization is the next step in the process of analyzing AMPs, after purification. Initially, to characterize AMPs they are cleavage into small fragments by tryptic digestion followed by mass spectrometry (MS).

MS is a sensitive technique used to detect, identify and quantitate molecules based on their mass and charge (*m/z*) ratio. The first step in the mass spectrometric analysis of compounds is the production of gas phase ions (positively or negatively charged) of the compound, for example by electron ionization. All mass spectrometers must function under high vacuum which is necessary to allow ions to reach the detector without undergoing collisions with other gaseous molecules (Hoffmann & Stroobant 2007). There are two main ionization techniques that are commonly used in MS: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (El-Aneed et al. 2009; Henzel et al. 2003; Thiede et al. 2005).

The success of ESI started in 1989 when Fenn and coworkers showed that multiple charged ions were obtained from proteins, allowing their molecular weight to be determined. At the beginning, ESI was considered as an ionization source dedicated only to protein analysis. Afterwards, its use was extended to other polymers and to the analysis of small polar molecules. Shortly, in ESI the sample (proteins or peptides) is nebulized when a high voltage is applied. ESI is ideally suited for biochemical analyses because it allows very high sensitivity to be reached and is easy to couple with separation techniques such as high-performance liquid chromatography (HPLC) (Hoffmann & Stroobant 2007).

MALDI-MS was first introduced in 1988 by Karas and Hillenkamp and soon it has become a widespread analytical tool for peptides, proteins, oligonucleotides, carbohydrates and lipids. The effective and directed energy transfer during a matrix-assisted laser-induced desorption event provides high ion yields of the intact analyte. Besides, the measurement of compounds is achieved with high accuracy and subpicomolar sensitivity (Cotter 1992).

Independently of the ionization source, the sensitivity of a mass spectrometer is related to the mass analyzer where ion separation occurs. Mass analyzers as quadrupole time-of-flight (QTOF) are commonly used and they can be configured together as QToF tandem mass spectrometric instruments. Tandem mass spectrometry (MS/MS) is a method where the gaseous

ions are subjected to two or more sequential stages of mass analysis, which can be separated according to their mass and charge ratio, *m/z* (El-Aneed et al. 2009).

3.2. Mode of action

AMPs can be used as antibiotics and preservatives. Therefore, it is essential to understand their mode of action. Most of experiments performed until now have focused primarily on the interaction of AMPs with model membrane systems. Nevertheless, the available information about the AMPs mode of action continues to increase.

The existence of two main groups of AMPs, cationic (positively charged) and anionic (negatively charged), leads to the hypothesis of different mechanisms of interactions between the different AMPs and the target cells.

3.2.1. Cationic AMPs

The mechanisms involved in the killer action of cationic AMPs predominantly include interactions between their positively charged residues and the anionic components of target cell membranes, resulting in their permeabilization, depolarization, leakage or lysis, and consequently in cell death (Brogden 2005; Giuliani et al. 2007; Matsuzaki 2009). Nevertheless, cell membranes are only one of the cationic AMPs cellular targets; AMPs can equally destabilize internal anionic cell constituents such as DNA, RNA, or cell wall components (Brogden 2005). Cationic AMPs typically exhibit minimal inhibitory concentrations (MIC) ranging from 10 to 100 μM (Matsuzaki 2009). Most of the amino acids of cationic AMPs are hydrophobic (50%), with a low proportion of both neutral polar and negatively charged amino acids. The mixed cationic and hydrophobic composition of cationic AMPs is a helpful characteristic for interact with microbial cytoplasmic membranes and to perturb them (Strom et al. 2002). The most potent cationic AMPs fold into molecules that have either amphipathic structures or cationic double wing structures with a hydrophobic core separating two charged segments. Such peptides are found in bacteria and animals (Hancock & Diamond 2000). Cationic AMPs such as nisin showed higher activity when added to others compounds (i.e. 1-propanol and EDTA) (Hancock & Lehrer 1998), leading one to suspect that there are other molecules that will act in synergy with cationic peptides.

Cationic AMPs can be grouped into four major classes based on their secondary structure. The most common are α-helical peptides and β-sheet peptides stabilized by 2–4 disulfide bridges (and occasionally containing a short α-helical stretch) (**Fig. 3**). Other structures less common are: extended peptides with a predominance of one or two amino acids (e.g. proline, tryptophan or histidine) and loop peptides formed by a single disulphide bond (**Fig. 3**) (Hancock & Chapple 1999; Hancock & Lehrer 1998).

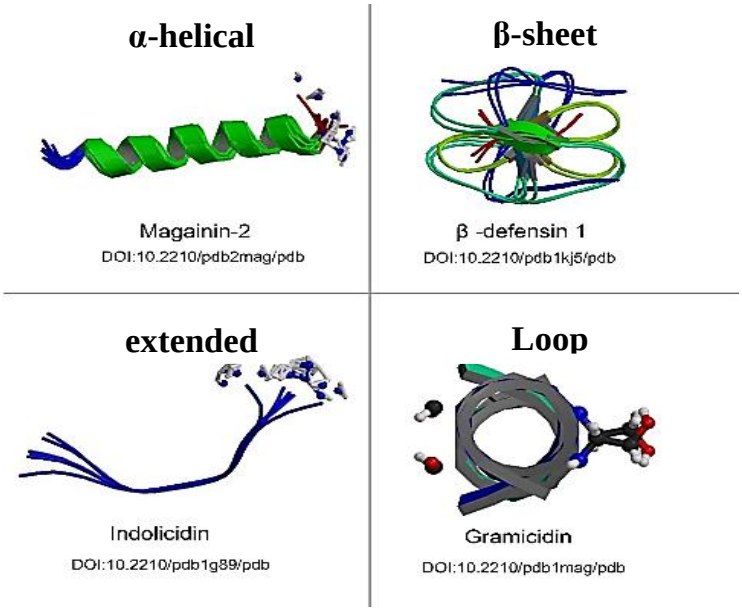

Fig. 3: Protein models representing the four structural classes of AMPs (withdrawn from Bahar & Ren 2013).

3.2.2. Anionic AMPs

The majority of known AMPs are cationic, anionic antimicrobial peptides (AAMPs) are very rare, it is believed that these peptides were developed in response to the bacterial resistance mechanisms toward cationic AMPs and therefore have a different mechanism of action (Lai et al. 2002; Li 2009) Some examples of AAMPs are the proenkephalin-derived peptides, bovine kappacin, peptide B and enkelytin, maximin H5 from the amphibian *Bombina maxima*, lysenins from the earthworm *Eisenia fetida* and dermcidin-derived peptide DCD-1L

from human skin (Bruhn et al. 2006; Harris et al. 2009; Goumon et al. 1998; Lai et al. 2002; Malkoski et al. 2001; Paulmann et al. 2012)

AAMPs generally exhibit weaker antimicrobial activity than cationic AMPs with MIC typically higher than 600 μM such as in kappacins, the first AAMPs isolated from bovine milk (Malkoski et al. 2001). Even though 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 membrane permeabilization via pore formation, which is the case of cyclotides (cyclotides are unique class of cysteine-rich macrocyclic peptides of about 30 amino acids in size that are defined by a head-to-tail cyclized backbone and three disulfide bonds in a knotted arrangement referred to as cyclic cystine-knot motif) (Craik et al. 1999; Harris et al. 2009). A recent study (Paulmann et al. 2012) showed that dermcidin-derived peptide DCD-1L, an anionic AMP from human eccrine sweat, upon interaction with lipid bilayers forms oligomeric complexes that are stabilized by Zn^{2+}. DCD-1L adopts a helical structure upon interaction with bacterial membrane phospholipids and can form ion channels in the membranes of the target cell. Moreover, investigation on the mode of action of the AAMPs kappacins showed that these peptides are strongly membranolytic at acidic pH, suggesting that divalent cations may facilitate its interaction 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). A disadvantage of many anionic antimicrobial peptides is that they often require cations, as cofactors for biocidal activity (Brogden et al. 1996).

3.2.3. Mechanism models of membrane disruptive AMPs

Most of the peptides α-helical structural are membrane disruptive, although some α-helical peptides don't disrupt the membrane of the target cells, i.e. buforin (Park et al. 1998) and a pleurocidin analogue (Patrzykat et al. 2002). Three mechanistic models have been developed to explain membrane disruption, the "carpet" model, "toroidal" pore model, "barrel-stave" model (**Fig. 4**) (Brogden 2005).

In the "carpet" model the cell membrane is fully covered by a carpet-like cluster of peptides that remain in contact with the lipid head groups. A saturation point is reached which

results in an extensive wormhole formation, triggering local disturbance in membrane stability, disruption of the membrane potential and finally, disintegration of the membrane (Costa et al. 2011; Powers & Hancock 2003).

In the “toroidal” pore model the peptides are oriented parallel to the plane of the plasma membrane and bind to the region of the phospholipid polar heads in a functionally inactive state. When a critical concentration is reached, the peptide molecules are reoriented perpendicularly to the plane of the bilayer penetrating the hydrophobic region (active state) and in conjunction with several surrounding lipids they invert themselves towards the interior of the membranes hydrophobic region then they adopt a multipored transitional state. This results in the irreversible rupture of the plasma membrane and an increase in the "transmembranal movement" of lipids. The transition between the inactive and active state of the peptide bound to the membrane is dependent the phospholipid composition of the bilayer and on AMPs concentration (Huang 2000).

The “barrel-stave” model proposes that a group of AMPs molecules with α-helical structures interact with each other on the surface of the plasma membrane to form a complex. Afterwards, the peptides are oriented perpendicular to the plane of the membrane which permits the hydrophobic region of the peptide to interact with the hydrophobic region of the bilayer, whereas the hydrophilic surface of the peptide is oriented inwards, forming a hydrophilic channel that magnifies along with the membrane. The formed protein complex behaves as a pore inserted into the membrane (Zhao et al. 2003).

Independently of which model is correct, the consequence of membrane disruption would be the fast depolarization of the cell leading to rapid cell death (Friedrich et al. 1999).

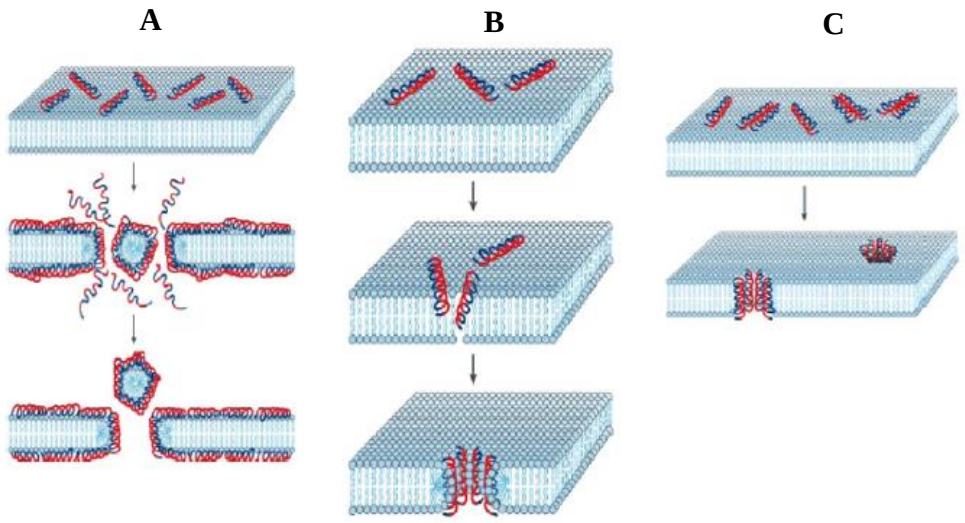

Fig. 4: Membrane-active AMPs mechanism of action models: (A) carpet model; (B) toroidal pore model; (C) barrel-stave model. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue (withdrawn from Brogden 2005).

3.2.4. Other AMPs mechanisms

Most of the AMPs interact with and influence the integrity of microbial membranes; however, it is not clear if membrane permeabilization is always the deadly event or if the membrane is the only site of action. AMPs that do not widely permeabilize microorganism's cell membranes may have effects on their viability that depend on interactions with intracellular components. Nevertheless, these peptides induce a considerably loss of viability slower than the membrane-acting peptides, which exert their antimicrobial effects within minutes (Giacometti et al. 1999). Has been proposed and demonstrated that these non-disruptive membrane AMPs translocate across membranes of the target cells (Park et al. 2000; Zhang et al. 2001), for instance, in the case of the skin frog AMP magainin 2, induces a transitory disruption rather than a large membrane perturbations and permeabilization does not occur (Park et al. 2000). Buforin II an AMP from the stomach of the toad *Bufo bufo gargarizans* (Park et al. 1996) translocate stochastically after the formation and disintegration of a non-permeabilizing pore-like structure (Kobayashi et al. 2004). These AMPs, once present in the microorganisms cytoplasm, are thought to interact with DNA, RNA and/or cellular proteins and to inhibit

synthesis of these molecules (Brogden 2005). For instance, buforin II penetrates into to the cell membranes, accumulates in the cytosol and bind to DNA and RNA killing the cells without cell lysing (Park et al. 1998).

Additionally, specific enzymatic targets have been identified for certain AMPs. An insect AMP, pyrrhocoricin, has been shown to bind the heat shock protein DnaK inhibiting chaperone-assisted protein folding (Kragol et al. 2001). While others AMPs induce the inhibition of peptidoglycan biosynthesis in bacteria such as the case of lantibiotic, mersacidin produced by Gram-positive species namely *Bacillis* sp. (Brötz et al. 1998).

Moreover, a recent study showed that the antimicrobial mechanism of lactoferrin and transferrin from human mucosa, inhibit the ATPase complex in *Pseudomonas aeruginosa* and *Lactococcus lactis*. As a result, the H^+-ATPase-mediated flux of protons is compromised leading to disorders in intracellular pH homeostasis and consequently resulting in cell death (Andrés & Fierro 2010).

Apoptosis can be triggered by AMPs that originate DNA damages. In animal cells, after DNA damage DNA repair and cell cycle checkpoints are activated to protect the damaged cell. A failure in DNA repair will cause the activation of the phosphoprotein p53, which can result in growth arrest or cell death in the damaged cell; this type of mechanism can also occur in yeast (Wang 2001).

Yeast cells undergo apoptosis by showing characteristic apoptotic makers such as externalization of phosphatidylserine to the outer leaflet of the plasma membrane, DNA fragmentation and chromatin condensation (Madeo et al. 1997). In the early stages of yeast apoptosis, the phosphatidylserine exposure serves as a sensitive marker. The most part (90%) of phosphatidylserine in *S. cerevisiae* is oriented towards the cytoplasm and is translocated to the outer leaflet of the plasma membrane when apoptosis is induced (Madeo et al. 1997). It can be detected with FITC labeled annexin V, which binds to phosphatidylserine with high affinity in the presence of Ca^{2+}, and then fluoresces (Madeo et al. 1997). However, the FITC-annexin V and propidium iodide double staining method is commonly used since phosphatidylserine translocation also occurs during necrosis (Cho et al. 2012).

DNA fragmentation is a late event in yeast apoptosis (Collins et al. 1997; Madeo et al. 1997) and is normally detected by the terminal dUTP nick-end labeling (TUNEL) method (Gavrieli et al. 1992; Gorczyca et al. 1993) that is a fast and sensitive way to visualize the

amount of DNA fragmentation in individual cells (Madeo et al. 1997). This method detected the 3´-OH termini produced by DNA cleavage. DNA strand breaks are detected by labeling free 3´-OH termini with FITC-labeled deoxyuridine, which is detected with alkaline phosphatase–coupled, anti-fluorescein antibody, and the formation of a dye precipitate with a phosphatase substrate (Gavrieli et al. 1992; Gorczyca et al. 1993).

Several AMPs have been reported to exert antimicrobial activity against yeast via different mechanism related to apoptosis, i.e: arenicin-1 (Cho & Lee 2011), coprisin (Lee et al. 2012), papiliocin (Hwang et al. 2011), melittin (Park & Lee 2010) and osmotin (Narasimhan et al. 2001; 2005). These AMPs induce particular phenomena in the target cells including reactive oxygen species (ROS) production, mitochondrial membrane depolarization, cytochrome c release, caspase activation, phosphatidylserine externalization, DNA fragmentation, nuclear condensation, apoptotic body formation, and membrane blebbing (**Fig. 5**) (Bortner & Cidlowski 2007; Cho et al. 2012; Madeo et al. 1997).

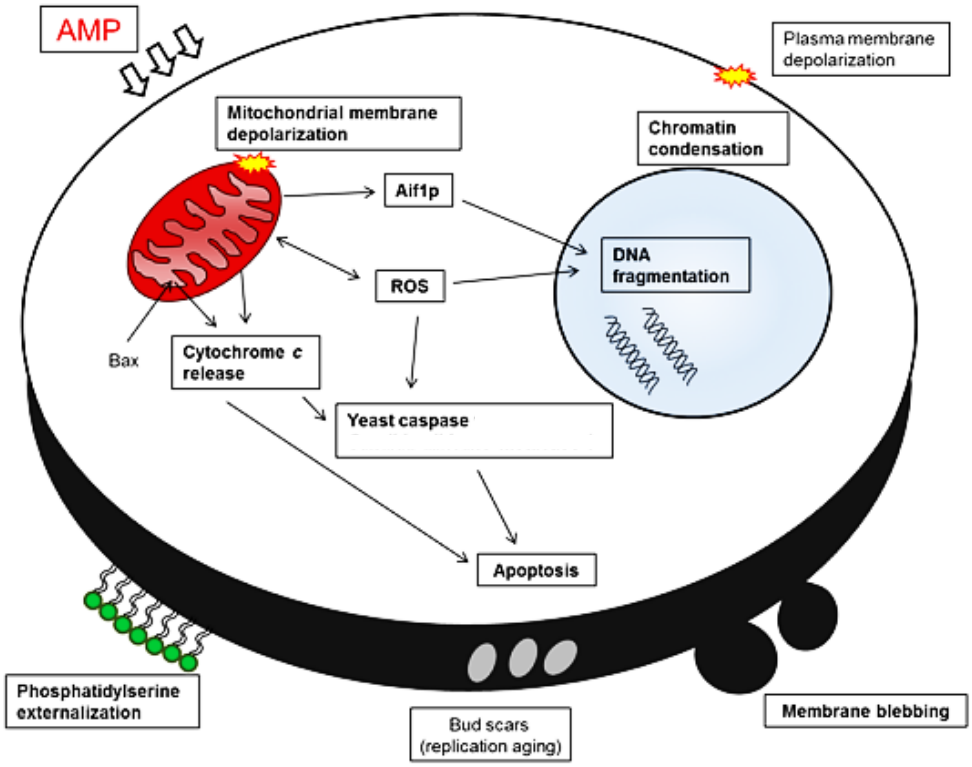

Fig. 5: Apoptotic phenomena induced by AMPs in yeast cells (withdrawn from Cho et al. 2012).

3.3. Large-scale production of AMPs

AMPs recently have received increasing attention as potential novel pharmaceutical agents. As a result, large quantities of AMPs are required, as well as reliable production methods. AMPs can be efficiently prepared by chemical synthesis, but this is extremely expensive. Isolation from natural sources rarely meet the requirements for quantity and preparative isolation is typically complicated and time-consuming, and therefore it is not an efficient way for obtaining AMPs in large amounts. (Li 2009; Park et al. 1998; Pyo et al. 2004; Xu et al. 2007).

The recombinant approach is relatively low cost and easy to scale up consequently is the more attractive methodology for large-scale production of AMPs (Li 2009). The host cells that

are available for production of recombinant proteins include bacteria, yeast, filamentous fungi, and unicellular algae. The choice of the host system depends on the protein of interest and all them have strengths and weaknesses (Adrio & Demain 2010). *Escherichia coli* and yeast are the two major systems used to produce recombinant antimicrobial peptides. *E. coli* is the most popular expression host due to its fast growth kinetics, its doubling time is about 20 min (Sezonov et al. 2007). However, the expression of a recombinant protein may cause a considerable decrease in generation time due to the metabolic burden on the microorganism (Bentley et al. 1990). *E.coli* is a good host cells for AMPs since post-translational modification is not required for the bioactivity of most AMPs (Li & Chen 2008). Nevertheless, if eukaryotic post-translational modifications are needed, a prokaryotic expression system may not be suitable (Sahdev et al., 2008). Even though, several AMPs have been produced in yeast with good yields (Li et al. 2005; Xu et al. 2008), several others were expressed in insignificant amounts (Hong et al. 2007; Zhou et al. 2005) or obtained as an inactive form (Beaulieu et al. 2005). Therefore, bacteria are used much more frequently than yeast for recombinant production of AMPs (Li & Chen 2008). The first yeast to be employed for the production of recombinant proteins was *S. cerevisiae* since there is a vast range of molecular tools available for *S. cerevisiae.* Besides, *S. cerevisiae* has a long history of use in the industrial production of bread and alcoholic beverages. Therefore, there is confidence that the organism is safe, and knowledge of industrial-scale fermentations facilitates production scale-up. However, *Pichia pastoris* is more often used for yeast expression system than *S. cerevisiae* since combines the advantages of relatively rapid expression times and low cost with eukaryotic co-translational and post-translational processing systems. Besides, *P. pastoris* is capable of utilise methanol as a carbon source and also provides a reliable means of accomplishing considerably amounts of protein from small culture volumes (Byrne 2015; Sudbery 1996)

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Chapter II

Identification of novel GAPDH-derived antimicrobial peptides secreted by *Saccharomyces cerevisiae* and involved in wine microbial interactions

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ABSTRACT

Saccharomyces cerevisiae plays a primordial role in alcoholic fermentation and has a vast worldwide application in the production of fuel-ethanol, food and beverages. The dominance of *S. cerevisiae* over other microbial species during alcoholic fermentations has been traditionally ascribed to its higher ethanol tolerance. However, recent studies suggested that other phenomena, such as microbial interactions mediated by killer-like toxins, might play an important role. Here we show that *S. cerevisiae* secretes antimicrobial peptides (AMPs) during alcoholic fermentation that are active against a wide variety of wine-related yeasts (e.g. *Dekkera bruxellensis*) and bacteria (e.g. *Oenococcus oeni*). Mass spectrometry analyses revealed that these AMPs correspond to fragments of the *S. cerevisiae* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein. The involvement of GAPDH-derived peptides in wine microbial interactions was further sustained by results obtained in mixed cultures performed with *S. cerevisiae* single mutants deleted in each of the GAPDH codifying genes (*TDH1-3*) and also with a *S. cerevisiae* mutant deleted in the *YCA1* gene, which codifies the apoptosis-involved enzyme metacaspase. These findings are discussed in the context of wine microbial interactions, biopreservation potential and the role of GAPDH in the defence system of *S. cerevisiae*.

Keywords: antimicrobial peptides; wine microbial interactions; alcoholic fermentation; biopreservation; metacaspases; glyceraldehyde-3-phosphate dehydrogenase;

1. INTRODUCTION

Alcoholic fermentation is the main biotransformation that occurs during winemaking, brewery and fuel-ethanol production. Since these industrial processes are conducted under non-sterile growth conditions, a huge variety of microorganisms is present and can participate in the fermentative process. Although several yeast and bacteria are able to perform alcoholic fermentation, *Saccharomyces cerevisiae* is the dominant microorganism in all those processes, being usually called the "wine yeast". During spontaneous wine fermentations, there is a consistent growth pattern in which the non-*Saccharomyces* species belonging to the natural microflora of grape musts (e.g. *Hanseniaspora guilliermondii*, *Hanseniaspora uvarum*, *Candida stellata*, *Kluyveromyces thermotolerans*, *Kluyveromyces marxianus* and *Torulaspora delbrueckii*) grow during the early stages of fermentation (up to 4–5 % v/ v of ethanol) but then begin to die-off giving way to *S. cerevisiae* strains to complete the fermentation process (Fleet and Heard 1993; Pretorius 2000).

The ability of *S. cerevisiae* to displace other microbial species during alcoholic fermentation has been always attributed to its higher fermentative power and capacity to withstand the increasingly adverse conditions established in the medium as the fermentation progresses, i.e. high levels of ethanol and organic acids, low pH values, scarce oxygen availability and depletion of certain nutrients (Bisson 1999; Bauer and Pretorius 2000; Hansen et al. 2001). However, the weight of these factors on microbial succession during wine fermentations has been recently questioned and other microbial interactions were proposed by different authors, such as growth arrest mediated by a cell–cell contact mechanism (Nissen and Arneborg 2003; Nissen et al. 2003; Arneborg et al. 2005) and death mediated by killer-like toxins (Comitini et al. 2005; Pérez-Nevado et al. 2006; Osborne and Edwards 2007; Albergaria et al. 2010).

The killer phenomenon has long been recognized among wine yeasts, although the relation between killer activity and the early disappearance of non-*Saccharomyces* yeasts from wine fermentations was never established because the killer toxins produced by *S. cerevisiae* that were identified up till now (K1, K2 and K28) are active only against strains of the same species (Pérez et al. 2001). Nevertheless, there are increasingly growing evidences suggesting the involvement of other killer-like toxins in the yeast–yeast and yeast–bacteria interactions in wine fermentations. Indeed, Comitini et al. (2005), as well as Osborne and Edwards (2007) and

Nehme et al. (2010), found that certain *S. cerevisiae* strains produce proteinaceous compounds that are active against malolactic bacteria. Likewise, in a previous work we demonstrated that *S. cerevisiae* CCMI 885 produces peptides (<10 kDa) that inhibit the growth of *H. guilliermondii*, *T. delbrueckii*, *K. marxianus* and *K. thermotolerans* (Albergaria et al. 2010). However, the identity of these antimicrobial peptides (AMPs) remained elusive.

In the present work, we purified the previously found AMPs and characterized them regarding their amino acid sequence, encoding genes and antimicrobial spectrum of action. The role of these AMPs in wine microbial interactions was also investigated using *S. cerevisiae* mutant strains deleted in the AMPs encoding genes.

2. MATERIALS AND METHODS

2.1. Strains and inoculum cultures

The following *S. cerevisiae* strains were used: CCMI 885 (Culture Collection of Industrial Microorganisms of ex-INETI, Lisbon, Portugal); BY4741 (*MATα his*3Δ1 *leu*2Δ0 *met*15Δ0 *ura*3Δ0) strain and its isogenic derivative strains *Δtdh1* (YJL052w::kanMX4), *Δtdh2* (YJR009c::kanMX4) and *Δtdh3* (YGR192c::kanMX4) (EUROSCARF, Frankfurt, Germany); BY4742 (*MATα his*3Δ1 *leu*2Δ0 *lys*2Δ0; *ura*3Δ0) strain and its isogenic derivative strain *Δyca1* (EUROSCARF, Frankfurt, Germany). The non-*Saccharomyces* strains used were: *Dekkera bruxellensis* ISA 1649, ISA 1700, ISA 1791, ISA 2104, ISA 2116, ISA 2211 (Instituto Superior de Agronomia, Lisbon, Portugal); *H. guilliermondii* NCYC 2380 (National Collection of Yeast Cultures, Norwich, United Kingdom); *K. marxianus* PYCC 2671 (Portuguese Yeast Culture Collection, New University of Lisbon, Portugal); *K. thermotolerans* PYCC 2908 and *T. delbrueckii* PYCC 4478. Two strains of *Oenococcus oeni* were used: ISA 4279 and DSM 2529 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Inoculums of all yeast strains were obtained by transferring one YEPD-agar slant of each strain (pre-grown at 30 °C for 48–72 h) into 50 ml of YEPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) and incubating cultures at 30 °C with 150 rpm of agitation during 16 h (for *Dekkera* strains incubation took 48–72 h). Inoculums of *O. oeni* strains were prepared by transferring 1ml of stock culture into 9 ml of MRS broth and incubating cultures at 25 °C without agitation for 48 h.

2.2. Purification of antimicrobial peptides from *S. cerevisiae* supernatants

AMPs were purified from *S. cerevisiae* CCMI 885 supernatants of alcoholic fermentations performed in synthetic grape juice (SGJ), prepared as described in Pérez-Nevado et al. (2006), at 25°C for 7 days. Cell-free supernatants (filtration by 0.22 μm Millipore membranes, Merck Millipore, Algés, Portugal) were first ultrafiltrated through centrifugal filter units (Vivaspin 15R, Sartorius, Göttingen, Germany) equipped with 10 kDa membranes and then concentrated (100-fold) with 2 kDa membranes. This concentrated fraction was first fractionated by gel filtration chromatography, using a Superdex-Peptide column (10/300 GL, GE Healthcare, London, UK) coupled to a High-Performance Liquid Chromatography (HPLC) system (Merck Hitachi, Darmstadt, Germany) equipped with an UV detector (Merck Hitachi, Darmstadt, Germany). One hundred microlitres of fraction was eluted with ammonium acetate 0.1 M at a flow rate of 0.7 ml/min. All fractions (Fig. 1a) were collected into 2 ml Eppendorf, freeze-dried and stored until utilisation. They were all screened for antimicrobial activity and fraction-II was identified as the most active fraction. Fraction-II was then further purified using a strong anion-exchange column (Q-Resource 6 ml, GE Healthcare, London, UK). Peptides were eluted at neutral pH using a gradient of ammonium acetate of 5–500 mM between 10 and 40 min at a flow rate of 1 ml/min. Fractions obtained were collected, vacuum-dried and screened for antimicrobial activity after resuspended in appropriated medium. Three anionic fractions (Fraction II-A, II-B and II-C in Fig. 1b) revealed antimicrobial activity against *H. guilliermondii* and were analysed by mass spectrometry.

2.3. Screening of antimicrobial activity and determination of the spectrum of action

Antimicrobial activity of fractions obtained in the different purification steps (gel filtration and anion-exchange chromatography) were tested against the sensitive strain *H. guilliermondii* using a total protein concentration of 1 mg/ml for gel filtration fractions (Fig. 1C) and 0.5 mg/ml for the anionic fractions (Fig. 1D). The spectrum of action of fraction-II was determined against *H. guilliermondii*, *K. marxianus*, *K. thermotolerans*, *T. delbrueckii*, *D. bruxellensis* and *O. oeni*, following the procedure described below.

All antimicrobial tests were performed in 96-well microplates using YEPD medium for yeast strains and MRS broth for *O. oeni* strains. Lyophilised fractions were resuspended in the respective growth medium (YEPD or MRS with 30 g/l of ethanol and pH 3.5) to a final protein

concentration of 1mg/ml in total volume of 100 µl. Control assays for each strain were performed using the respective growth medium without addition of the AMPs. The initial cell density in the antimicrobial tests was 10^5 cells/ml for yeasts and 10^6 cells/ml for bacteria strains. The microplates were incubated in a thermo-Shaker (Infors HT, Bottmingen, Switzerland) at 30 °C under 700 rpm of agitation for yeasts and at 25 °C without agitation for *O. oeni*. Growth of the cultures was followed by absorbance measurements at 590 nm using a microplate reader (Dinex Technologies Inc., Chantilly, USA) and also by the enumeration of colonies forming units (CFU) using the classical plating method (as described in the "Cell growth" sub-section), during the time-course of the experiments. All antimicrobial tests were performed in triplicates.

2.4. Mass spectrometry (LC-ESI-MS/MS) and sequence analysis

Peptides present in the three anionic fractions were sequenced by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). For LC-ESI-MS/MS analysis of peptide mixtures, on-line nano-flow liquid chromatography was performed using the Ultimate 300 RSLC (Dionex, Voisins le Bretonneux, France) with 15 cm nanocapillary columns of an internal diameter of 75 µm (Acclaim PepMap RSLC, Dionex). The solvent gradient from 4% to 50% acetonitrile in 0.5% formic acid was run at a flow rate of 300 nl/min for 30 min. The eluate was electrosprayed into an LTQ Velos mass spectrometer (Thermo Fisher Scientific, Courtaboeuf, France) through a nanoelectrospray ion source. The LTQ Velos was operated in a CID top 10 mode (i.e. one full scan MS from which 10 major peaks are selected for MS/MS). Raw data files were processed with search engines installed in-house, Mascot (version 2.2, Matrix Science, London, UK) and PEAKS studio (version 5.3, Bioinformatics Solutions Inc., Waterloo, Canada). For peptide identification, the UniProt taxonomy *S. cerevisiae* (6,650 sequences) protein database was used and the parameters for searching were: none enzyme and possible oxidation of methionine. Peptide mass tolerance and fragment mass tolerance were set to 1.5 and 0.8 Da, respectively. Peptides identification was validated when significant Mascot and PEAKS scores were obtained with false discovery rate <1%. In addition, a manual validation of MS/MS spectra was performed to be sure of the peptide sequence.

2.5. Alcoholic fermentations performed with mixed cultures of *H. guilliermondii* and *S. cerevisiae*

Alcoholic fermentations were performed in 150 ml of SGJ (supplemented with 200 mg/l of L-leucine, 120 mg/l of L-histidine, 180 mg/l of L-methionine and 120 mg/l of uracil) using mixed cultures of *H. guilliermondii* with each of the following *S. cerevisiae* strains: BY4741 and its isogenic derivatives *Δtdh1*, *Δtdh2*, *Δtdh3*; and BY4742 and its isogenic derivative *Δyca1*. Fermentations were carried out at 25 °C under slow agitation (80 rpm) with an initial cell density of 10^5 cells/ml of each species. A single culture fermentation of *H. guilliermondii* was also performed under the same growth conditions to compare its cell viability under single and mixed culture. All fermentations were performed in duplicates and daily samples were taken to determine cell viability, sugars consumption and ethanol production.

2.6. Cell growth

Culturability was determined by the classical plating method both in the antimicrobial tests and alcoholic fermentations. Briefly, samples were plated onto YEPD-agar plates, after appropriate dilution (decimal serial dilution method) and incubated at 25 °C in a Vertical Incubator (Infors, Anjou, Canada) and the number of colony forming units (CFU) enumerated after 2–6 days. In the mixed culture fermentations, the CFU counts of *H. guilliermondii* were obtained on 0.01% cycloheximide YEPD-agar plates and CFU counts of *S. cerevisiae* as the difference between total CFU counts on YEPD-agar plates and CFU counts of *H. guilliermondii*. Enumerations of CFU counts in the antimicrobial tests were determined on YEPD-agar plates both for yeasts and bacteria.

2.7. Sugars consumption and ethanol production

Glucose, fructose and ethanol concentrations in alcoholic fermentations were analysed using a High-Performance Liquid Chromatography (HPLC) system (Merck Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany). Fermentation samples were first filtrated by 0.45 μm Millipore membranes (Merck Millipore, Algés, Portugal) and then injected on a Sugar-Pak column (Waters Hitachi, Milford, USA) and eluted with a degassed aqueous mobile phase of CaEDTA (50 mg/l) at 90 °C using a flow rate of 0.5 ml/min. All samples were analysed in duplicate.

3. RESULTS

3.1. Purification and identification of AMPs from *S. cerevisiae* fermentation supernatants

In the previous work (Albergaria et al. 2010), we found that *S. cerevisiae* CCMI 885 supernatants obtained from alcoholic fermentations performed in SGJ, contained a peptidic fraction (<10 kDa) active against some wine-related yeasts. This peptidic fraction was first fractionated by gel filtration chromatography (Fig. 1A) and all fractions were collected, lyophilised and then screened for antimicrobial activity. Results revealed that fraction-II exhibited strong antimicrobial activity (Fig. 1C) and thus this fraction was further purified by ion-exchange chromatography. Since most AMPs are cationic in nature fraction-II was first pooled into a cation-exchange column (S-Resource) but none of the fractions obtained exhibited antimicrobial activity against *H. guilliermondii* (data not shown). Thus, fraction-II was pooled into a strong anion-exchange column Fig. 1B and the three anionic (at neutral pH) fractions obtained (fractions II-A, II-B and II-C) were screened for antimicrobial activity against *H. guilliermondii* (Fig. 1D).

Since all anionic fractions showed antimicrobial activity peptides were sequenced by liquid chromatography electrospray ionization-tandem mass spectrometry (LC-ESIMS/MS). Sequence analysis revealed that all peptides present in each anionic fraction correspond to fragments of the *S. cerevisiae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) isoenzymes, GAPDH2/3 and GAPDH1 (Table 1), which are encoded by the *TDH2*, *TDH3* and *TDH1* genes, respectively.

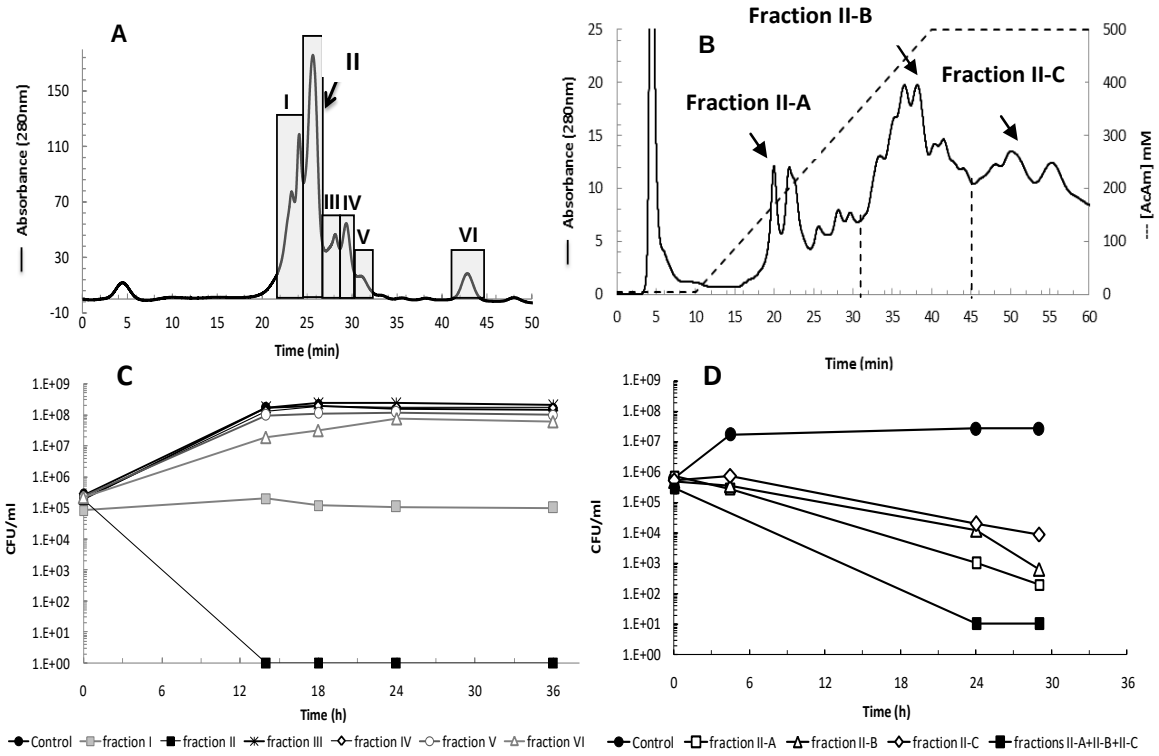

Fig. 1: Chromatographic steps used in the purification process of the AMPs. First, the concentrated peptidic (<10 kDa) fraction obtained from *S. cerevisiae* CCMI 885 supernatants was fractionated by gel filtration chromatography (A) and fractions were collected and checked for antimicrobial activity (B); Since only fraction-II exhibited strong antimicrobial activity, this fraction was further fractionated using a strong anion-exchange column (C) and the three anionic fractions obtained were tested for antimicrobial activity against *H. guilliermondii* (D).

Table 1: Sequence analysis by LC-ESI-MS/MS of the peptides present in each of the anionic bioactive fractions obtained from the anion-exchange chromatography (Fig. 1B).

Fractions	Sequence	Protein Accessions*	Number of MS/MS	Ion Score	Exp Value	Charge	m/z [Da]	MH+[Da] observ	MH+[Da] theor	ΔM [ppm]
Fraction II-A	**VSWYDNEYGYSTR**	**P00358**	**19**	**88**	**7.438E-06**	**2**	**820.51358**	**1640.0199**	**1639.70100**	**193.62**
	ISWYDNEYGYSAR	**P00360**	**12**	**88**	**7.05811E-06**	**2**	**811.84664**	**1622.6860**	**1623.70700**	**-629.48**
	VSWYDNEYGYSTRV	P00358	10	87	9.46623E-06	2	869.93185	1738.8564	1738.77000	49.26
	ISWYDNEYGYSARV	P00360	5	70	0.000514842	2	861.93159	1722.8559		46.46
	VSWYDNEYGYSTRVV	P00358	4	61	0.003617934	2	919.51678	1838.0263		101.79
	FRVPTVDVSVVD	P00360;P00358	1	53	0.026088308	2	666.92529	1332.8433		95.62
	FRVPTVDVSVVDL	P00360;P00358	1	50	0.050345078	2	723.42711	1445.8470		32.52
Fraction II-B	**ISWYDNEYGYSAR**	**P00360**	**8**	**82**	**2.94727E-05**	**2**	**812.38477**	**1623.76225**	**1623.70700**	**33.76**
	VSWYDNEYGYSTR	**P00358**	**5**	**71**	**0.000398533**	**2**	**820.51358**	**1640.01989**	**1639.70100**	**193.62**
	VSWYDNEYGYSTRV	P00358	5	86	1.21949E-05	2	869.93185	1738.85642	1738.77000	49.26
	LVSWYDNEYGYSTR	P00358	5	76	0.000120382	2	877.01541	1753.02354	-	135.26
	ISWYDNEYGYSARV	P00360	3	77	9.23986E-05	2	862.01493	1723.02257	-	143.19
Fraction II-C	**ISWYDNEYGYSAR**	**P00360**	**6**	**77**	**9.07985E-05**	**2**	**812.93001**	**1624.85274**	**1623.70700**	704.86
	VSWYDNEYGYSTR	**P00358**	**5**	**74**	**0.000218754**	**2**	**820.93026**	**1640.85325**	**1639.70100**	701.40
	ISWYDNEYGYSARV	P00360	3	77	9.45517E-05	2	861.83356	1722.65984	-	67.35-
	VSWYDNEYGYSTRV	P00358	2	72	0.000288878	2	870.51520	1740.02312	1738.77000	719.73

* Protein accession number in the UniProt protein database (http://www.uniprot.org/) for taxonomy *S. cerevisiae*; P00358 corresponds to GAPDH2/3 and P00360 to GAPDH1

Two main peptides with molecular weights of 1.638 and 1.622 kDa and the following amino acid residues VSWYDNEYGYSTR and ISWYDNEYGYSAR were identified in each fraction (Fig. 2). The theoretical isoelectric point (pI) of these peptides estimated by the ExPASy software (http://www.expasy.ch/tools/peptide-mass.html) is 4.37.

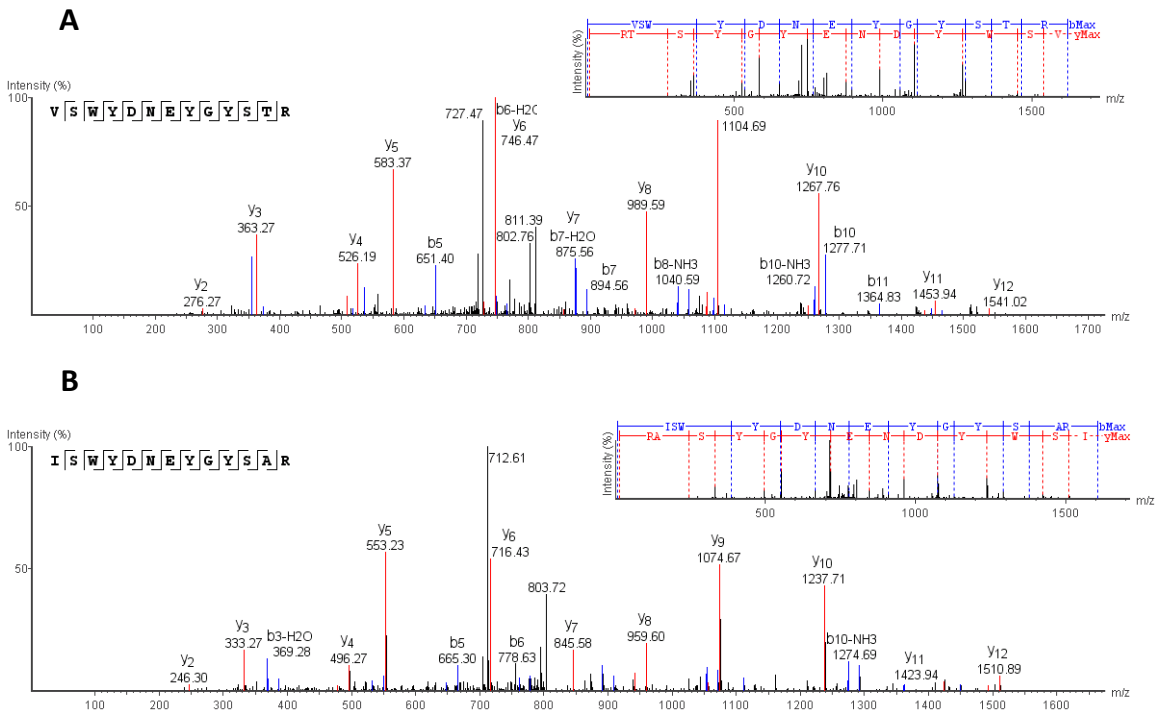

Fig. 2: Mass spectrometry spectra (MS/MS) of the two main peptides (double charged) found and validated in each of the anionic fractions obtained by anion-exchange chromatography (fractions II-A, II-B and IIC indicated in Fig. 1B). (A) Peptide VSWYDNEYGYSTR, m/z 820.24, $[M+2H]^{2+}$.(B) Peptide ISWYDNEYGYSAR, m/z 812.24, $[M+2H]^{2+}$.

3.2. Spectrum of action of the AMPs

The spectrum of action of the antimicrobial peptides (AMPs) was determined against several wine-related yeasts (*H. guilliermondii*, *K. thermotolerans*, *T. delbrueckii*, *K. marxianus* and *D. bruxellensis*) and bacteria (*O. oeni*). Results showed that under the conditions tested (YEPD or MRS, at pH 3.5) the AMPs inhibited the growth of all these microbial species (Fig. 3), although for some yeast strains (e.g. *T. delbrueckii*) only a fungistatic effect was observed, while on others a fungicide effect was shown. *H. guilliermondii* showed to be the most sensitive yeast, with total death occurring after 14 h of incubation with the AMPs, followed by *K. marxianus* and *D. bruxellensis* strain ISA 2211 with total death established within 44 h and 96 h, respectively. The present results revealed that these AMPs are active against a wide variety of microorganisms

associated with wine fermentations, although the sensitivity of these microbial species towards the AMPs is strain-specific, as shown by the results obtained for different strains of *D. bruxellensis* and *O. oeni* (Fig. 4). Moreover, these AMPs also showed inhibitory activity when tested in a modified-SGJ (20 g/l of sugars, 30 g/l of ethanol) at pH 3.5 (data not shown).

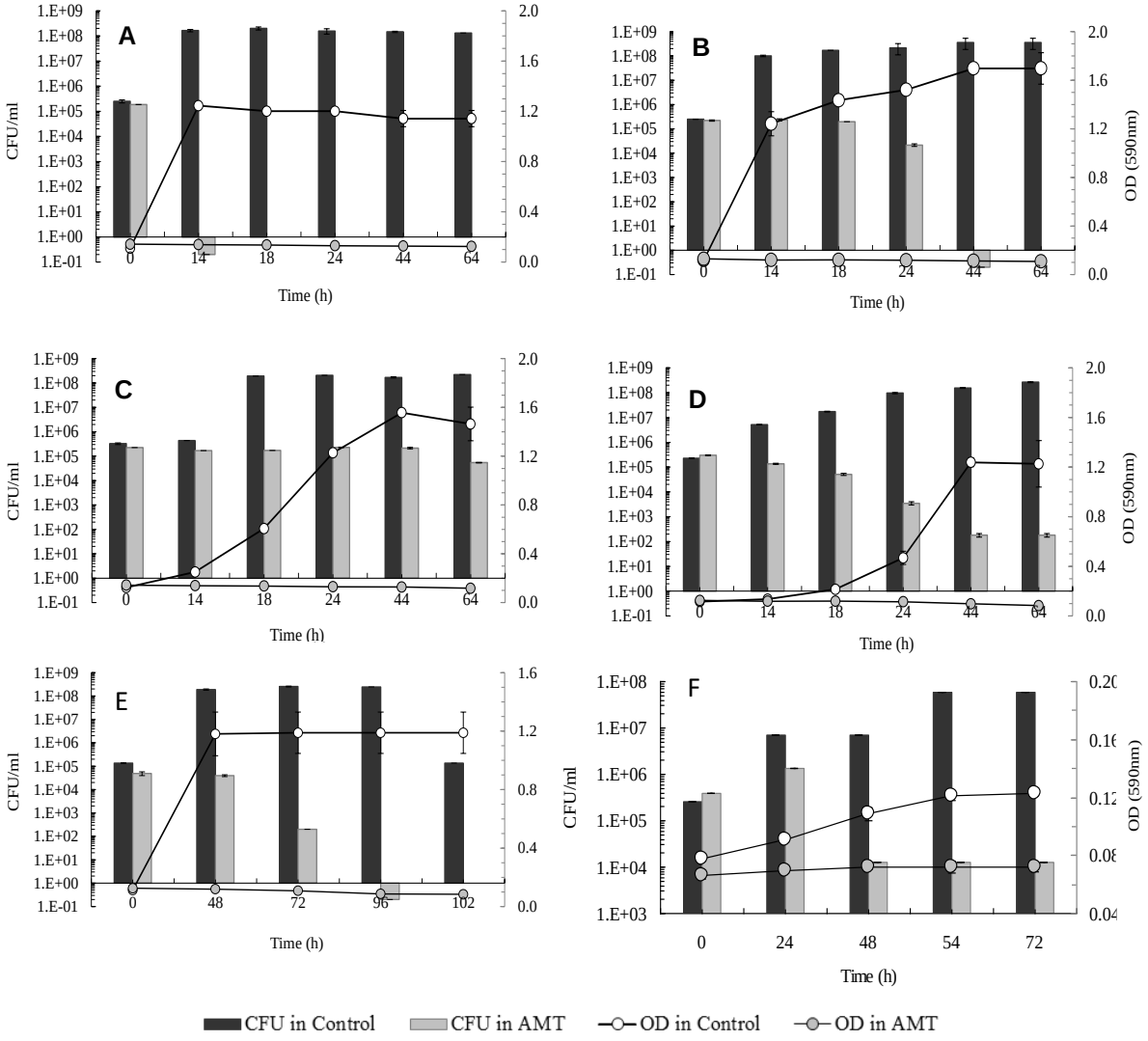

Fig. 3: Viable cells (CFU) and optical density (OD) of *Hanseniaspora guilliermondii* (A), *kluyveromyces marxianus* (B), *Torulaspora delbrueckii* (C), *Kluyveromyces thermotolerans* (D), *Dekkera bruxellensis* strain ISA 2211 (E) and *Oenoccocus oeni* strain DSM 2529 (F) in the antimicrobial tests (AMT) performed with YEPD medium (at pH 3.5) for yeasts and MRS broth (at pH 3.5) for bacteria, without addition of fraction-II (Control) and with addition of 1 mg/ml of fraction-II (AMT). Data represented correspond to mean values of triplicate independent assays ± SD (error bars).

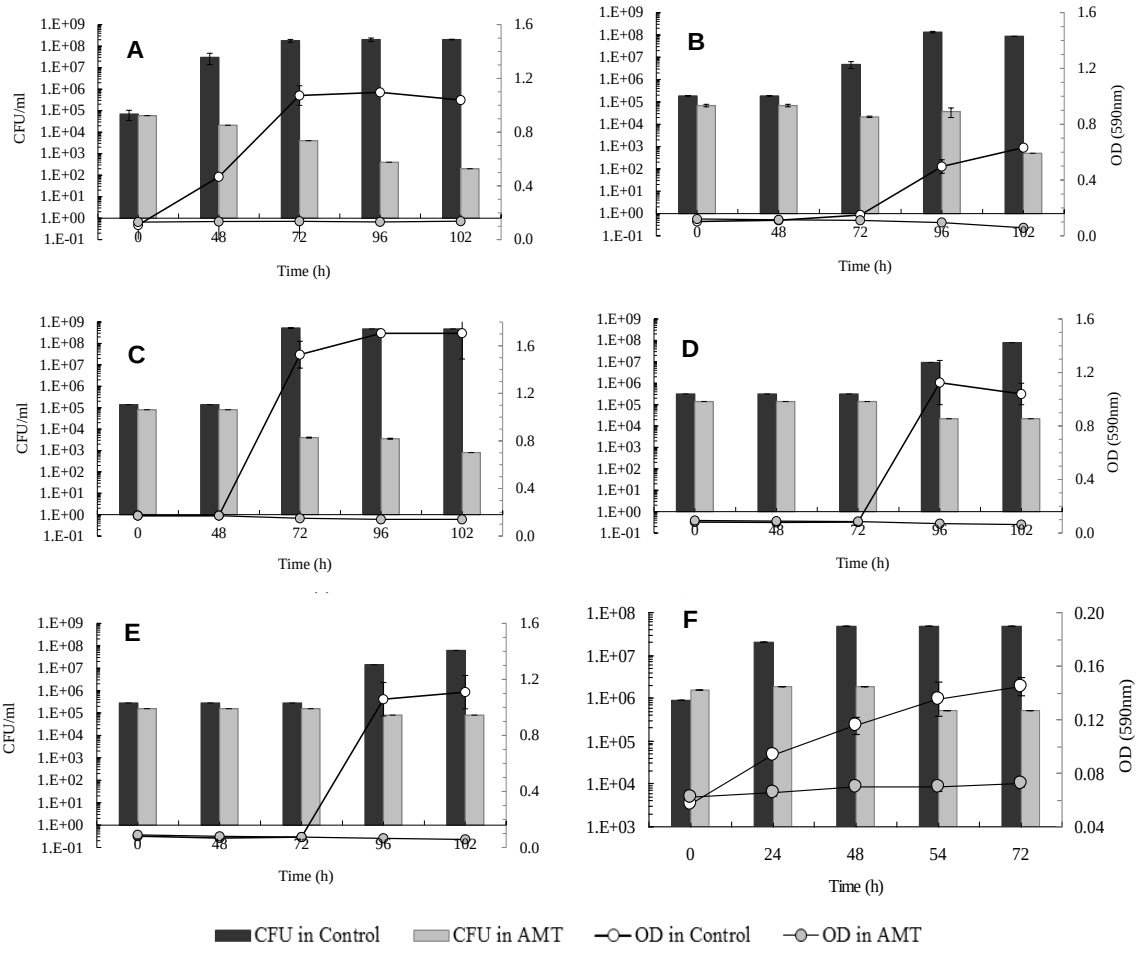

Fig. 4: Viable cells (CFU) and optical density (OD) of *Dekkera bruxellensis* strains ISA 1649 **(A)**, ISA 1700 **(B)**, ISA 1791 **(C)**, ISA 2104 **(D)** and ISA 2116 **(E)**, and of *Oenoccocus oeni* ISA 4279 **(F)** in the antimicrobial tests (AMT) performed with YEPD medium for *D. bruxellensis* strains and with MRS broth for *O. oeni* without addition of fraction-II (Control) and with addition of fraction-II (AMT). Data represented correspond to mean values of triplicate independent assays ± SD (error bars).

3.3. Role of GAPDH-derived peptides in *S. cerevisiae* antagonism

In order to further confirm that GAPDH-derived peptides are involved in the early death of non-*Saccharomyces* during wine fermentations, we performed alcoholic fermentations with mixed cultures of *H. guilliermondii* and *S. cerevisiae* mutant strains deleted in each of the *TDH1-3* genes. *S. cerevisiae* wild-type strain BY4741 was used in mixed culture with *H. guilliermondii* as positive control and *H. guilliermondii* in single culture for negative control. Growth profiles of both yeasts (Fig. 5A, B) showed that all strains were able to grow during the first 1–2 days of fermentation, but then *H. guilliermondii* began to die off (Fig. 5B) while *S. cerevisiae* kept its cell density at about 10^7 CFU/ml until the end of fermentation (Fig. 5A). However, death rate of *H. guilliermondii* significantly varied whether in single or in mixed culture and was faster in the mixed cultures performed with the wild-type strain of *S. cerevisiae*, particularly after the 3rd day of fermentation. Interestingly, the death rate of *H. guilliermondii* was much slower in the mixed cultures performed with the *Δtdh1*, *Δtdh2* and *Δtdh3* mutants than in the mixed culture performed with the wild-type strain (Fig. 5B). Although ethanol levels had varied among fermentations, when we compare the ethanol profile of the mixed fermentation performed with the wild-type strain and those of *Δtdh1* mutants (Fig. 5C), it can be clearly seen that ethanol cannot explain the differences observed in the death rates of *H. guilliermondii* on those fermentations (Fig. 5B).

Recently, Silva et al. (2011) reported that GAPDH is a specific substrate of yeast metacaspase and showed that the in vivo cleavage of GAPDH by metacaspase originated several GAPDH-derived peptide fragments, namely some equal to the ones identified in the present work. In view of this information, we wondered if a *S. cerevisiae* mutant deleted on the metacaspase *YCA1* gene would not prevent the production of GAPDH-derived peptides and thus avoid death of non-*Saccharomyces* in fermentations with *S. cerevisiae*. To confirm this hypothesis, we performed alcoholic fermentations with *H. guilliermondii* in co-culture with *S. cerevisiae* wild-type strain BY4742 and its isogenic mutant *Δyca1*. Since cycloheximide is a well-known inhibitor of apoptosis, we also performed fermentation with *H. guilliermondii* and *S. cerevisiae* wild-type strain, adding cycloheximide after the first day of fermentation. Fermentation of *H. guilliermondii* in single culture was used as negative control. Results showed (Fig. 6B) that the use of both cycloheximide and *Δyca1* strain in mixed cultures with *H. guilliermondii* significantly prevented death of the non-*Saccharomyces* strain, which was able to keep its culturability at relatively high levels (ca 10^4 CFU/ml) until the end of fermentation (7

days). In both cases, the culturability of *H. guilliermondii* after 5 days was four orders of magnitude higher than in the mixed culture fermentation performed with the wild type strain (without cycloheximide) and comparable to the one observed in the *H. guilliermondii* single culture fermentation. It is important to notice that ethanol profiles in the mixed cultures performed with the *Δyca1* strain and with the wild-type strain (without cycloheximide) were quite similar (Fig. 6C), which confirmed that minor death of *H. guilliermondii* in the presence of the *S. cerevisiae Δyca1* strain was not caused by ethanol.

In order to confirm the presence/absence of the previously identified GAPDH-derived anionic AMPs in these mixed cultures, we analysed the chromatographic profiles of *S. cerevisiae* BY4742 and *Δyca1* supernatants (5 days-old), using the same purification procedure. Results showed (Fig. 7) that the anion-exchange chromatographic profile of *S. cerevisiae* BY4742 is very similar to the one exhibited by *S. cerevisiae* CCMI 885 (Fig. 1B) but quite different from that of *S. cerevisiae Δyca1*. Moreover, antimicrobial tests performed with these anionic fractions showed that all BY4742 anionic fractions killed *H. guilliermondii*, while the *Δyca1* anionic fraction only exerted a minor inhibitory effect over the same strain (data not shown). These results confirmed that the bioactive anionic peptides are absent, or at least are present at much lower amounts, in *S. cerevisiae Δyca1* supernatants what explains that *H. guilliermondii* dies less in co-cultivation with Δyca1 than with BY4742 or CCMI 885 strains (Fig. 6B).

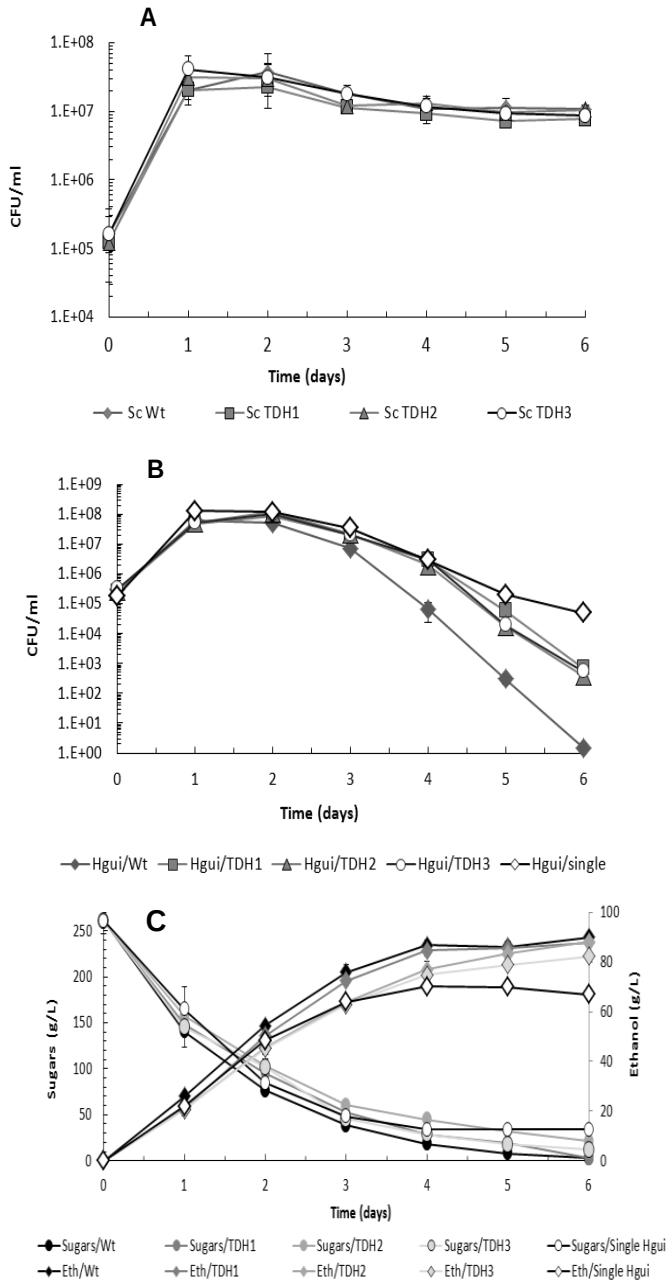

Fig. 5- Cell growth of *S. cerevisiae* (A) and *H. guilliermondii* (B) and sugar consumption and ethanol production (C) during alcoholic fermentations performed with *H. guilliermondii* in single and mixed cultures with each of the following *S. cerevisiae* strains: wild-type BY4741 (Wt), *Δtdh1* (*TDH1*), *Δtdh2* (*TDH2*) and *Δtdh3* (*TDH3*). Data represented correspond to mean values of duplicate independent assays±SD (error bars)

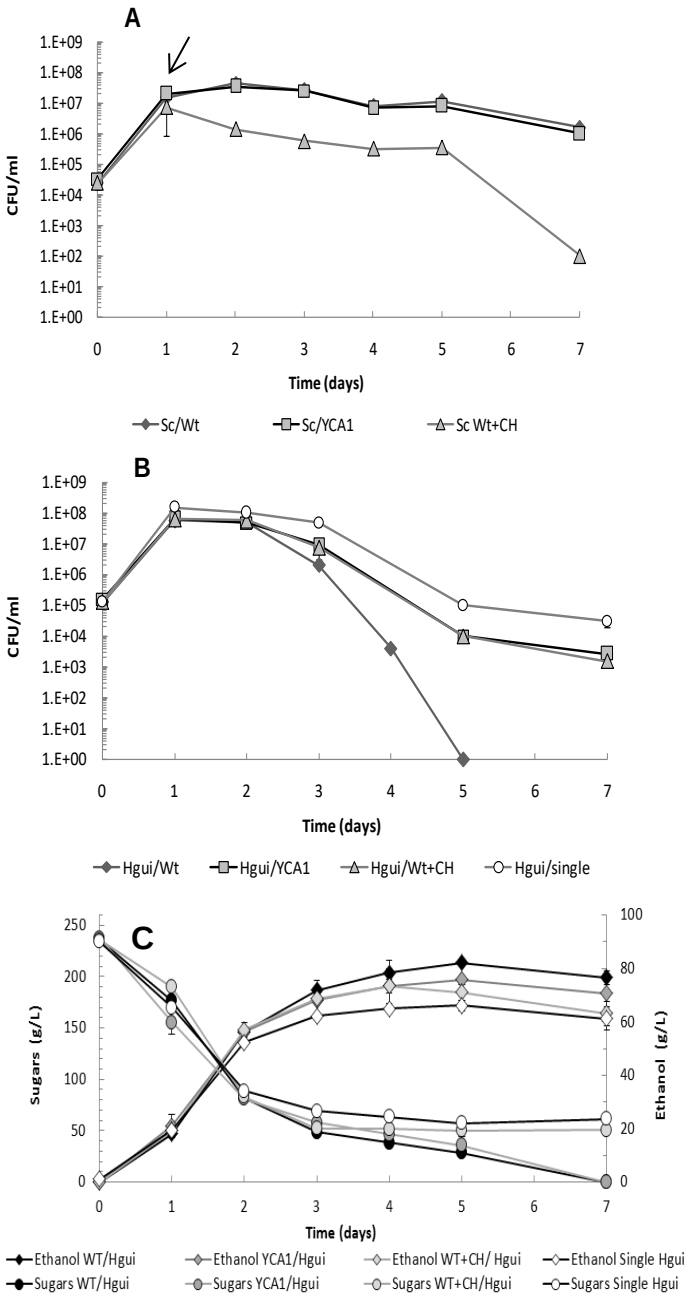

Fig. 6- Cell growth of *S. cerevisiae* (A) and *H. guilliermondii* (B) and sugar consumption and ethanol production (C) during alcoholic fermentations performed with *H. guilliermondii* in single and mixed cultures with *S. cerevisiae* wild-type strain BY4742 (Wt), wild-type strain in the presence of cycloheximide (Wt+CH) and mutant strain *Δyca1* (*YCA1*). Arrow indicates the point at which 0.001 % of cycloheximide was added to the culture. Data represented correspond to mean values of duplicate independent assays±SD (error bars)

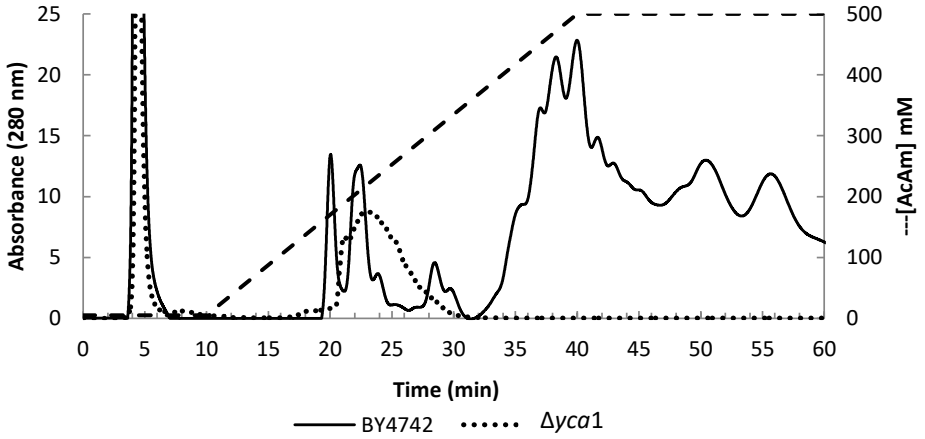

Fig. 7- Chromatographic profiles of the anionic peptides present in *S. cerevisiae* BY4742 and *Δyca1* supernatants (5 days old), obtained by pooling fraction-II of each strain (from gel filtration) into a strong anion exchange column (Q-Resource 6 ml, GE Healthcare, London, UK)

4. DISCUSSION

We had previously reported that *S. cerevisiae* CCMI 885 produces antimicrobial peptides (AMPs<10 kDa) that inhibit the growth of *H. guilliermondii*, *T. delbrueckii*, *K. marxianus* and *K. thermotolerans* during alcoholic fermentations performed with mixed cultures (Albergaria et al. 2010). In the present work, we show that these AMPs are derived from the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Although surprising, this finding is supported by several studies showing that GAPDH, besides its glycolytic role, displays several other activities in different subcellular locations (membrane, cytosol and nucleus), including a primary role in apoptosis and in a variety of critical nuclear pathways (Sirover 2005; Nakajima et al. 2009; Silva et al. 2011). More importantly, in two recent publications, GAPDH-derived AMPs with antifungal activity were isolated, one from the human placental tissue (Wagener et al. 2013) and another from the skin of yellowfin tuna (Seo et al. 2012). Those AMPs correspond to small cationic peptides that match the N-terminal (2-32) amino acid sequence of the human and fish GAPDH protein, respectively, and are active against the pathogenic yeast *Candida albicans*. Conversely, the AMPs here identified are anionic (at neutral pH), match the C-terminal (309-321) amino acid sequence of the *S. cerevisiae* GAPDH protein and are active against

several wine-related yeasts (e.g. *D. bruxellensis*) and bacteria (e.g. *O. oeni*). Regarding the anionic nature of these AMPs it is important to emphasize that at the acidic pH conditions of wine fermentations (pH ranging 3.0–3.5), peptides with p*I* =4.37 are not negatively charged. In spite of these differences, the above-mentioned findings clearly indicate that GAPDH plays an important role in the defense system of different organisms.

In the yeast *S. cerevisiae*, three related but not identical GAPDH isoenzymes with different specific activities are encoded by unlinked genes designated *TDH1*, *TDH2* and *TDH3* (McAlister and Holland 1985a). McAlister and Holland (1985b) have also shown that none of these *TDH* genes are individually essential for cell viability, but a functional copy of either *TDH2* or *TDH3* is required for growth since *Δtdh2 Δtdh3* cells are not viable. For this reason, in the present work, we have used single mutant strains of *S. cerevisiae* deleted in each of the *TDH1-3* genes in mixed cultures with *H. guilliermondii* to evaluate their impact on the early death of the non-*Saccharomyces* yeast. Our results show that deletion of each of these genes in *S. cerevisiae* reduces its antagonism against *H. guilliermondii*, thus further demonstrating that GAPDH is involved in wine microbial interactions.

Delgado et al. (2001) found that each of the three GAPDH polypeptides encoded by the *TDH1-3* genes is associated with the cell wall of *S. cerevisiae*. The same authors also demonstrated that GAPDH accumulates in the cell wall of *S. cerevisiae* in response to starvation and temperature upshift (Delgado et al. 2003). Beyond this stress response, specifically related to the cell-wall-associated GAPDH, a recent work by Silva et al. (2011) identified GAPDH as a specific target of metacaspase in *S. cerevisiae*, thus proving GAPDH is associated with apoptosis in *S. cerevisiae*. In a previous work (Albergaria et al. 2010), we showed that *S. cerevisiae* begins to secrete AMPs to the extracellular medium at the end of the exponential growth phase (1–2 days) in alcoholic fermentations. In addition, our current work also shows that a mutant strain of *S. cerevisiae* deleted in the metacaspase *YCA1* gene significantly prevents death of *H. guilliermondii* during alcoholic fermentation. Taken together, these findings suggest that the presence of GAPDH-derived peptides in the extracellular media at the end of exponential growth phase might be due to apoptotic cells of *S. cerevisiae* inducing the cleavage of GAPDH by metacaspases. However, to definitively establish this connection between apoptosis and secretion of AMPs further investigation must be carried out.

Most industrial processes involving alcoholic fermentations with *Saccharomyces* strains, such as wine, beer or fuel-ethanol production, are carried out under non-sterile growth conditions due to technical and economic reasons, with high risks of microbial contamination. Wine contamination problems can occur at multiple stages of the winemaking process, and can lead to stuck fermentations, low levels of ethanol and the presence of off-flavours in wine. Likewise, contaminations of industrial fuel-ethanol fermentations by yeasts and bacteria have a negative impact on ethanol yield and productivity (Liberal et al. 2007). In both fermentation processes, spoilage microorganisms can include a wide variety of yeasts, namely those of the species *D. bruxellensis* and *Zygosaccharomyces bailii* and bacteria, such as lactic acid and acetic acid bacteria (Loureiro and Malfeito-Ferreira 2003; Liberal et al. 2007). Chemical preservatives such as sulphur dioxide (SO_2) are commonly used in winemaking to prevent the development of spoilage microorganisms. However, some wine contaminants such as *Pichia* spp. and *Dekkera* spp. can resist to the SO_2 levels used on those processes (Barata et al. 2008; Basílio et al. 2008) that cannot be too high in order to allow fermentation by *S. cerevisiae* strains. In this context, proteins and peptides exhibiting antimicrobial properties might have a remarkable potential for food preservation and control of spoilage microorganisms, although in the winemaking process selectivity is required for not affecting beneficial microorganisms. The use of killer toxins produced by *Pichia anomala* and *Kluyveromyces wickerhamii* with fungicidal effects against *D. bruxellensis* in wine has been reported (Comitini et al. 2004; Comitini and Ciani 2011). In the present work, we found that *S. cerevisiae* secretes AMPs during alcoholic fermentation that are active at oenological growth conditions against a wide variety of wine-related microbial species, including *D. bruxellensis* strains. Thus, the possibility of using these AMPs as natural alternative biopreservatives in alcoholic fermentations, wine and/or other food products looks promising and will be further assessed.

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Chapter III

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 a fluorescence ratio imaging microscopy (FRIM) technique and culturability by a 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 ($\Delta pH = pHi - pHext$). 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 subpopulation 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 $0 < \Delta pH < 3$). 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 that facilitate these interactions. The peptide 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 characterized 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 μM such as in kappacins, the first AAMPs isolated from bovine milk (Malkoski et al., 2001), while cationic AMPs typically Exhibit MIC ranging from10 to 100 μM (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; Siegumfeldt 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 subpopulations of cells simultaneously. Several authors (Hornbæk et al., 2002; Mortensen et al., 2006, 2008; Smigic et al., 2009; Vindelov 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 rpmfor 16 h. Allmediawere 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, $CaCl_2$, 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 Na_2HPO_4 (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 Section 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 µl 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 µl of a 9.16 mM CDCFDA-SE solution or 4.48 mM CFDA-SE to 990 µl of cell suspension that was incubated at 25 °C for 3 h. After 2 h and 30 min of incubation with the probes, 10 µl 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 µl 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 Axioskop 50, Germany) equipped with a Zeiss Neofluar 40× objective (numerical aperture 0.75) and a HBO 50Whalogen lamp to provide excitation of the probes (CFDA-SE and CDCFDASE). Stained cells were excited for 3 s 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 RS Image software (Roper Scientific, version 1.9.2). For PI staining measurements, cells were excited for 3 s 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 programme (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 pHi, 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 to500µl 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 Na_2HPO_4 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 (pHi), 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 R470/440 calculated as described above and plotted against the respective pHi 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.

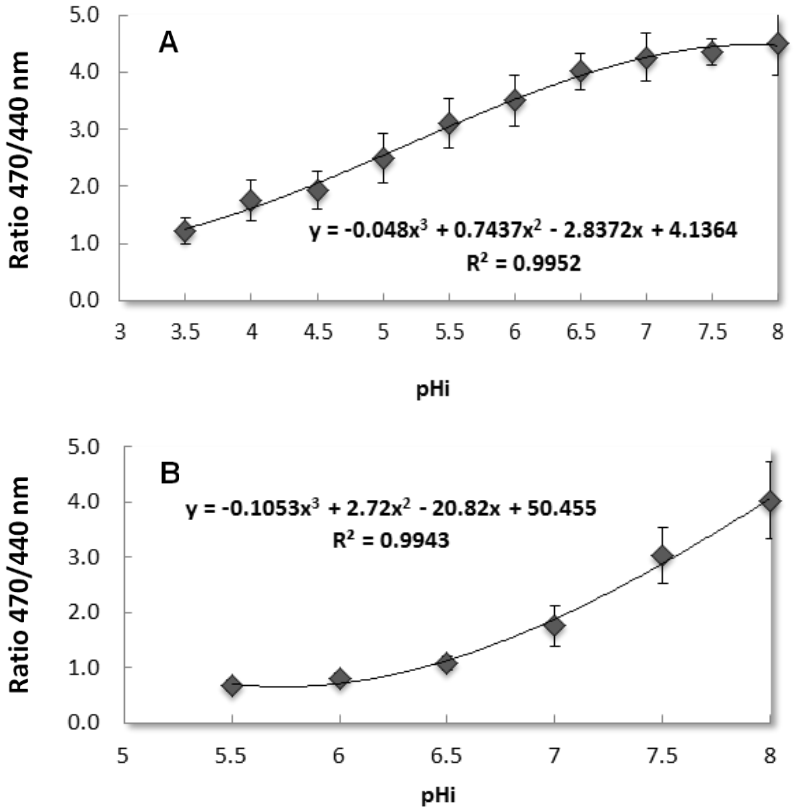

Fig. 1: Calibration curves of the fluorescence ratio (R470/440 nm) emitted by *H. guilliermondii* cells stained with the pH-sensitive probes CDCFDA-SE (A) and CFDA-SE (B) at different pHi values. Values represented correspond to the mean of 50 single cells measurements and error bars to standard deviation (±SD). Calibration points were fitted by a third-degree polynomial curve.

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. 3B). These results indicate that for *H. guilliermondii* cells under this stress conditions, CDCFDA-SE does not measure the vacuolar pH (pH_{vac}) 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. 3A). Hence, in the control assay the information obtained after 24 h by using the CDCFDA-SE probe refers mainly to pH_{vac}. 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)

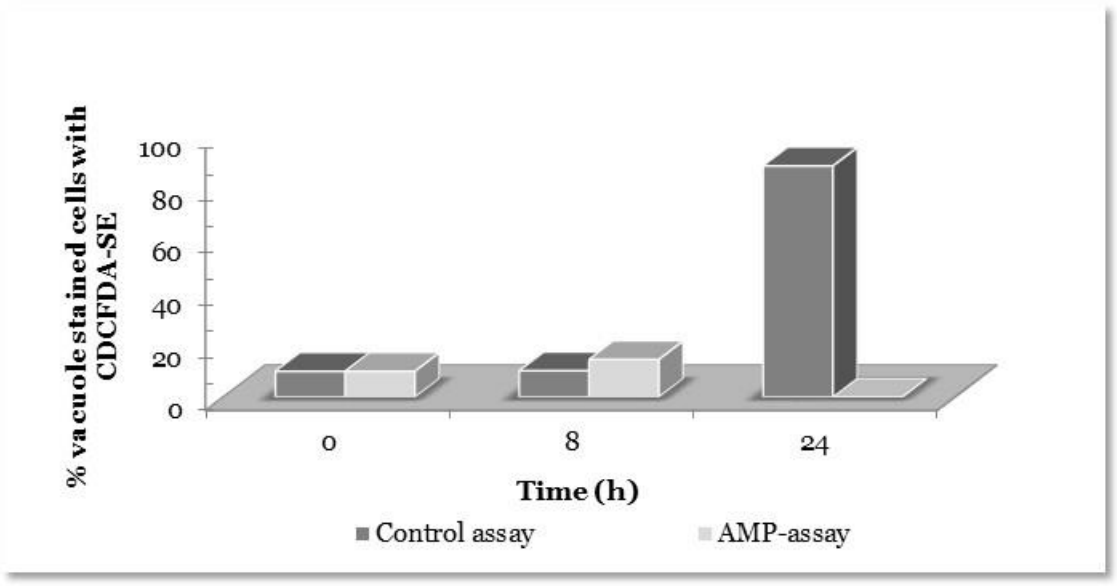

Fig. 2: Percentage of *H. guilliermondii* cells exhibiting CDCFDA-SE fluorescence exclusively in the vacuole after 0 h, 8 h and 24 h of incubation in the absence (Control assay) and in the presence of AMPs (AMP-assay). Percentages were calculated based on the microscopic observation of 100 individual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

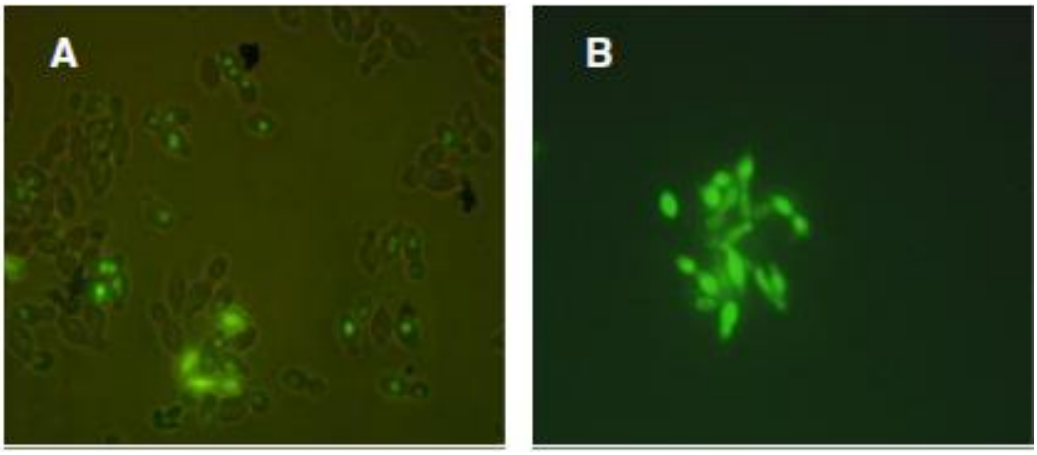

Fig. 3: Microscope images (WB-filter) of *H. guilliermondii* cells stained with CDCFDA-SE after 24 h of incubation in the absence of AMPs (control assay) (A) and in the presence of the AMPs (AMP-assay) (B). Objective amplification of 100×.Microscope observation of the AMP-assay sample showed the same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

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 one 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 the cells into three subpopulations according to the pH gradient ($\Delta pH = pHi - pHext$) 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; Guldfeldt and Arneborg, 1998; Imai et al., 1994; Imai and Ohno, 1995; Rowe et al., 1994) for healthy cells of *S. cerevisiae* (7.0), the following subpopulations were defined: healthy cells for $\Delta pH \geq 3.0$ ($=6.5 - 3.5$); sub-lethally injured cells for $0 < \Delta pH < 3.0$; severely injured cells for $\Delta pH = 0$. 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 ($\Delta pH \geq 3.0$) and their culturability rapidly decreased (Fig. 4-D), with the subpopulation of severely injured cells ($\Delta pH = 0$) 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 ($\Delta pH = 0$) 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% sublethally injured (with $0 < \Delta pH < 3$) (Fig. 4-D). 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 pHi 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 cellswere able to recover their culturability and pHi (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 10^7 CFU/ml within 24 h once the specific growth rate under the same growth conditions (control assay) is just 0.28 h^{-1}.

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 to their ΔpH (=pHi−pHext) range and pHi values determined by the FRIM method using the CFDA-SE probe.

	% Healthy cells (ΔpH≥1.5)	**% Sub-lethally injured cells (0<ΔpH<1.5)**	**% Severely injured cells (ΔpH=0)**	**%cytoplasm-stained cells**
Control-assay	2.0	96.0	2.0	100
AMP-assay	5.9	9.8	84.3	100

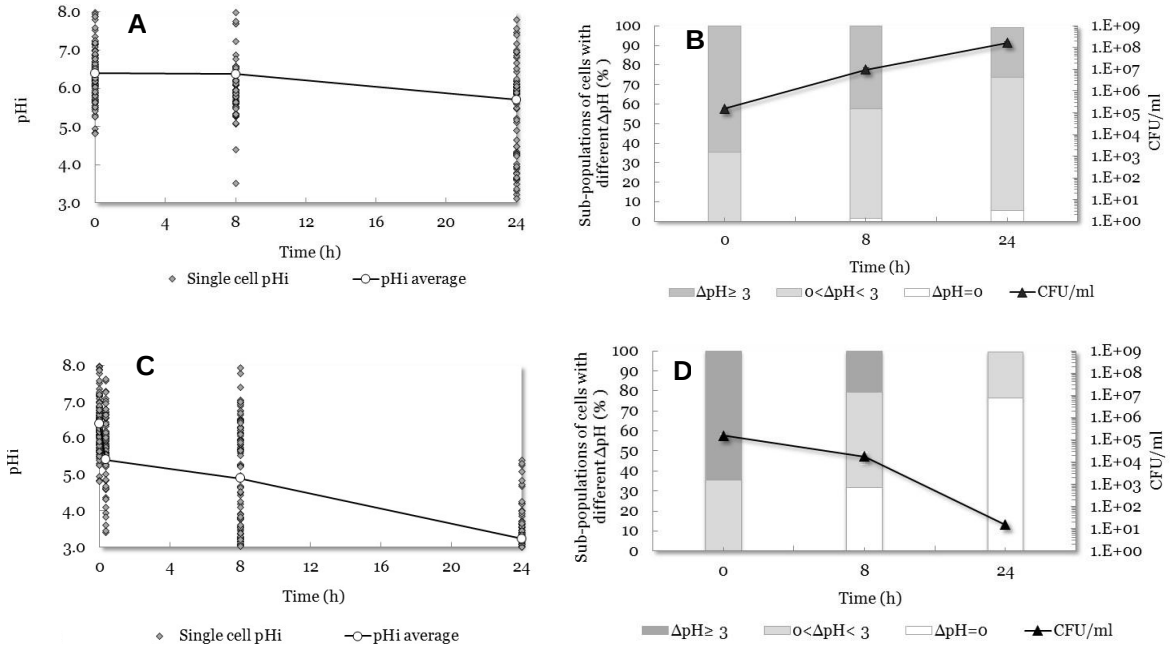

Fig. 4: Changes in the culturability (CFU/ml) and pHi, as determined by the FRIM method with the CDCFDA-SE probe, of *H. guilliermondii* cells during the control (A, B) and AMP-assay (C, D). Evolution of pHi of individual cells (50 cells analysed per sample) and of the pHi average value (A, C), as well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

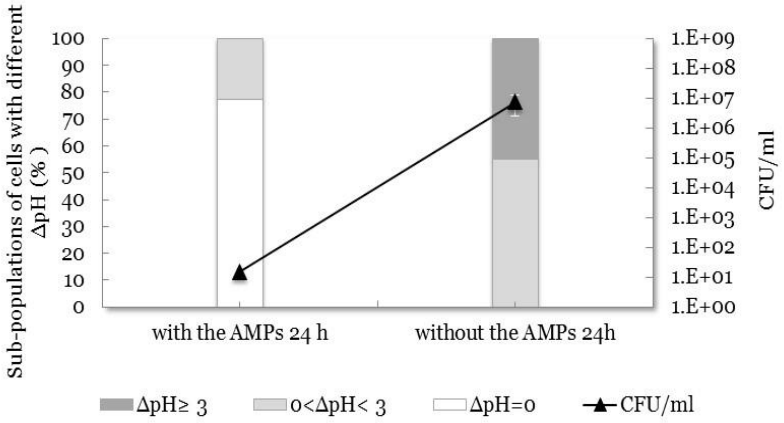

Fig. 5. Culturability (CFU/ml) and sub-populations of *H. guilliermondii* cells with ΔpH ≥ 3, healthy; 0 < ΔpH < 3, sub-lethally-injured; ΔpH=0, severely injured after being exposed to AMPs for 24 h (with the AMPs 24 h) and after being allowed to recover for 24 h in YEPD medium (without the AMPs 24 h).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

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 pHi values measured for cells in the control after 24 h (Fig. 4-A) refers mainly to the vacuolar pH. This might explain why pHi 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., 1997), 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 pHi 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 pHi (i.e., between 5.5 and 8.0) (Fig. 1-A), the following subpopulations were defined: healthy cells for ΔpH ≥1.5 (=7.0−5.5); sub-lethally injured cells for 0 < ΔpH <1.5; severely injured cells for ΔpH=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 obtainedwith the CFDASE 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 pHi and the culturability exhibited by cells, as previously reported by several other authors (Fang et al., 2006; Gaggìa et al., 2010; Imai and Ohno, 1995; Rechinger and Siegumfeldt, 2002). Even if some compounds can have a pronounced antimicrobial effect without targeting membrane integrity (Powersand and Hancock, 2003) our results show that these *S. cerevisiae* AMPs actually disturb the membrane integrity of cells. They also demonstrate that pHi 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. 3-B), 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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Oral communications

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Chapter IV

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 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 andAMP2/3), 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.

Keywords: Antimicrobial peptides; Wine fermentation; Non-*Saccharomyces* yeasts; Glyceraldehyde 3-phosphate dehydrogenase; Apoptosis/necrosis; Cell-penetrating peptides

1 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 theAMP1, with the amino acid residues VSWYDNEYGYSTR 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).

2 MATERIALS AND METHODS

2.1. 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.

2.2. 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.

2.3. 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.

2.4. 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.

2.5. 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 section 2.4.

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/ml 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.

2.6. Internalization of AMPs fluorescently-labeled with fluorescein (FITC)

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 ºC) 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/ml, and four different media were used: 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 ×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 Axioskop 50, 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).

2.7. 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 AMPs-treated cells by the epifluorescent microscopic methods described in the following section.

2.7.1. 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 ºC under agitation (150 rpm), and permeabilized with sodium citrate 0.1 M for 30 min at 70 ºC. 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 ºC 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).

2.7.2. 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 ºC 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).

2.7.3. DAPI staining

Chromatin condensation was accessed by microscopic observations of cells stained with the fluorescent dye 4,6 diamidino-2-phenylindole (DAPI). Briefly, AMPs-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).

3 RESULTS

3.1. 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 *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. 2).

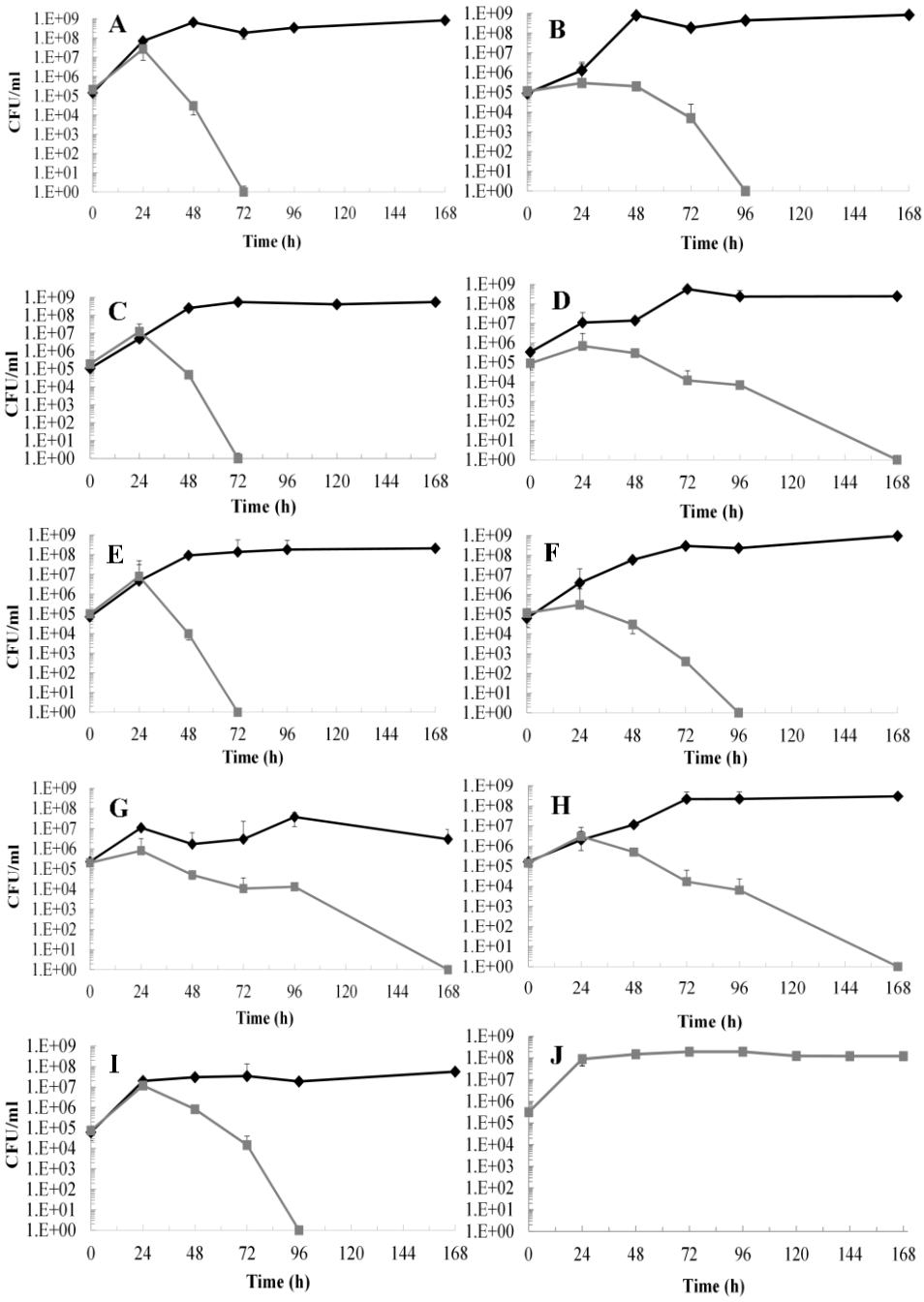

Fig. 1 Cell density profiles (CFU/ml) of *S. cerevisiae* (diamonds) and *H. guilliermondii* (squares) during mixed-culture fermentations performed with *S. cerevisiae* strains CCMI 885 (A), ISA 1000 (B), ISA 1028 (C), ISA1029 (D), ISA 1046 (E), ISA 1063 (F), ISA 1200 (G), S101 (H), ATCC 6269 (I), and during the single-culture fermentation of *H. guilliermondii* (J). Values represented are means of triplicate measurements ± SD (error bars) of two independent biological experiments

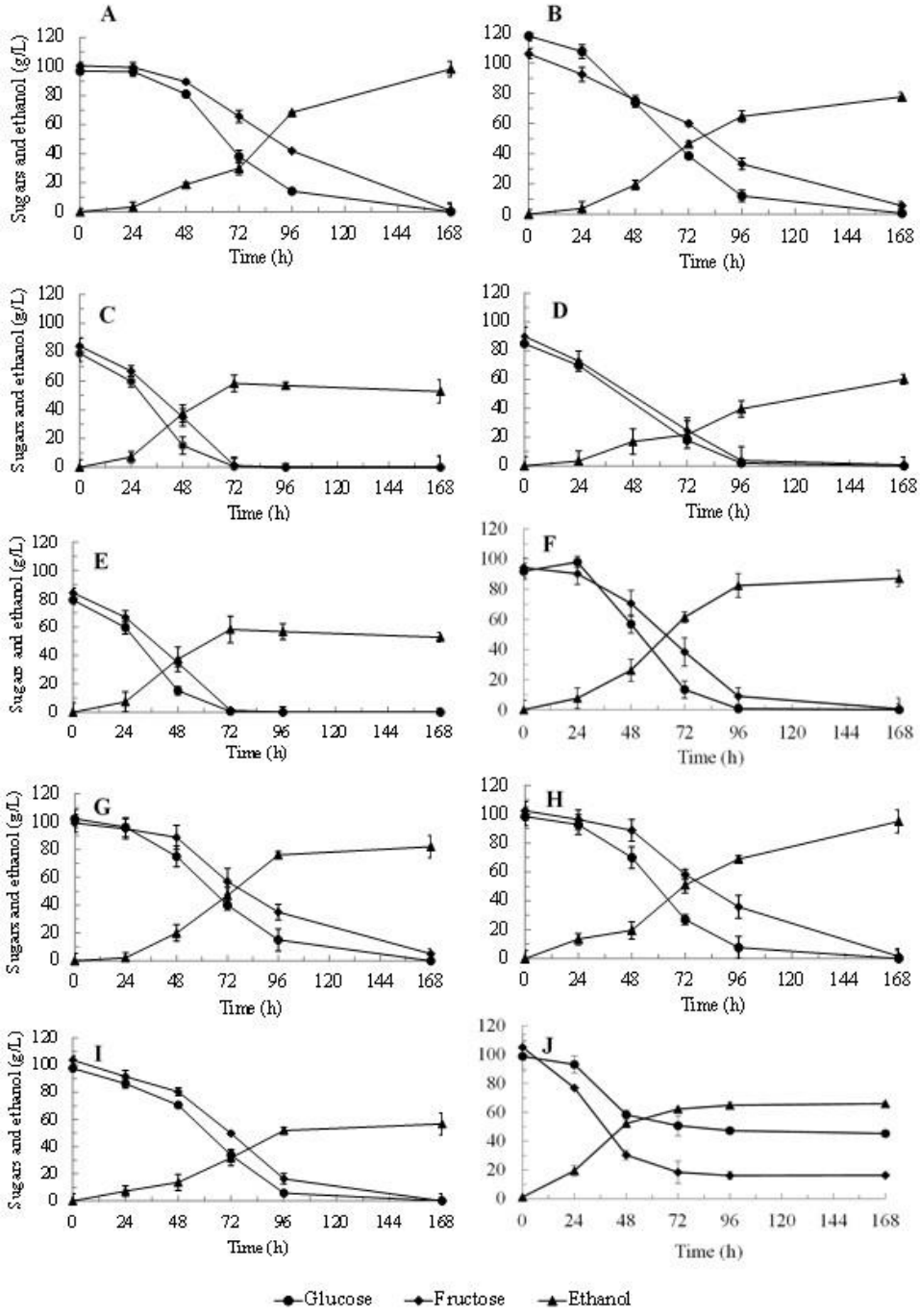

Fig. 2: Sugars consumption and ethanol production during alcoholic fermentations performed with mixed-cultures of *H. guilliermondii* with *S. cerevisiae* CCMI 885 **(A)**, ISA 1000 **(B)**, ISA 1028 **(C)**, ISA1029 **(D)**, ISA 1046 **(E)**, ISA 1063 **(F)**, ISA 1200 **(G)**, S101 **(H)**, ATCC 6269 **(I),** as well as during a single-culture fermentation of *H. guilliermondii* **(J)**. Data presented correspond to means of triplicate measurements ± SD (error bars) of two independent biological assays.

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. 3). 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 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.

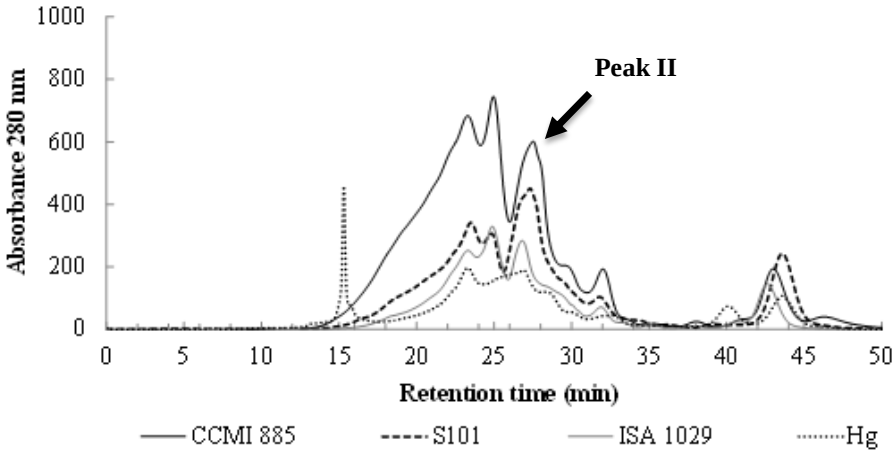

Fig. 3: Gel-filtration chromatographic profiles of the peptidic fractions (2–10 kDa) of supernatants obtained from the mixed-culture fermentations performed with *H. guilliermondii* and different *S. cerevisiae* strains (CCMI 885, S101, and ISA 1029), and from the single-culture fermentation of *H. guilliermondii* (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).

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. 3) 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

3.2. 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. 4). Results show a high homology among the GAPDH sequences in the AMP2/3 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.

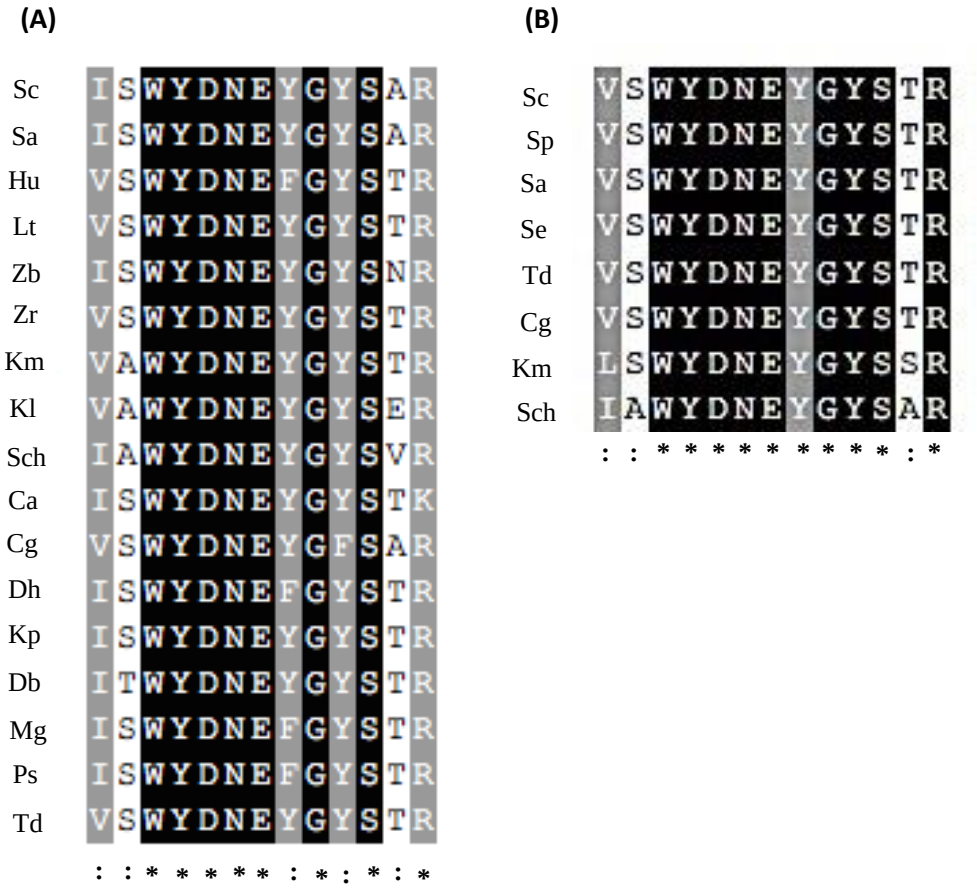

Fig. 4: Amino acid sequence alignments of the GAPDH isoenzymes of several wine-related yeasts, in the region that matches the AMPs secreted by *S. cerevisiae* AMP1 **(A)** and AMP2/3 **(B)**. Peptides were aligned using CLUSTAL OMEGA (McWilliam et al. 2013) with HHalign algorithm (Söding 2005). The peptide sequences correspond to the region matching the AMPs secreted by *S. cerevisiae* (309-321) from GAPDH1 and GAPDH2/3. All amino acid sequences were retrieved from the available web databases. Accession numbers of presented protein sequences in panel **(A)** are: Sc (NP_012483), Sa (EJS43172), Hu (KKA0346), Lt (KLTH0B01958p), Zb (CDH09398), Zr (C5E0E4), Km (BAP71733), Kl (P17819), Sch (ABN68431), Ca (AAC49800), Cg (KTA97609), Dh (Q6BMK0), Kp (AAC49649), Db

(EIF47503), Mg (A5DDG6), Ps (G8YFD4), Td (G8ZVS4). Accession numbers of presented protein sequences in panel **(B)** are: Sc (NP_011708), Sa (EJS43025), Se (KOG98603), Cg (KTA96251), Km (BAP70297), Sch (ABN64899). *Ca: Candida albicans, Cg: Candida glabrata, Dh: Debaryomyces hansenii, Db: Dekkera bruxellensis, Hu: Hanseniaspora uvarum, Km: Kluyveromyces marxianus, Kl: Kluyveromyces lactis, Kp: Komagataella pastoris, Lt: Lachancea thermotolerans, Mg: Meyerozyma guilliermondii, Ps: Pichia sorbitophila, Sa: Saccharomyces arboricola, Sc: Saccharomyces cerevisiae, Sch: Scheffersomyces stipites; Se: Saccharomyces eubayanus, Sp: Saccharomyces pastorianus, Td: Torulaspora delbrueckii, Zb: Zygosaccharomyces bailii, Zr: Zygosaccharomyces rouxii.*

3.3. Antimicrobial properties of the native and syntheticAMPs

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).

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).

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 (p*I*=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 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. 5) 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. 5).

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/ml to a final value determined when the respective control-assay reached the stationary growth phase.

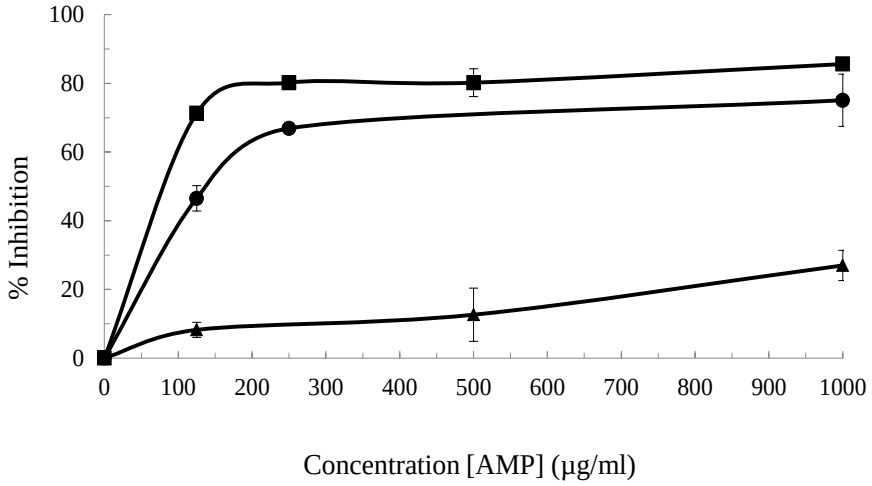

Fig. 5: Growth inhibition of *H. guilliermondii* (relative to control) in the assays performed in YEPD (pH = 6.0) with increasing concentrations of the synthetic AMP2/3 alone (triangles), of the synthetic AMP1 alone (circles) and of a mixture of AMP2/3 and AMP1 (squares) at a ratio of 1:1. Values represented are means of triplicate measurements ± SD (error bars) of two independent biological experiments.

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. 6).

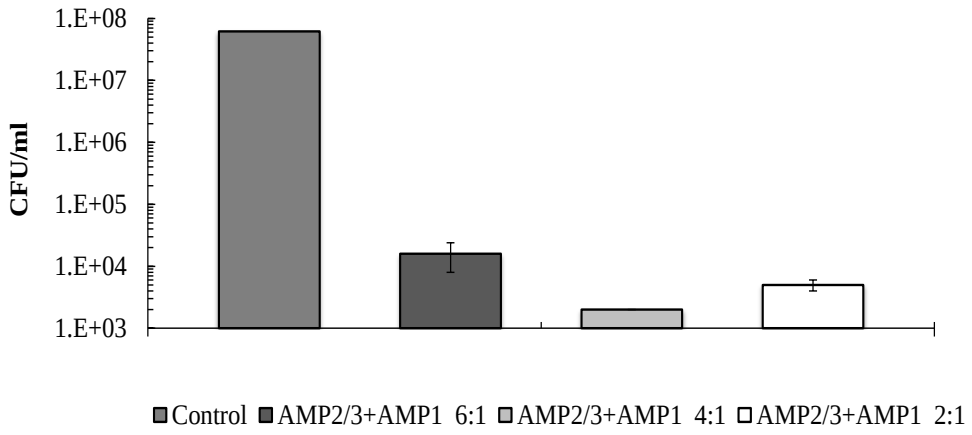

Fig. 6: Cell viability (CFU/ml) of *H. guilliermondii* after 18 h of incubation in YEPD (pH = 6.0) without AMPs (control) and with mixtures of the synthetic AMP2/3 and AMP1 (final concentrations of 1000 μg/ml) at ratios of 6:1, 4:1, and 2:1. The initial cell density was 10^5 CFU/ml in all the assays. Values are means of triplicate measurements ± SD (error bars) of two biological independent assays.

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. 7). 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. 6), 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).

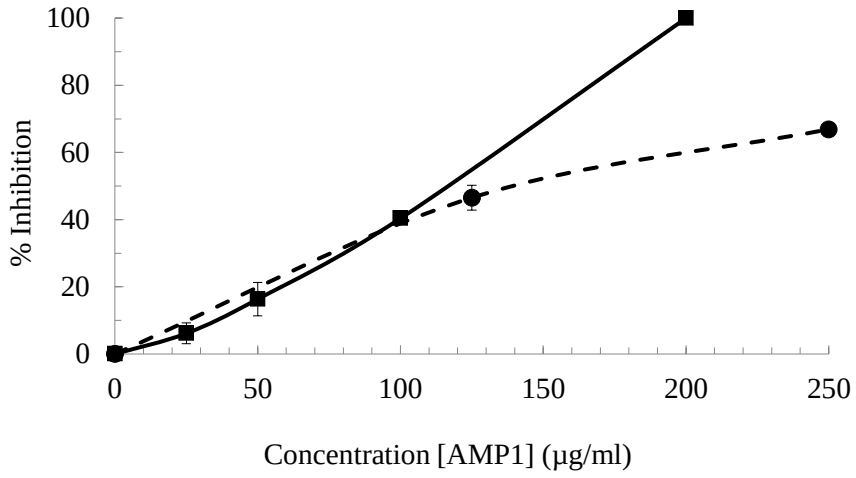

Fig. 7: Growth inhibition of *H. guilliermondii* (relative to control) in assays performed in YEPD (pH=6.0) with the synthetic AMP1 alone (●) and with the AMP1 mixed with the AMP2/3 (■) at a ratio of 4:1 (AMP2/3: AMP1), in function of AMP1 concentration. Values correspond to means of triplicate measurements ± SD (errors bars) of two independent biological experiments.

3.4. 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 labelled with FITC (AMPs-FITC). Results showed that the synthetic AMPs were able to enter in cells of both yeasts (Figs. 8 and 9). 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. 9 A, B). On the other hand, ethanol had no impact on the ability of the AMPs to penetrate *H. guilliermondii* cells (Fig. 9A), whereas an increased internalization was observed in *D. bruxellensis* cells (Fig. 9B). 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. 9A, B).

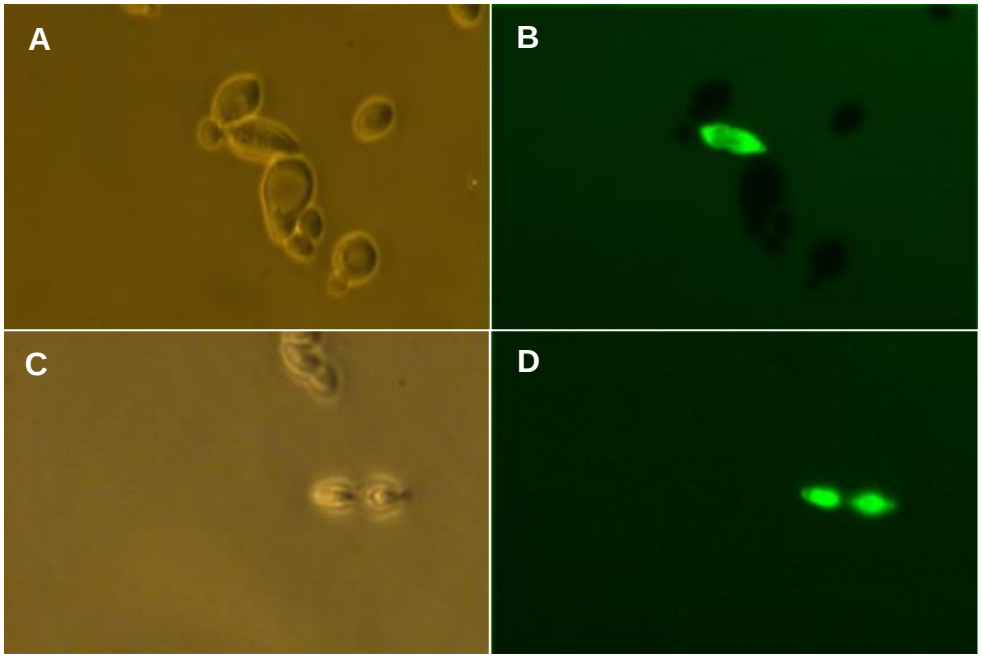

Fig. 8: Internalization of the synthetic AMPs fluorescently labeled with FITC by *D. bruxellensis* (A, B) and *H. guilliermondii* (C, D) cells. Microscopic observation in bright field (A, C) and in fluorescent filter (470 nm) (b, d) using a ×100 objective amplification

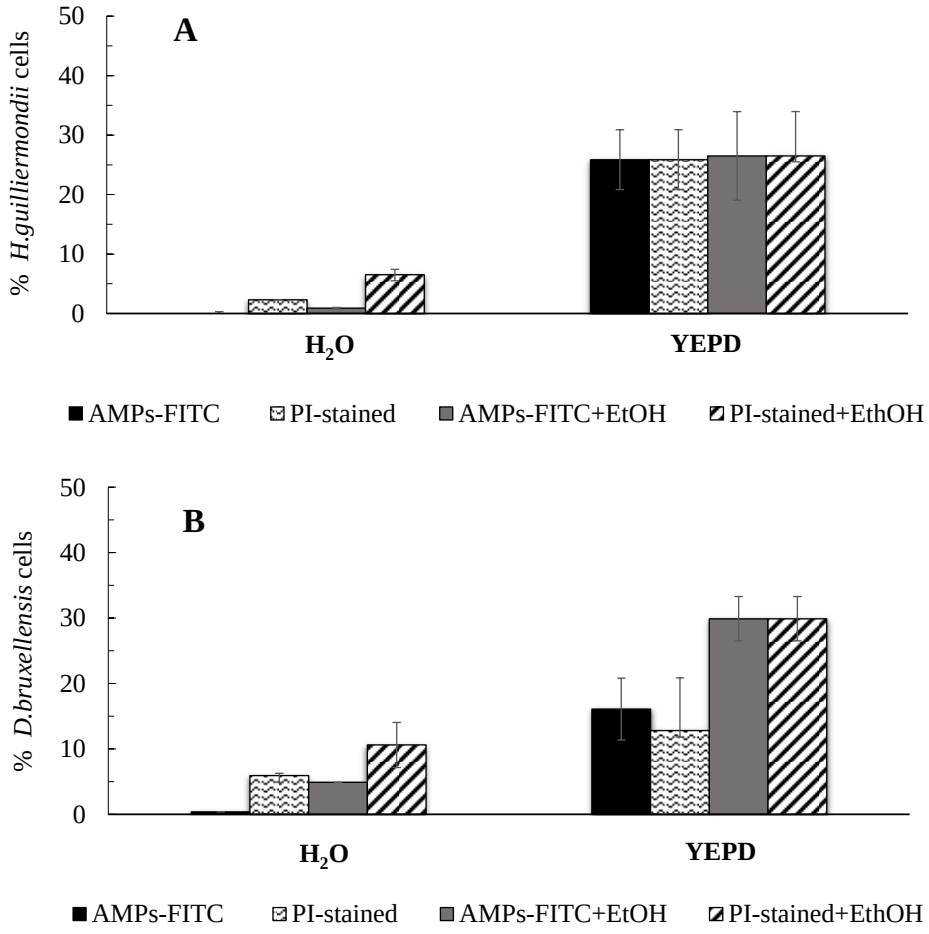

Fig. 9: Percentage of *H. guilliermondii* (a) and *D. bruxellensis* (b) cells that internalized the synthetic AMPs fluorescently labeled with FITC (AMPs-FITC) and that lost membrane integrity (PI-stained) after incubation in deionized water and in YEPD, without ethanol (AMPs-FITC/PI-stained) and with 30 g/l of ethanol (AMPs-FITC + EthOH/ PI-stained + EthOH). Values correspond to means of triplicate measurements ± SD (error bars) from two biological independent assays.

3.5. 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/mlof 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. 10) 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. 10). 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. 10), our results suggest that the AMPs indeed induce apoptosis in the sensitive yeast *H. guilliermondii*.

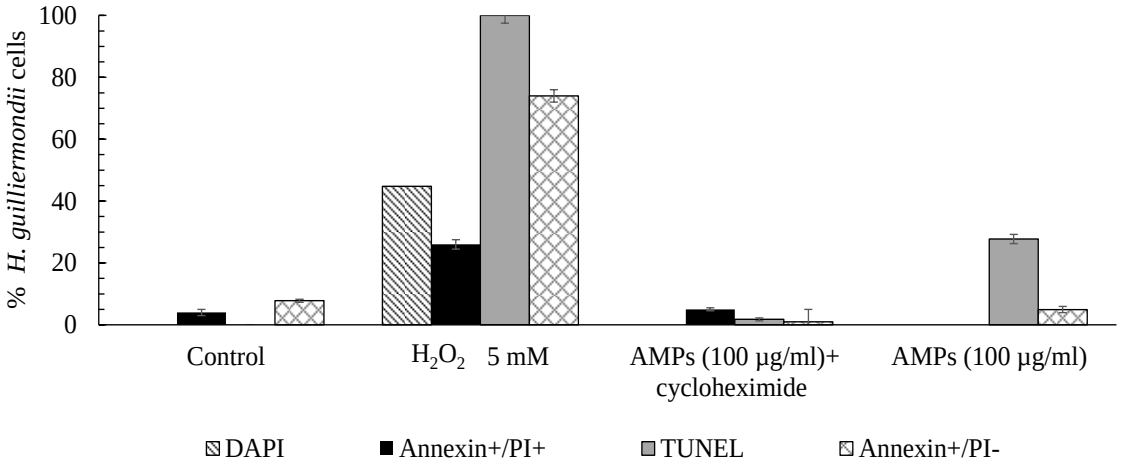

Fig. 10: Percentage of *H. guilliermondii* cells exhibiting apoptotic/necrotic molecular markers after incubation in YEPD medium, for 2 h, at different conditions: without AMPs (control); with 5 mM of H_2O_2 (positive control); with 100 µg/ml of AMPs plus 0.01 % cycloheximide (negative control); with 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

4. 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.

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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Chapter V

Saccharomyces cerevisiae accumulates GAPDH-derived peptides on its cell surface that induce death of non-*Saccharomyces* yeasts by cell-to-cell contact

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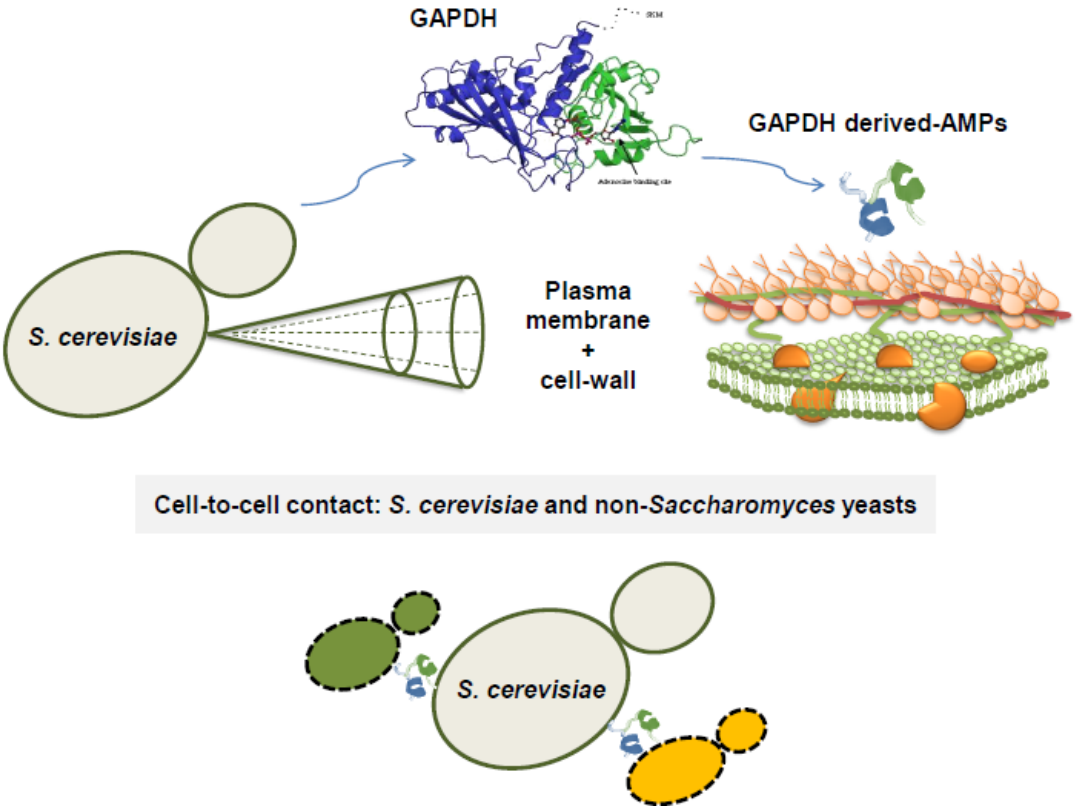
ABSTRACT

During wine fermentations *Saccharomyces cerevisiae* starts to excrete into the growth medium antimicrobial peptides (AMPs) that induce death of non-*Saccharomyces* yeasts at the end of exponential growth phase (24-48 h). Those AMPs were found to derive from the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). On the other hand, the early death of non-*Saccharomyces* yeasts during wine fermentations was also found to be mediated by a cell-to-cell contact mechanism. Since GAPDH is a cell wall-associated protein in *S. cerevisiae*, we put forward the hypothesis that the GAPDH-derived AMPs could accumulate on the cell surface of *S. cerevisiae*, thus inducing death of non-*Saccharomyces* yeasts by cell-to-cell contact. Here we show that 48 h-grown (stationary phase) cells of *S. cerevisiae* induce death of *Hanseniaspora guilliermondii* and *Lachancea thermotolerans* by direct cell-to-cell contact, while 12 h-grown cells (mid-exponential phase) do not. Immunological tests performed with a specific polyclonal antibody against the GAPDH-derived AMPs revealed their presence in the cell-wall of *S. cerevisiae* cells grown for 48 h, but not for 12 h. Taken together, our data shows that accumulation of GAPDH-derived AMPs on the cell surface of *S. cerevisiae* is one of the factors underlying death of non-*Saccharomyces* yeasts by cell-to-cell contact.

Keywords: antimicrobial peptides; glyceraldehyde-3-phosphate dehydrogenase; cell surface proteins; microbial interactions; ecological dominance; wine fermentation;

GRAPHICAL ABSTRACT



1 INTRODUCTION

For a long time, the early death of non-*Saccharomyces* yeasts, such as *Hanseniaspora uvarum*, *Hanseniaspora guilliermondii*, *Lachancea thermotolerans* and *Torulaspora delbrueckii*, during wine fermentation was thought to be primarily due to their low ability to withstand the selective growth factors of wine environment (Bauer and Pretorius 2000). Throughout the last decade, however, several studies have demonstrated that antagonistic interactions, mediated both by a cell-to-cell contact mechanism (Nissen and Arneborg 2003; Nissen, Nielsen and Arneborg 2003; Renault, Albertin and Bely 2013) and by the excretion of antimicrobial peptides (AMPs) into the medium (Albergaria *et al.* 2010; Branco *et al.* 2014, 2017), play an important role in this phenomenon.

Evidence that direct microbial interactions (i.e. by cell-to-cell contact) are involved in the early death of non-*Saccharomyces* yeasts was first reported by Nissen and Arneborg (2003) and Nissen, Nielsen and Arneborg (2003). Those authors found that the early death of *L. thermotolerans* and *T. delbrueckii* during mixed-culture fermentations with *S. cerevisiae* is mediated by a cell-to-cell contact mechanism, and not by any toxic compound excreted into the medium. Although, the mechanism/s underlying cell-to-cell contact death has remained unknown, further studies confirmed the involvement of this phenomenon in the early death of some non-*Saccharomyces* yeasts during wine fermentations (Renault, Albertin and Bely 2013; Kemsawasd *et al.* 2015). On the other hand, Pérez-Nevado *et al.* (2006) demonstrated that the early death of *H. guilliermondii* and *H. uvarum* during mixed-culture fermentations with *S. cerevisiae* is due to toxic compounds secreted by *S. cerevisiae.* Later, Albergaria *et al.* (2010) discovered that those toxic compounds correspond to small peptides (2-10 kDa) that are secreted by *S. cerevisiae* and excreted into the fermentation medium at the end of the exponential growth phase (24-48 h). Finally, Branco *et al.* (2014) identified those AMPs as being fragments of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Subsequent studies (Kemsawasd *et al.* 2015; Branco *et al.* 2017) have now firmly confirmed that different *S. cerevisiae* strains secrete these GAPDH-derived AMPs during wine fermentation, which induce death of several non-*Saccharomyces* yeasts. Moreover, Kemsawasd *et al.* (2015) showed that death of *L. thermotolerans* during mixed-culture fermentations with *S. cerevisiae* is induced both by a cell-to-cell contact-mediated mechanism and the presence of GAPDH-derived AMPs in the

fermentation medium. However, the mechanism/s by which *S. cerevisiae* induces death of non-*Saccharomyces* yeasts by cell-to-cell contact has remained unknown.

Since GAPDH is not only a glycolytic enzyme located in the cytosol, but also a cell wall-associated protein in *S. cerevisiae* (Delgado *et al.* 2001; Delgado, Gil and Gozalbo 2003), we wonder if accumulation of GAPDH-derived peptides on the surface of *S. cerevisiae* cells could induce death of non-*Saccharomyces* yeasts by direct cell-to-cell contact. Thus, the aim of the present study was to investigate if death of non-*Saccharomyces* yeasts by cell-to-cell contact with *S. cerevisiae* is due to the presence of the GAPDH-derived AMPs on their cells surface.

2 MATERIAL AND METHODS

2.1. Strains and growth conditions

S. cerevisiae strains used in the present work were: CCMI 885 (Culture Collection of Industrial Microorganisms of ex-INETI, Lisbon, Portugal); BY4741 (*MAT*α *his*3Δ1 *leu*2Δ0 *met*15Δ0 *ura3*Δ0) and its isogenic derivative strain *Δtdh3* (YGR192c::kanMX4); BY4742 (*MAT*α *his*3Δ1 *leu*2Δ0 *lys*2Δ0; *ura*3Δ0) and its isogenic derivative strain Δ*yca1*; S101 (Saint Georges S101, Bio Springer, France). The non-*Saccharomyces* strains used were: *L. thermotolerans* CBS 2803 (*Centraalbureau voor Schimmelcultures*, the Netherlands) and *H. guilliermondii* NCYC 2380 (National Collection of Yeast Cultures, Norwich, United Kingdom). The origin and main characteristics of these yeasts are listed in the supplementary **Table S1**.

Inocula of each yeast strain were obtained by transferring one YEPD-agar slant of each strain (pre-grown at 30 °C for 48-72 h) into 50 ml of YEPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) and incubating cultures at 30 °C and 150 rpm of agitation for 16 h.

2.2. Mixed-cell- and dialysis-tube assays

S. cerevisiae strains were first incubated in synthetic grape juice (SGJ) medium, at 25ºC and 150 rpm of agitation for 72 h (see growth curves in supplementary **Fig. S1**). The SGJ (110 g/l of glucose plus 110 g/l of fructose, pH 3.5) was prepared as described in Pérez-Nevado et al. (2006), and it was supplemented with 200 mg/l of L-leucine, 120 mg/l of L-histidine, 180 mg/l of L-methionine and 120 mg/l of uracil for strains BY4741, BY4742, *Δtdh*3 and *Δyca*1. Then, 12-h grown cells and 48 h-grown cells of the strains CCMI 885, S101 BY4741, Δ*tdh*3, BY4742, and Δ*yca1* were centrifuged, and cells washed with deionized-water and resuspended in a

carbohydrate-free medium with ethanol (SGJ without sugars with 30 g/l of ethanol). *H. guilliermondii* and *L. thermotolerans* were first incubated in YEPD (20 g/l of glucose, 20 g/l of peptone, 10 g/l of yeast extract), at 25ºC and 150 rpm of agitation, for 16 h. Then, cultures were centrifuged and cells washed with deionized-water and resuspended in the same carbohydrate-free medium.

Mixed-cell assays were performed in 250 ml Blue-Cap flasks (Duran®, Mainz, Germany), containing 245 ml of the carbohydrate-free medium with 30 g/l of ethanol to which *H. guilliermondii* and *L. thermotolerans* were added together with *S. cerevisiae* cells pre-grown for 12 h (mid-exponential phase) and 48 h (stationary phase), respectively, at a final cell density of 10^7 cells/ml of each yeast species. Each flask was fitted with a butyl stopper and a fermentation lock in tygon tubing containing 50% (v/v) sterile glycerol. Flasks were kept at 25ºC, with 150 rpm of agitation, during 30 h. Mixed-cell assays were carried out in duplicates.

Dialysis-tube assays were performed with *H. guilliermondii* and *S. cerevisiae* cells grown for 12 h (mid-exponential phase) and for 48 h (stationary phase), respectively, separated by a dialysis tube membrane in the system described by Kemsawasd *et al.* (2015). Briefly, the dialysis tube system consisted of an outer compartment, composed by a 250 ml Blue-Cap flask (Duran®, Mainz, Germany) and an inner compartment, composed by a 5 ml dialysis device made of cellulose membranes with a molecular weight cut-off of 1000 kDa (Spectra/Por® Float-A-Lyzer®, SpectrumLabs). The outer compartment contained 245 ml of the carbohydrate-free medium that was inoculated with *S. cerevisiae* at a final cell density of 10^7 cells/ml. In the inner compartment 5 ml of the carbohydrate-free medium was inoculated with *H. guilliermondii* at a final cell density of 10^7 cell/ml. Each system was fitted with a butyl stopper and a fermentation lock in tygon tubing containing 50% (v/v) sterile glycerol. Dialysis-tube assays were performed in the same conditions of the mixed-cell assays, i.e. at 25ºC, with 150 rpm of agitation, during 30 h. Dialysis-tube assays were carried out in duplicates.

In both assays, samples were taken at 0, 5, 24 and 30 h, respectively, to determine the number of colony forming units (CFU) of yeast strains. In the mixed-cell assays performed with *S. cerevisiae* and *H. guilliermondii*, CFU counts of *H. guilliermondii* were obtained on 0.01% cycloheximide YEPD-agar plates (only *H guilliermondii* grows) and CFU counts of *S. cerevisiae* as the difference between total CFU counts on YEPD-agar plates and CFU counts of *H. guilliermondii.* In the mixed-cell assays performed with mixed-cultures of *S. cerevisiae* CCMI

885 and S101 together with *L. thermotolerans*, the CFU counts were enumerated using the Wallerstein laboratory nutrient (WLN) agar (Oxoid) medium. CFU counts were performed in triplicates.

2.3. Analysis of the cell surface proteins of *S. cerevisiae*: extraction and fractionation by gel filtration chromatography

Cell surface proteins of *S. cerevisiae* cells were extracted using the protocol described by Van Leeuwen *et al.* (1991), with some modifications. This method was applied to *S. cerevisiae* cells pre-grown for 12 h (strain CCMI 885) and for 48 h (strains CCMI 885, BY4741, BY4742 and *Δtdh3*). Yeast strains were incubated in SGJ, and the same amount of cells (*ca* 10^9) was collected after 12 h and 48 h of cultivation. Cells were washed twice with ice-cold distilled water and once with buffer A (0.1 M Glycine, 0.3 M KCL, pH 7.0) and pellets were frozen with liquid nitrogen and kept at -80 ºC. 10-15 g (wet weight) of each yeast cell pellet was resuspended in 15 ml of buffer A containing 0.1 mM of phenyl-methyl-sulfonyl-fluoride (PMSF). Afterwards, cell suspension was transferred to a French press to disrupt cells, and then the suspension was centrifuged once again at 4 ºC and 2100×*g*, for 10 min. The supernatant was filtered through a Sartorius glass fiber filter with a GF/C Whatman membrane and centrifuged at 4 ºC and 2100 ×*g*, for 20 min. The pH was adjusted to 4.9 by slowly addition of 10 ml of buffer A, with constant stirring, and 0.4 ml HCl 0.1 M in order to precipitate the mitochondrial membranes. When the pH of the solution reached 4.9, cells were centrifuged immediately at 4 ºC and 2100 ×*g*, for 10 min. After that, the supernatant was transferred to a fresh centrifuge tube and the pH adjusted to 7.0 with 10 ml of buffer A and 0.4 ml KOH 1 M and kept on ice. This fraction contains both the proteins incorporated in the plasma membrane and those bound to the cell-wall (van Leeuwen et al. 1991).

The cell surface protein fractions obtained were first ultrafiltrated by centrifugal filter units equipped with 10 kDa membranes (Vivaspin 15R, Sartorius, Germany) and then the respective peptidic fractions (proteins <10 kDa) were fractionated by gel filtration chromatography using a High-Performance Liquid Chromatographic (HPLC) system (Merck Hitachi, Germany), coupled with a Superdex Peptide column (10/300 GL, GE Healthcare, London, UK) that was equilibrated and eluted with a 0.1 M ammonium acetate solution. Elution was performed at a flow rate of 0.7 ml/min and proteins detected by absorbance at 280 nm using

an UV detector (Merck Hitachi, Germany). All chromatographic fractions (see **Fig. 4**) were collected, lyophilized and stored at -20 ºC.

Cell-wall bound proteins of *S. cerevisiae* cells grown for 12 h and 48 h (of the strains CCMI 885, S101, BY4741 and *Δtdh3*) were extracted using the protocol described by Delgado *et al.* (2001). Briefly, intact cells were collected, centrifuged for 10 min at 2100 ×*g*, washed once with sterile distilled-water, and then resuspended in sterile distilled water containing 1% (v/v) of β-mercaptoethanol. The cell suspension was then incubated for 30 min at 37 °C, with shaking to release the cell-wall bound proteins. After this treatment, cells were sedimented, and the supernatant fluid was recovered, filtered by 0.22 μm Millipore membranes (Merck-Millipore, Algés, Portugal) and concentrated by freeze-drying. The cell-wall extracts were kept at -20 ºC to be further used in the enzyme-linked immunosorbent assay (ELISA).

2.4. Antimicrobial tests of gel-filtration peptidic fractions

All lyophilized gel-filtration fractions were resuspended in 100 μl of YEPD (with 30 g/l of ethanol and pH 3.5) and their antimicrobial activity tested against *H. guilliermondii*. Antimicrobial tests were performed in 96 wells-microplates, with each well filled with 100 μl of YEPD with the gel-filtration fractions, inoculated with 10^5 cells/ml of *H. guilliermondii*. Cultures were incubated in a Thermo-Shaker (Infors HT, Bottmingen, Switzerland) at 30 °C, under strong agitation (700 rpm). Control assays were performed in YEPD without the gel-filtration fractions and growth was followed by absorbance measurements at 590 nm in a Microplate Reader (Dinex Technologies Inc., Chantilly, USA). The percentage of survival of yeasts in these assays was calculated as the cell viability in each assay relative to the respective control assay (100% of survival). Samples (10 μl) were taken at 0 h, 16 h and 24 h in all assays for CFU counts. Briefly, 100 μl of culture samples were spread onto YEPD-agar plates, after appropriate dilution, and plates incubated at 25°C. CFU counts were made after 2-6 days. All tests were performed in triplicate.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The presence of the GAPDH-derived AMPs on the surface of *S. cerevisiae* cells was analysed by ELISA, using a specific polyclonal antibody against the GAPDH1 (309-321) peptide (i.e. anti-AMP1). Polyclonal rabbit antiserum raised against the AMP1 conjugate was obtained

by multiple intradermic injections into rabbits. The experiments on rabbits were carried out by GenScript Inc. Company (GenScript HK Limited, Hong Kong).

The samples analysed by ELISA were: 1) the gel-filtration fractions-I (**Fig. 4**), containing the surface proteins extracted from *S. cerevisiae* cells pre-grown for 12 h and 48 h, of the strain CCMI 885); 2) the cell-wall bound proteins directly extracted from *S. cerevisiae* cells, pre-grown for 12 and 48 h, of the strains CCMI 885, S101, BY4741 and *Δtdh*3.

First, 100 µl of samples were used for coating each well of the 96-wells microplate MICROLON® high-binding (Greiner Bio-One, Germany). Then, the 96-wells microplate was incubated overnight at 4ºC. Afterwards, 100 µl of 6 M urea was added to samples in order to denature proteins and improve protein detection by ELISA, as previously described by Hnasko *et al.* (2011). The microplate was thereafter washed 4 times using a phosphate-buffered saline (PBS)-Tween washing solution (0.05% Tween 20 in 0.01 M PBS). Then, samples were blocked during 2 h at room temperature by adding 200 µl of blocking solution containing bovine serum albumin (BSA 1%) in PBS and washed 4 times with washing solution. Next, 100 µl of the primary polyclonal rabbit antibody (GenScript HK Limited, Hong Kong) specific to the AMP1 (anti-GAPDH-1(309-321), diluted in 1% BSA to a final concentration of 10 µg/ml, was added to each well and incubated for 2 h at 37ºC. After removing the unbound material, by washing the microplate 4 times with PBS-Tween solution, the secondary antibody (anti-rabbit IgG-fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) was diluted (1.0 µg/ml in 1% BSA) and 100 µl were added to each well followed by 2 h of incubation at 37 ºC. Subsequently, the microplate was washed 4 times with PBS-Tween solution. Then, 100 µl/well of alkaline phosphatase substrate (100 mM Tris-HCL, 100 mM Nacl, 5 mM MgCl2, 1 mg/ml para-Nitrophenylphosphate (PnPP) was added to the microplate and incubated for 10 to 30 min at room temperature in the dark. The enzyme-substrate reaction was stopped by adding 100 µl of 3 N NaOH to each well. The optical density (OD) was measured at 405 nm using a microplate reader (Bio-Rad, Benchmark, USA). All samples were analysed in triplicate.

To establish the relationship between absorbance and concentration, a standard curve was constructed using the synthetic AMP1. For that, 1 mg/ml of GAPDH-1 (309-321) (AMP1), chemically-synthetized by GenScript (GenScript HK Limited, Hong Kong), was diluted to 1:32, 1:64, 1:128, 1:256, 1:512 in PBS at pH 7.2. Then, three replicates of 100 µl were taken from each diluted sample and transferred to the 96-wells microplate MICROLON® high binding. The

ELISA procedure above-described was applied to the microplate and a linear regression equation calculated using the absorbance values obtained and the respective concentration (µg/ml) of the AMP1.

2.6. Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Cell surface proteins, extracted by the Van Leeuwen *et al.* (1991) method from *S. cerevisiae* cells pre-grown for 48 h (strain CCMI 885) were resolved by two dimensional polyacrylamide gel electrophoresis (2D-PAGE). Prior to 2D-PAGE, samples were cleaned from contaminants by a precipitation method using a 2D-Clean-Up Kit (GE, Healthcare, London, UK). The obtained pellet was air-dried for 5 min and the proteins resuspended in the rehydration solution (7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer (pH 3-10), 0.002% (w/v) bromophenol blue and 0.28% (w/v) dithiothreitol (DTT). The protein concentration was determined by the Bradford method (BIO-RAD Protein assay, California, USA).

First, gel strips with 7 cm in length and linear 4-7 pH gradient (Immobiline DryStrip, GE Healthcare, London, UK) were rehydrated for 16 h with 125 µl of rehydration solution, containing 35 µg of the above-mentioned surface proteins samples. Then, the first dimension electrophoresis was run in a Ettan IPGphor III system (GE Healthcare, London, UK) using the following conditions: 200 V for 1 h; 500 V for 30 min; voltage gradient up until 1000 V for 30 min; voltage gradient up until 5000 V for 1 h 30 min; 5000 V for 1 h 30 min; for a total of 12825 V/h at 20 ˚C. After isoelectric focusing, equilibration of the strips was performed in two steps. In the first one, strips were equilibrated in equilibration buffer (6 M urea, 50 mM tris-HCl pH 8.8, 30% (v/v) glycerol, 2% (m/v) SDS and bromophenol blue) with 10 mg/ml of Dithiothreitol (DTT) for 20 min. In the second step, the procedure was repeated with 25 mg/ml of iodoacetamide instead of DTT. Both equilibration steps were performed in a rocking platform shaker (VWR International, USA).

In the second dimension electrophoresis, the strips were placed onto gradient 4-12% Bis-Tris SDS-PAGE gels (NuPAGE® NOVEX® Zoom® Protein Gels, 1.0 mm, IPG well, Life Technologies, Thermo Fisher Scientific, USA). The gels were also loaded with molecular weight markers (diluted 1:10) (Mark12™ Unstained Standard, Life Technologies, Thermo Fisher Scientific, USA). The electrophoresis was run at 150 V for 1 h 25 min in a XCell SureLock™ Mini-Cell Electrophoresis System (Life Technologies, Thermo Fisher Scientific, USA), using

MES running buffer (1 M MES, 1 M Tris Base, 69.3 mM SDS, 20.5 mM EDTA, pH 7.3 – stock solution). After SDS-PAGE electrophoresis, gels were silver stained according to the protocol described by Heukeshoven and Dernick (1985).

2.7. Peptides identification by mass spectrometry (MALDI-TOF/MS)

Spots 2 and 3 of the 2D-PAGE gel (indicated in **Fig. 5**) were manually excised and the gel pieces placed in microfuge tubes, washed, dried and digested by trypsin as described in Santos *et al.* (2007). The trypsin digested gel samples were then analyzed by Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) (Bruker–Daltonics equipped with a LIFT cell and N2 laser). Peak list and spectral processing were done in Flex-Analysis 3.0 (Bruker, Daltonics, cidade, país). Protein identification was carried out using MASCOT software (Matrix Science, London, UK) and the identified proteins were then analyzed using the IMG system program (http://img.jgi.doe.gov).

3 RESULTS

3.1. Death of non-*Saccharomyces* yeasts by cell-to-cell contact with *S. cerevisiae*

In order to investigate the factors underlying the death of non-*Saccharomyces* yeasts by cell-to-cell contact with *S. cerevisiae*, we conceived assays in which *H. guilliermondii* and *L. thermotolerans* were placed in direct contact with 12 h-grown and 48 h-grown cells of *S. cerevisiae* (mixed-cell assays), in a carbohydrate-free medium to avoid cells from producing growth metabolites, and at high cell density (10^7 cells/ml) to promote physical contact. Results (**Fig. 1**) show that when *H. guilliermondii* remained alone (**Fig. 1A**), or was placed in direct contact with 12 h-grown cells of the *S. cerevisiae* strains CCMI 885 (**Fig. 1B**), BY4741 (**Fig. 1D**) and BY4742 (**Fig. 1G**), its cell viability remained unchanged during 30 h. Conversely, when *H. guilliermondii* was placed in direct-contact with 48 h-grown cells of the same *S. cerevisiae* strains (**Fig. 1C, E** and **H**) its cell viability decreased rapidly, exhibiting just 19%, 35% and 65%, respectively, of its initial cell density after just 5 h. However, when *H. guilliermondii* was placed in direct contact with 48 h-grown cells of the *S. cerevisiae* null mutants Δ*tdh*3 (**Fig. 1F**) and Δ*yca1* (**Fig. 1I**), its cell viability remained unchanged after 5 h. Comparing survival rates of *H. guilliermondii* when placed in direct contact with 48 h-grown cells of the *S. cerevisiae* mutant

strains with the respective wild-type strains (i.e. comparing **Fig. 1E** with **F** and **Fig. 1H** with **I**), it is clear that deletion of either *TDH3* or *YCA1* genes, significantly prevents death of *H. guilliermondii*.

To cross-check that death of *H. guilliermondii* when placed in direct contact with *S. cerevisiae* cells (mixed-cell assays) is mediated just by a cell-to-cell contact mechanism and not by any toxic compound present in the medium, dialysis-tube assays were performed using the same medium and conditions of the mixed-cell assays, but with *H. guilliermondii* physically separated from *S. cerevisiae* cells by a cellulose membrane. In the dialysis-tube system yeasts cells are physically-separated but sense the same medium metabolites since these can cross the membrane (Kemsawasd et al. 2015). Results (**Fig. 2**) show that, in this situation, *H. guilliermondii* cell viability remained unchanged throughout the entire time of the assay (30 h) both for 12 h- and 48 h-grown cells of *S. cerevisiae* cells. In fact, by comparing results obtained in the mixed-cell assays with those obtained in the dialysis-tube assays (i.e. comparing **Fig. 1C** with **Fig. 2B** and **Fig. 1E** with **Fig. 2D**) we can conclude that 48 h-grown cells of *S. cerevisiae* cells induce death of *H. guilliermondii* exclusively by a cell-to-cell contact-mediated mechanism.

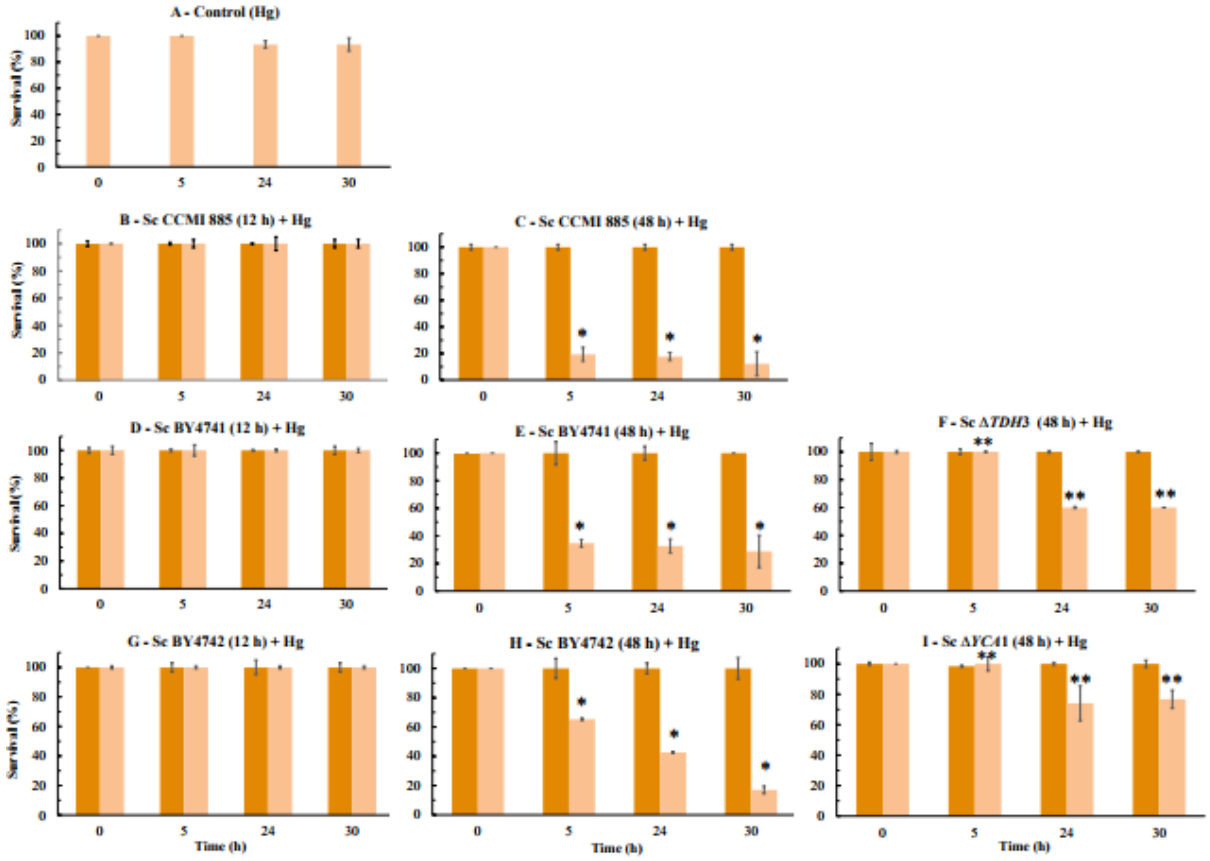


Fig. 1: Percentage of survival (relative to the initial cell density) of *S. cerevisiae* (dark orange bars) and *H. guilliermondii* (light orange bars) during mixed-cell assays performed in a carbon-free medium, at high cell density (10^7 cells/ml). Assays were performed with *H. guilliermondii* (Hg) cells alone (A), with *H. guilliermondii* cells mixed with 12 h-grown (B,D,G) and 48 h-grown (C,E,H) cells of the *S. cerevisiae* strains CCMI 885 (B,C), BY4741 (D,E) and BY4742 (G,H), and with H. guilliermondii cells mixed with 48 h-grown cells of the *S. cerevisiae* mutants *Δtdh3* (F) and *Δyca1* (I). Values represented are means of triplicate measurements ± SD (error bars) of two independent biological experiments. Data obtained for each strain in the different mixed-cell assays was analysed by ANOVA.*, statistically different values ($P<0.05$) between the assays performed with 48 h-grown and 12 h-grown cells of each *S. cerevisiae* strain; **, statistically different values ($P<0.05$) between assays performed with 48 h-grown cells of the *S. cerevisiae* mutant strains *(Δtdh3* and *Δyca1*) and with 48 h-grown cells of the respective wild-type strains (BY4741 and BY4742).

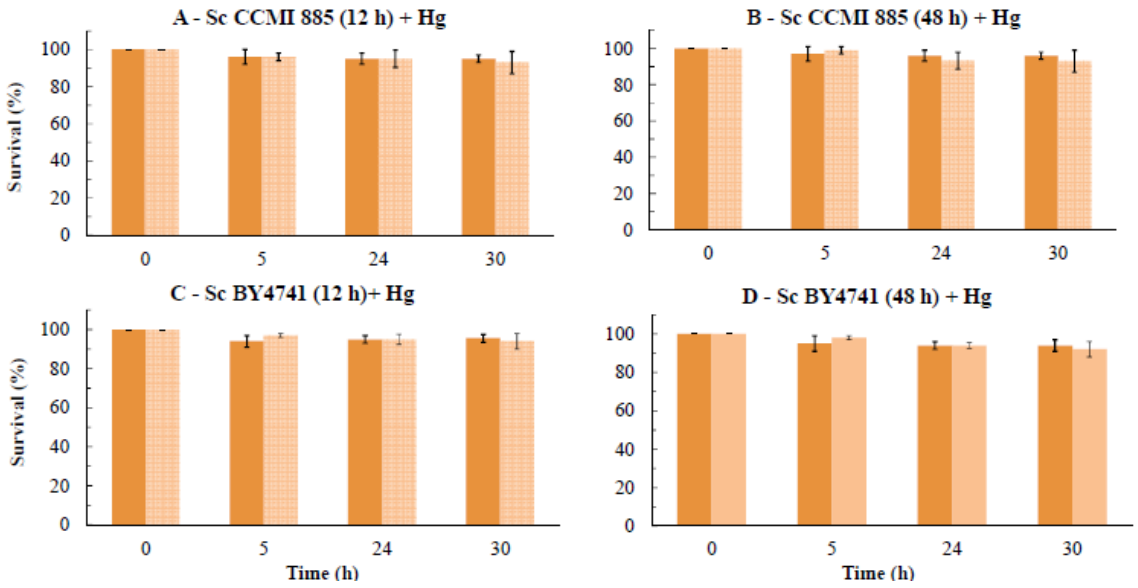


Fig. 2: Percentage of survival (relative to initial cell density) of *S. cerevisiae* (dark orange bars) and *H. guilliermondii* (light orange bars) during the dialysis-tube assays performed in a carbon-free medium, at high cell density (10^7 cells/ml). Assays were performed with *H. guilliermondii* cells separated by a cellulose membrane from *S. cerevisiae* cells pre-grown for 12 h (A,C) and for 48 h (B,D) of the strains CCMI 885 (A,B) and BY4741 (C,D), respectively. Values represented are means of triplicate measurements ±SD (error bars) of two independent biological experiments. Data obtained for each strain in the dialysis-tube assays performed with 12 h-grown and 48 h-grown cells of each *S. cerevisiae* strain was analysed by ANOVA and all values were found to be non-statistically different (P>0.05).

Kemsawasd *et al.* (2015), using the same dialysis-tube system but a different medium (SGJ with 200 g/l of sugars), had previously demonstrated that *S. cerevisiae* (strain S101) induces death of *L. thermotolerans* by direct cell-to-cell contact. In the present work, we wanted to check-out if 48 h-grown cells of *S. cerevisiae* would induce death of *L. thermotolerans* by direct cell-to-cell contact, while 12 h-grown cells would not, as it happened with *H. guilliermondii.* Therefore, we performed mixed-cell assays with *L. thermotolerans* in direct contact with *S. cerevisiae* cells (strains CCMI 885 and S101) pre-grown for 12 h and 48 h, respectively, in the carbohydrate-free medium. Results (**Fig. 3**) showed that *L. thermotolerans*

was able to keep its cell viability unchanged during 30 h when it remained alone (**Fig. 3A**) and also when it was placed in direct contact with *S. cerevisiae* cells pre-grown for 12 h of both strains (**Fig. 3B** and **D**). Quite the reverse, when placed in direct contact with *S. cerevisiae* cells pre-grown for 48 h of both strains, *L. thermotolerans* lost 65% and 52%, respectively, of its initial cell viability after 30 h (**Fig. 3C** and **E**).

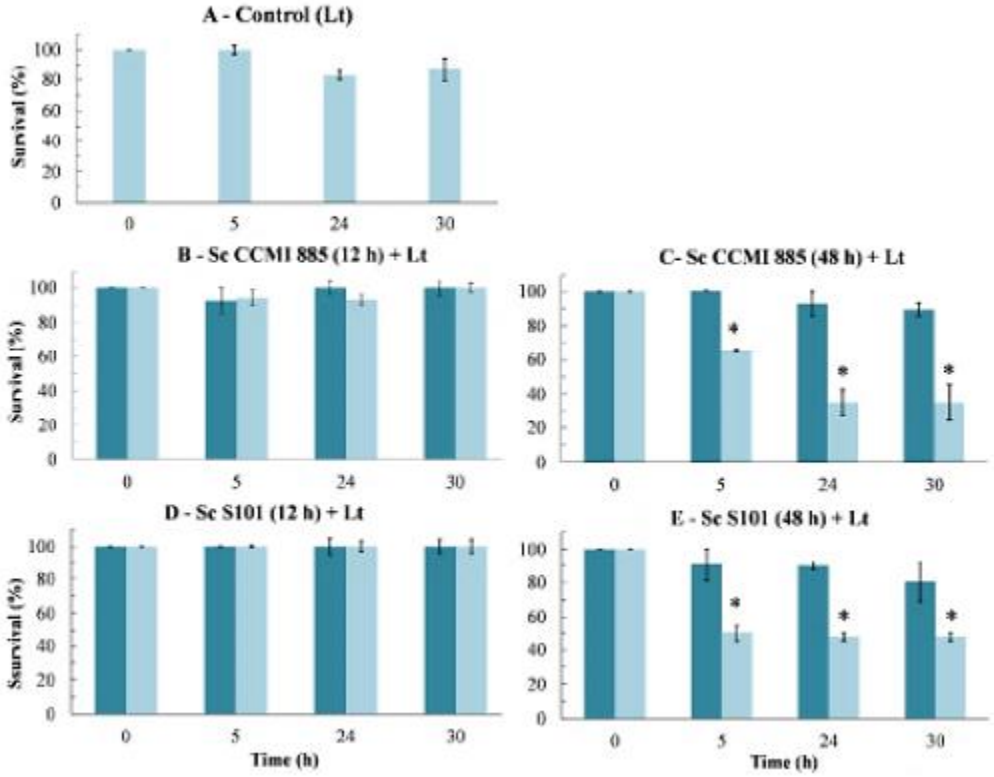


Fig. 3: Percentage of survival (relative to initial cell density) of *S. cerevisiae* (dark blue bars) and *L. thermotolerans* (light blue bars) during the mixed-cell assays performed in a carbon-free medium, at high cell density (10^7 cells/ml). Assays were performed with *L. thermotolerans (Lt)* cells alone (A), and with *L. thermotolerans* mixed 12 h-grown (B,D) and 48 h-grown (C,E) cells of the *S. cerevisiae* (Sc) strains CCMI 885 (B,C) and S101 (D,E). Values represented are means of triplicate measurements ±SD (error bars) of two independent biological experiments. Data obtained for each strain in the different mixed-cell assays was analysed by ANOVA. *, statistically different values ($P<0.05$) between the assays performed with 48 h-grown and 12 h-grown cells of each *S. cerevisiae* strain.

3.2. Analysis of cells surface proteins of *S. cerevisiae*: extraction and fractionation by gel filtration chromatography

In view of the results obtained in the mixed-cell and dialysis-tube assays (**Figs. 1**-**3**), which show that 48 h-grown cells of *S. cerevisiae* induce death of non-*Saccharomyces* yeasts by cell-to-cell contact while 12-h grown cells do not, we extracted the surface proteins of those cells and analysed them by gel-filtration chromatography. It should be mentioned here that the surface proteins were extracted using the protocol described by Van Leeuwen *et al.* (1991), which proved to effectively separate mitochondrial from plasma membranes.

The surface proteins extracted (containing both the proteins incorporated in the plasma membranes and those bound to the cell-wall) were first ultrafiltrated and, then, fractionated by gel filtration chromatography (**Fig. 4**). All strains exhibited similar chromatographic profiles, showing one peak at about 25 min (fraction-I indicated in **Fig. 4B**) in all chromatograms. The retention time of this peak (25 min) is close to that of the gel-filtration fraction from where the GAPDH-derived AMPs were previously identified by Branco *et al.* (2014) in fermentation supernatants. Moreover, the intensity of fraction-I is much lower in *S. cerevisiae* (strain CCMI 885) cells pre-grown for 12 h (**Fig. 4A**) than in cells pre-grown for 48 h (**Fig. 4B**). Also, the intensity of fraction-I is much lower in *S. cerevisiae* cells pre-grown for 48 h of the mutant strains *Δtdh3* and *Δyca1* (**Fig. 4C**,**E**) than that in the respective wild-type strains (**Fig. 4D**,**F**).

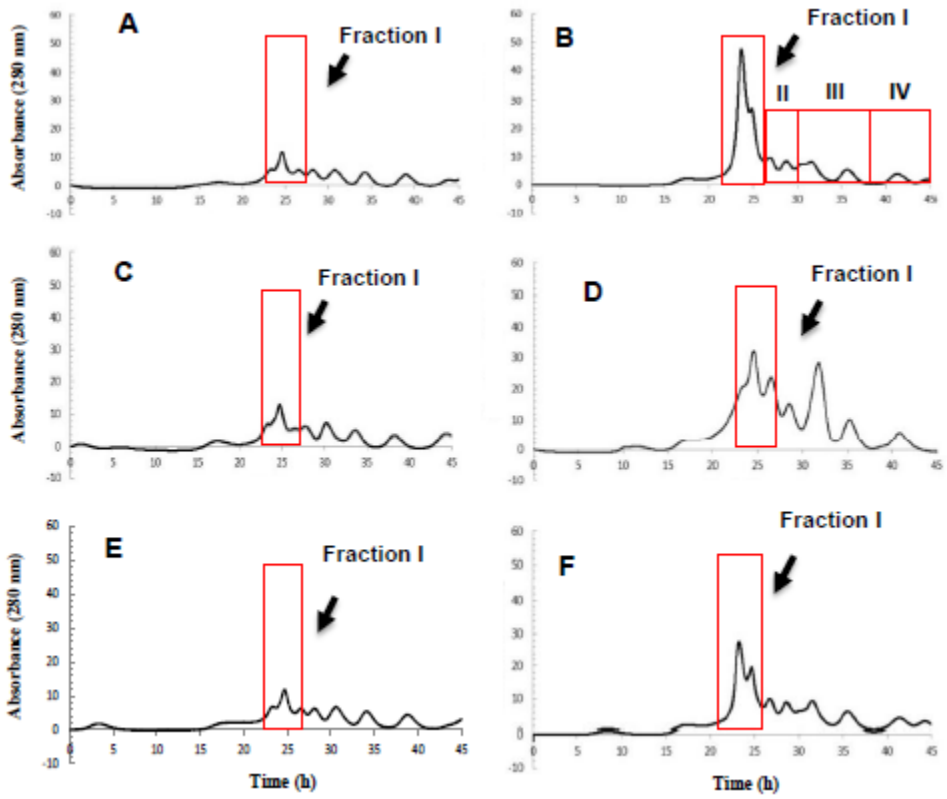


Fig. 4: Gel-filtration chromatography of surface proteins (fractions <10 kDa) extracted from: *S. cerevisiae* CCMI 885 cells pre-grown for 12 h (**A**) and for 48 h (**B**), respectively; *S. cerevisiae* cells pre-grown for 48 h of the BY4741 mutant *Δtdh3* (**C**) and the respective wild-type strain (**D**); *S. cerevisiae* cells pre-grown for 48 h of the BY4742 mutant *Δyca1* (**E**) and the respective wild-type strain (**F**). Fraction-I highlighted in all graphs was found to contain the GAPDH-derived AMPs.

S. cerevisiae cells that induced death of the non-*Saccharomyces* yeasts by cell-to-cell contact (i.e., 48 h-grown cells of the strains CCMI 885, BY4741 and BY4742) (**Fig. 1B**,**E**,**H**) also exhibited higher amounts of fraction-I on their surface (**Fig. 4B**,**D**,**F**). Thus, fraction-I was a good candidate to contain the bioactive peptides. Nevertheless, the antimicrobial activity of all gel-filtration fractions (i.e. fractions I, II, II and IV) was tested against *H. guilliermondii*. Results (supplementary **Table S2**) showed that only fraction-I exhibited significant antimicrobial activity against *H. guilliermondii*. Indeed, gel-filtration fractions-I obtained from *S. cerevisiae* cells pre-

grown for 48 h of the strains CCMI 885, BY4741 and BY4742 inhibited the growth of *H. guilliermondii* by 68 %, 51% and 75, respectively (supplementary **Table S2**). Conversely, gel-filtration fraction-I obtained from 12 h-grown cells of *S. cerevisiae* CCMI 885, as well as from 48 h-grown cells of the mutant strains Δ*tdh3* and Δ*yca1* were only able to inhibit the growth of *H. guilliermondii* by 12%, 25% and 20%, respectively (supplementary **Table S2**).

Antimicrobial tests confirmed that *S. cerevisiae* pre-grown for 48 h contains AMPs on their cells surface, which induce death of *H. guilliermondii*. Furthermore, our results also indicate that the *TDH3* and *YCA1* genes are involved in the accumulation of those AMPs on the surface of *S. cerevisiae* cells, since the gel-filtration fractions-I isolated from the surface of *S. cerevisiae* cells pre-grown for 48 h of the null mutants Δ*tdh3* and Δ*yca1* induced just a slight inhibition of *H. guilliermondii* growth (supplementary **Table S2**) by comparison with the respective wild-type strains (i.e., 25% and 20% compares with 51% and 75%, respectively).

3.4. Identification of GAPDH-derived AMPs on the cell surface of *S. cerevisiae*

3.4.1 Enzyme-linked immunosorbent assay (ELISA)

The bioactive gel-filtration fraction-I obtained by fractionation of the surface proteins extracted from *S. cerevisiae* (strain CCMI 885) cells pre-grown for 12 h and for 48 h, respectively, were analysed by enzyme-linked immunosorbent assays (ELISA) using a specific polyclonal antibody raised against the GAPDH-derived AMPs. Immunological tests confirmed the presence of the GAPDH-derived AMPs in both gel-filtration fractions, but with higher concentration in 48 h-grown cells (15.7±0.4 µg/ml) than in 12 h-grown cells (6.8±0.3 µg/ml). These results demonstrated that *S. cerevisiae* cells accumulate the GAPDH-derived AMPs on their membranes during growth, i.e. from mid-exponential (12 h) to stationary phase (48 h).

Given that the above-mentioned samples contained both the proteins incorporated in the plasma membrane and those loosely bound to the cell-wall, we also performed ELISA tests with samples containing just the cell-wall bound proteins of *S. cerevisiae* cells pre-grown for 12 h, and for 48 h, of the strains CCMI 885, BY4741, S101 and Δ*tdh*3. It should be mentioned here that the cell-wall proteins were solubilized from intact cells using the β-mercaptoethanol treatment, a method that releases yeast cell-wall molecules without significant intracellular contamination (Chaffin *et al.* 1998). Results (**Table 1**) showed that the GAPDH-derived AMPs

are present in the cell-wall of the *S. cerevisiae* strains CCMI 885, BY4741 and S101 pre-grown for 48 h, but were not detectable in the cell-wall of the same strains pre-grown for 12 h. Moreover, the AMPs were also not detectable in the cell-wall of the *S. cerevisiae* mutant Δ*tdh*3 pre-grown for 48 h.

3.4.2 Two dimensional polyacrylamide gel electrophoresis (2D-PAGE)

To further confirm the identification of the GAPDH-derived AMPs, surface proteins of *S. cerevisiae* (strain CCMI 885) cells pre-grown for 48 h were resolved by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The proteome obtained (**Fig. 5**) revealed an intense spot (spot 1 indicated in Fig. 5) at a molecular weight (MW) close to 36 kDa and an isoelectric point (p*I*) ranging from 6.5-7.0, which probably contains the GAPDH protein. In fact, GAPDH is a cell-wall associated protein in *S. cerevisiae* (Delgado *et al.* 2001) whose MW is 36 kDa and the p*I* ranges from 6.59-6.98, depending of its isoforms. Moreover, the 2D-gel also exhibits two other intense spots (spots 2 and 3 indicated in **Fig. 5**) that fall in the range of 8-10 kDa for the MW and of 4.0-4.5 for the p*I*. Since the GAPDH-derived AMPs identified by Branco *et al.* (2014) have an apparent MW of 8.0 kDa and a p*I* of 4.37, spots 2 and 3 were manually excised from the 2D-gel and further analysed by mass spectrometry. The peptides identified by mass spectrometry (MALDI-TOF/ MS) are listed in supplementary **Table S3** and confirmed the presence in both spots of peptides derived from the GAPDH isoenzyme-1, namely one peptide exhibiting exactly the same amino acid sequence of the AMP1 (ISWYDNEYGYSAR).

Table 1: Concentration (μg/ml) of the GAPDH-derived AMPs determined by ELISA in the cell-wall extracts of different *S. cerevisiae* strains pre-grown for 12 h and 48 h. Values presented are means (±SD) of triplicates.

Strains	GAPDH-derived AMPs [μg/ml]	
	12 h-grown cells	48 h-grown cells
CCMI 885	nd*	0.43±0.07
S101	nd*	0.26±0.05
BY4741	nd*	0.21±0.09
Δ*tdh*3	nd*	nd*

* Non-detectable

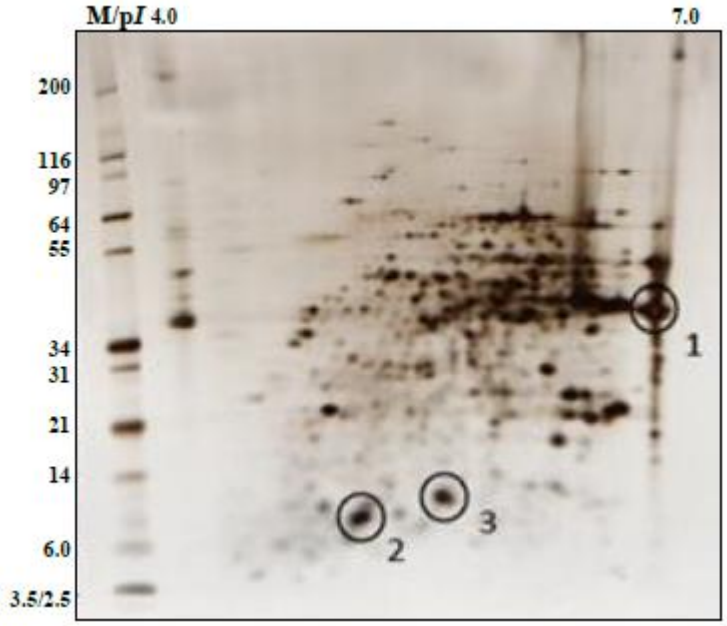


Fig. 5: Proteome of the surface proteins extracted from *S. cerevisiae* cells pre-grown for 48 h, resolved by 2D-PAGE. M: Molecular weight markers (kDa); p*I*: isoelectric point of the strip (linear 4-7 pH gradient); Spots 2 and 3 were excised from the gel, and peptides identified by mass spectrometry.

4. DISCUSSION

We had previously reported that different *S. cerevisiae* strains secrete AMPs derived from the glycolytic enzyme GAPDH during wine fermentations that are active against several yeasts and bacteria (Albergaria and Arneborg 2016; Branco *et al.* 2017). Besides, we also demonstrated that death of *L. thermotolerans* in mixed-culture fermentations with *S. cerevisiae* is due to the combined effect of cell-to-cell contact and GAPDH-derived AMPs (Kemsawasd *et al.* 2015). However, the mechanisms underlying the death of non-*Saccharomyces* yeasts by cell-to-cell contact with *S. cerevisiae* have remained unknown.

In the present work, we demonstrate that 48 h-grown, conversely to 12 h-grown, cells of *S. cerevisiae* induce death of *L. thermotolerans* and *H. guilliermondii* by direct cell-to-cell contact (Figs. 1-3). These results are in agreement with previous reports (Nissen and Arneborg 2003; Nissen, Nielsen and Arneborg 2003; Renault, Albertin and Bely 2013; Kemsawasd *et al.*

2015) claiming that death of *L. thermotolerans* and *T. delbrueckii* during mixed-culture fermentations with *S. cerevisiae* is mediated by a cell-to-cell contact mechanism. They also agree with the fact that non-*Saccharomyces* yeasts begin to die-off during wine fermentation only after 24-48 h, i.e. after cells attain the stationary growth phase (Nissen and Arneborg 2003; Pérez-Nevado *et al.* 2006; Xufre *et al.* 2006).

Furthermore, *S. cerevisiae* starts to excrete the GAPDH-derived AMPs into the extracellular medium at the end of the exponential growth phase (Albergaria *et al.* 2010; Branco *et al.* 2014, 2017). In *S. cerevisiae*, the *TDH3* gene codifies the synthesis of GAPDH (McAlister and Holland 1985) and the *YCA1* gene codifies the synthesis of metacaspase who cleaves the GAPDH in apoptotic cells (Silva *et al.* 2011). Interestingly, our results show that *S. cerevisiae* cells pre-grown for 48 h of the null mutants Δ*tdh*3 and *Δyca*1 did not trigger death of the non-*Saccharomyces* yeasts, conversely to the respective wild-type strains. On the other hand, GAPDH is a cell-wall-associated protein in *S. cerevisiae* (Delgado *et al.* 2001). Thus, surface proteins of several *S. cerevisiae* cells were extracted and analysed by ELISA, using a specific polyclonal antibody against the GAPDH-derived AMPs secreted by *S. cerevisiae* and identified by Branco *et al.* (2014). Immunological tests confirmed the presence of the GAPDH-derived AMPs on the cell-wall of *S. cerevisiae* cells pre-grown for 48 h, but not for 12 h (**Table 1**). Besides, the GAPDH-derived AMPs were also identified by mass spectrometry in the surface proteome of *S. cerevisiae* cells pre-grown for 48 h (**Fig. 5** and supplementary **Table S3**). In summary, our work shows that accumulation of GAPDH-derived AMPs on the cell surface of *S. cerevisiae* is one of the factors that triggers death of non-*Saccharomyces* yeasts by cell-to-cell contact. Moreover, several *S. cerevisiae* strains (i.e., CCMI 885, S101, BY474 and BY4742) that induced death of *H. guilliermondii* and *L. thermotolerans* by cell-to-cell contact also accumulated the GAPDH-derived AMPs on their cells surface, what seems to indicate that this is a more general phenomenon of the *S. cerevisiae* species.

The involvement of GAPDH-derived peptides in the antagonism exerted by *S. cerevisiae* against other microbial species highlights the multifunction feature of this protein (Sirover 2011). Indeed, GAPDH is the prototype of a "moonlighting" protein that exhibits several other activities beside its glycolysis role (Sirover 2011). Those activities include a primary role in apoptosis and in a variety of critical nuclear pathways that occur in different subcellular locations, including membrane, cytosol and nucleus (Sirover 2005; Nakajima *et al.* 2009; Silva *et*

al. 2011). Moreover, GAPDH has been found on the cell surface of *Streptococcus pyogenes* (Pancholi and Fischetti 1992) *Kluyveromyces marxianus* (Fernandes *et al.* 1992), *Candida albicans* (Gil-Navarro *et al.* 1997) and *S. cerevisiae* (Delgado *et al.* 2001; Delgado, Gil and Gozalbo 2003). Fernandes *et al.* (1992) found that, in *Kluyveromyces marxianus*, an up-shift of the growth temperature from 26 to 40 ºC induces flocculation of cells and the accumulation of GAPDH in the cell wall. In *S. cerevisiae*, GAPDH was found to accumulate in the cell-wall in response to starvation and temperature upshift (Delgado, Gil and Gozalbo 2003), although its function has remained unknown. In the present work we show that the death of non-*Saccharomyces* yeasts by direct contact with *S. cerevisiae* stationary-grown cells is related with the accumulation of GAPDH-derived AMPs on their surface. Therefore, our work unveiled at least one of the functions of the GAPDH accumulation in the cell-wall of *S. cerevisiae*, although we do not know yet how the GAPDH is transported to the cell-wall, nor what triggers the production of the GAPDH-derived AMPs.

Silva *et al.* (2011) reported that, in yeast, GAPDH is a specific substrate of metacaspases and that the in vivo cleavage of GAPDH in apoptotic cells originates several GAPDH-derived fragments, namely some fragments equal to the AMPs identified by Branco *et al.* (2014). In the present study, we found that 48 h-grown cells of the *S. cerevisiae* mutant deleted in the metacaspase gene (Δ*yca*1) did not induce the death of non-*Saccharomyces* yeasts, conversely to its wild-type strain. These results suggest that accumulation of GAPDH-derived peptides on the cell surface of *S. cerevisiae* at the end of the exponential growth phase might result from the cleavage of GAPDH by metacaspases in apoptotic cells. In case this hypothesis is confirmed, the existence of a programmed-cell death process in yeast could gain a new meaning: that of producing defensive weapons for the microbial population.

In conclusion, our work shows that death of wine-related non-*Saccharomyces* yeasts by direct contact with stationary-grown cells of *S. cerevisiae* is mediated, at least partly, by the accumulation of GAPDH-derived AMPs on their surface.

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SUPPLEMENTARY DATA

Supplementary Table S1: Origin and characteristics of yeast strains used in the present work

Yeast strains	Origin	Type of strain/Specific mutations
S. cerevisiae CCMI 885	isolated from Alentejo wines, Portugal	Wild yeast
S. cerevisiae S101	isolated from Beaujolais wines, France	Wild yeast
S. cerevisiae BY4741	isogenic derivative strain from *S. cerevisiae* S288C (Winston et al., 1995)	laboratory strain (*MAT*α *his3*Δ1 *leu2*Δ0 *met*15Δ0 *ura3*Δ0)
S. cerevisiae Δtdh3	isogenic derivative strain from *S. cerevisiae* BY4741 (Winzeler et al., 1999)	laboratory strain deleted in the *TDH3* gene that codifies for the synthesis of the GAPDH3 isoenzyme
S. cerevisiae BY4742	isogenic derivative strain from *S. cerevisiae* S288C **(**Winston et al., 1995**)**	laboratory strain (*MAT*α *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0)
S. cerevisiae yca1	isogenic derivative strain from *S. cerevisiae* BY4742 (Winzeler et al., 1999)	laboratory strain deleted in the *YCA1* gene, which codifies the apoptosis-involved enzyme metacaspase
L. thermotolerans CBS 2803	isolated from grapes, Verona, Italy	Wild yeast
H. guilliermondii NCYC 2380	isolated from Douro wines, Portugal	Wild yeast

Winzeler EA, Shoemaker DD, Astromoff A, Liang H, *et al.* Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. Science 1999; 6:901-906.

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Supplementary Table S2: Percentage of *H. guilliermondii* growth inhibition (relative to control) by the gel-filtration fractions (indicated in **Fig. 4**) obtained by fractionation of the surface proteins extracted from *S. cerevisiae* strains cultivated during 12 h (strain CCMI 885) and 48 h (all strains).

Values correspond to means (±SD) of triplicate growth assays.

		Growth inhibition of *H. guilliermondii* (%)			
***S. cerevisiae* strains**	fractions from cells grown for	**fraction I**	**fraction II**	**fraction III**	**fraction IV**
CCMI 885	12 h	12 (±2)	3.0 (±0.2)	10(±1)	2.4 (±0.4)
	48 h	68 (±2)	2.2 (±0.1)	0 (±0)	0 (±0)
BY4741	48 h	51 (±3)	5.0 (±0.2)	11.2(±0.5)	2.6 (±0.5)
Δ*tdh*3	48 h	25 (±5)	3.3 ±(0.3)	4.5 (±0.6)	4.5 (±0.4)
BY4742	48 h	75 (±2)	4.4 (±0.4)	12(±1)	8 (±1)
Δ*yca*1	48 h	20 (±3)	3.4 (±0.2)	10(±2)	9 (±1)

Supplementary Table S3: Primary structure (amino acid sequence) of the peptides present in spots 2 and 3 that were excised from the 2D-PAGE gel (Fig. 5) and identified by MALDI-TOFMS.

Gel spots	Protein Hits	Nominal Mass (M_r)	Protein Score	Sequence Coverage (%)	Identified peptides
Spot 2	Glyceraldehyde-3-phosphate dehydrogenase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	35728	43	17	-.MIRIAINGFGR.I, R.VPTVDVSVVDLTVK.L, K.IVSNASCTTNCLAPLAK.V, K.LISWYDNEYGYSAR.V, K.KIVSNASCTTNCLAPLAK.V
Spot 3	Pyruvate decarboxylase isozyme 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	61457	92	11	K.LTAATNAKQ.-, K.LIDLTQFPAFVTPMGK.G, R.WAGNANELNAAYAADGYAR.I, K.NPVILADACCSRHDVKAETK.K
Spot 3	Pyruvate decarboxylase isozyme 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	61873	92	10	K.NPVILADACASRHDVK.A, R.WAGNANELNAAYAADGYAR.I, K.STPANTPMKQEWMWNHLGNFLR.E
Spot 3	Pyruvate decarboxylase isozyme 3 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	61542	92	9	K.FALQNLLK.V, K.NPVILSDACASRHNVK.K, R.WAGNANELNAAYAADGYAR.I, K.VKNATFLGVQMKFALQNLLK.V
Spot 3	Glyceraldehyde-3-phosphate dehydrogenase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	35728	68	17	R.VPTVDVSVVDLTVK.L, K.ELDTAQKHIDAGAK.K, K.LISWYDNEYGYSAR.V, K.IVSNASCTTNCLAPLAK.V

Protein score is -10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 52 are statistically significant ($p<0.05$).

Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits

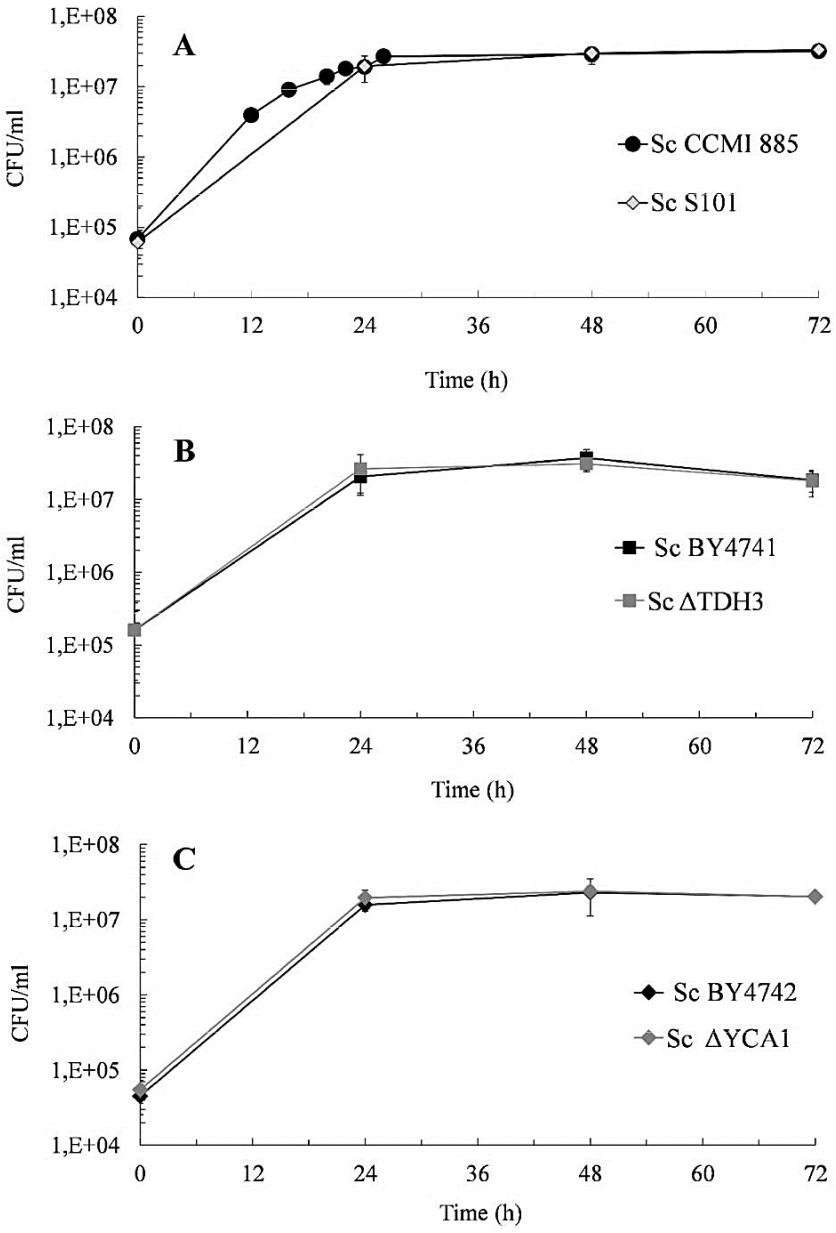

Fig. S1: Growth curves (CFU/ml) of *S. cerevisiae* strains (A-CCMI 885 and S101; B-BY4741 and *TDH3*; C-BY4742 and *YCA1*) during synthetic grape juice (pH 3.5) fermentations, performed at 25ºC and 150 rpm of agitation. Values correspond to means ±SD (errors bars) of two independent experiments.

The results presented in this Chapter are published in:

Peer-Reviewed Journal

Branco P, Kemsawasd V, Santos L, Diniz M, Caldeira J, Almeida MG, Arneborg N, Albergaria H. (2017) Accumulation of GAPDH-derived peptides on the surface of *Saccharomyces cerevisiae* cells induces death of non-*Saccharomyces* yeasts by cell-to-cell contact. FEMS Microbiol Ecol 93:1-10. doi: 10.1093/femsec/fix055.

Oral presentation

Branco P, Kemsawasd V, Santos L, Diniz M, Caldeira J, Almeida MG, Arneborg N, Albergaria H (2016) Death of non-*Saccharomyces* yeasts induced by cell-cell contact with *Saccharomyces cerevisiae*: role of the cell wall-associated GAPDH protein 6th Conference on Physiology of Yeast and Filamentous Fungi (PYFF6), *Instituto Superior Técnico*, University of Lisbon, July 11-14, Lisbon, Portugal.

Branco P, Kemsawasd V, Arneborg N, Albergaria H (2014) Cell-cell interactions in yeast cultures at high cell density: role of the *Saccharomyces cerevisiae* cell-wall associated protein GAPDH and physiological alterations induced in sensitive yeast cells. XX Jornadas das Leveduras Professor Nicolau Van Uden, *Instituto Superior Técnico*, University of Lisbon, July 11-12, Lisbon, Portugal.

Poster presentation

Branco P, Kemsawasd V, Arneborg N, Albergaria H (2013) Interactions between *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii*: cell-cell contact mechanism. MicroBiotec'13, December 6-8, *Aveiro*, Portugal (work selected for "flash presentation").

Chapter VI

Effect of GAPDH-derived antimicrobial peptides on sensitive yeast cells: plasma membrane permeability, intracellular pH and H^+-influx/-efflux

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ABSTRACT

Saccharomyces cerevisiae secretes antimicrobial peptides (AMPs) derived from glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which induce death of several non-*Saccharomyces* yeasts. Previously, we demonstrated that the naturally-secreted GAPDH-derived AMPs (i.e., saccharomycin) caused a loss of culturability and decreased the intracellular pH (pHi) of *Hanseniaspora guilliermondii* cells. In this study, we show that chemically-synthesized analogues of saccharomycin also induce a pHi drop and loss of culturability in *H. guilliermondii*, although to a lesser extent than saccharomycin. To assess the underlying causes of the pHi drop, we evaluated the membrane permeability to H^+ cations of *H. guilliermondii* cells, after being exposed to saccharomycin or its synthetic analogues. Results showed that the H^+-efflux decreased by 75.6% and the H^+-influx increased by 66.5% in cells exposed to saccharomycin at pH 3.5. Since H^+-efflux via H^+-ATPase is energy-dependent, reduced glucose consumption would decrease ATP production and consequently H^+-ATPase activity. Glucose uptake rates in cells exposed to saccharomycin or to its synthetic analogues were, however, not affected, suggesting that the AMPs rather than affecting glucose transporters affect plasma membrane H^+-ATPase. Thus, our study revealed that both saccharomycin and its synthetic analogues induced cell death of *H. guilliermondii* by increasing the proton influx and inhibiting the proton efflux.

Keywords: pH homeostasis, proton fluxes, H^+-ATPase, glucose transporters; saccharomycin; *Saccharomyces cerevisiae*

1. INTRODUCTION

Saccharomyces cerevisiae secretes antimicrobial peptides (AMPs) that induce death of several wine-related non-*Saccharomyces* yeasts, as a strategy to combat those microbial species during alcoholic fermentations (Albergaria et al., 2010; Albergaria and Arneborg, 2016; Branco et al., 2014, 2017a). The natural biocide (i.e. saccharomycin) is composed by two main anionic (isoelectric point of 4.37) peptides (AMP1 and AMP2/3), derived from the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Branco et al., 2014).

AMPs in nature are important defensive weapons that exhibit rapid and efficient toxicity against a wide range of microorganisms (Ganz and Lehrer, 1999). In higher eukaryotes, AMPs are generally located at sites exposed to microbial invasion, such as the epithelia of mammals, amphibians and insects (Harris et al., 2009). Interestingly, we recently found that the GAPDH-derived AMPs secreted by *S. cerevisiae* accumulate on its cells surface, inducing death of other competitor species by direct cell-to-cell contact (Branco et al. 2017b). This means that they share a common characteristic with AMPs from higher eukaryotes, i.e. they accumulate in cells tissues that are in direct contact with external environment. The majority of natural AMPs are cationic (Marshall et al., 2003). Nevertheless, a significant number of anionic AMPs have been found which are involved in the innate immune response of eukaryotic cells (Harris et al., 2009).

Most AMPs exert their microbicide effect via disruption of the cell membrane (Brogden, 2005; Harris et al., 2009; Yeaman and Yount, 2003). However, membrane damage is only one of many mechanisms that AMPs possess to induce death of microbial cells. Several studies (Brogden, 2005; Straus and Hancock, 2006) reported that certain AMPs interact with intracellular targets such as DNA, RNA and proteins, inhibiting their synthesis. In addition, AMPs action can be influenced by external factors such as osmolarity, temperature, external pH (e.g. kappacins are strongly membranolytic under acidic pH) (Dashper et al., 2005; Yeaman and Yount, 2003), or the presence of divalent metal cations. For instance, the effect of dermcidin-derived peptide DCD-1L, an anionic AMP from human eccrine sweat, is influenced by Zn^{2+}, which stabilizes the interaction of oligomeric complexes of those peptides with the lipid bilayers of the target cell membranes (Paulmann et al., 2012).

Even though microbial efflux pumps are energy-dependent transporters that extrude toxic compounds, including antibiotics (Piddock, 2006), a recent study showed that lactoferrin and transferrin from human blood and mucosal surface inhibit the ATPase complex in *Pseudomonas*

aeruginosa and *Lactococcus lactis*. As a result, the H^+-ATPase-mediated flux of protons is compromised leading to deficiencies in intracellular pH homeostasis and consequently resulting in cell death (Andrés and Fierro, 2010).

Yeast plasma membrane H^+-ATPase is a membrane enzyme that plays an essential role in the physiology of yeast; its physiological function is to pump protons out of the cell. This enzyme hydrolyses much of the ATP generated by cells that originates an electrochemical gradient of protons (Portillo and Serrano, 1989). The activity of plasma membrane H^+-ATPase can be correlated with the proton efflux and is implicated in the intracellular pH homeostasis (Opekarova and Sigler, 1982). The intracellular pH (pHi) of yeasts can be determined by the fluorescence ratio imaging microscopy (FRIM) method, which is a technique that measures the pHi of cells by using fluorescent pH sensitive probes (Arneborg et al., 2000; Siegumfeldt et al., 1999). This technique, based on the linear response between the ratiometric intensity of fluorescence emitted by the probe and the pHi of cells, gives information at the single-cell level, which allows determining the pHi of different subpopulations of cells simultaneously.

In a previous work (Branco et al., 2015) we showed that the natural GAPDH-derived AMPs secreted by *S. cerevisiae* (i.e., saccharomycin) kill *H. guilliermondii* by affecting the pHi and membrane permeability of cells. The aim of the present study was to evaluate if chemically-synthesized analogues of saccharomycin (synthetic AMPs) induce similar physiological changes in *H. guilliermondii*. Additionally, we investigated if the drop of pHi to a deadly value, previously observed in *H. guilliermondii* cells exposed to saccharomycin (Branco et al., 2015), is exclusively due to an increase of cell membrane permeability, what in an acidic medium as in grape musts (pH 3.5) would increase the H^+-influx, or if saccharomycin also affects the efflux of protons via H^+-ATPase. Since H^+-ATPase is an energy-dependent transporter, the putative effect of ATP decrease due to glucose uptake disturbance or sugars depletion was also evaluated, in the absence/presence of the AMPs.

2. MATERIALS AND METHODS

2.1 Strains and growth conditions

In this work, we used the following yeast strains: *Saccharomyces cerevisiae* CCMI 885 (Culture Collection of Industrial Microorganisms, LNEG, Portugal) and *Hanseniaspora guilliermondii* NCYC 2380 (National Collection of Yeast Cultures, Norwich, United Kingdom). Strains were maintained in YEPD-agar slants (20 g/l glucose, 20 g/l peptone, 10 g/l yeast extract and 20 g/l agar, pH 6) and stored at 4 º C. Inoculums of *S. cerevisiae* and *H. guilliermondii* were obtained by transferring one YEPD-agar slant of each strain (pre-grown at 30 °C for 48–72 h) into 100 ml of YEPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose) and incubating cultures at 30 °C with 150 rpm of agitation during 16 h.

2.2 Purification of saccharomycin by gel filtration chromatography

The naturally-secreted GAPDH-derived AMPs (i.e. saccharomycin) were purified from supernatants of a synthetic grape juice (SGJ) fermentation performed with *S. cerevisiae* CCMI 885, at 25 ºC, for 7 days. The SGJ (pH 3.5) contained 220 g/l of sugars (110 g/l of glucose and 110 g/l of fructose), and was prepared as described in Pérez-Nevado et al. (2006). First, the cell-free fermentation supernatant (filtration by 0.22 μm Millipore membranes) was ultrafiltrated through centrifugal filter units (Vivaspin 15R, Sartorius, Germany) equipped with 10 kDa membranes and concentrated (10-fold) with 2 kDa membranes. Then, 100 μl of this (2-10) kDa peptidic fraction was fractionated by gel filtration chromatography, using a Superdex-Peptide column (10/300 GL, GE Healthcare, UK) coupled to an HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with an UV detector (Merck Hitachi, Darmstadt, Germany), and eluted with ammonium acetate 0.1 M at a flow rate of 0.7 ml/min. The gel-filtration fraction-I indicated in **Fig. S1** (see supplementary data) was collected, lyophilized and stored frozen at -20 ºC. The presence of the GAPDH-derived AMPs that compose saccharomycin (i.e., AMP2/3 and AMP1) in the gel-filtration faction-I was confirmed by performing an enzyme-linked immunosorbent assay (ELISA), using a specific polyclonal antibody as described in Branco et al. (2017b). In the assays performed with saccharomycin (section 2.5), this gel-filtration fraction was used after being resuspended in YEPD with 30 g/l of ethanol (at pH 3.5 and at pH 6.0) to a final total protein concentration of 250 μg/ml, which corresponds to the minimal inhibitory concentration (MIC) determined against *H. guilliermondii* in Branco et al. (2017a).

2.3. Preparation of synthetic analogues of saccharomycin (AMP2/3 and AMP1)

Synthetic analogues of the AMPs that compose saccharomycin, i.e. AMP2/3 (VSWYDNEYGYSTR) and AMP1 (ISWYDNEYGYSAR), were chemically synthetized according to standard procedures and purchased from GenScript Inc. Company (GenScript HK Limited, Hong Kong). The synthetic peptides (AMP2/3 and AMP1) were obtained in lyophilized form (purity>98%) and stock solutions of each peptide were prepared by dissolving 2 mg of lyophilized powder in 1 ml of deionized water, and adjusting the pH to 8.0 with a sodium hydroxide solution until total solubilization was attained. In previous work (Branco et al. 2017a), we found that the activity of these synthetic peptides is maximal when the two peptides (i.e. AMP2/3 and AMP1) are mixed at a ratio of 4:1 (AMP2/3:AMP1). Therefore, mixtures of AMP2/3+AMP1 at a ratio of 4:1 in a final concentration of 1000 µg/ml, which corresponds to the MIC against *H. guilliermondii* determined by Branco et al. (2017a), were prepared.

2.4 Effect of the synthetic AMPs on culturability, intracellular pH and membrane permeability of *H. guilliermondii* cells

The effect of the synthetic AMPs on culturability, intracellular pH **(**pHi) and membrane permeability of *H. guilliermondii* cells was determined during growth assays performed in 25 ml of YEPD medium (with 30 g/l of ethanol, at pH 6.0), without (Control assay) and with the synthetic AMPs (Synthetic AMPs assay) using the mixtures described in section 2.3 (i.e. AMP2/3+AMP1 at a ratio of 4:1 at a final concentration of 1000 µg/ml). Both assays (Control and AMPs assays) were inoculated with 10^5 cells/ml of *H. guilliermondii* and incubated at 30 ºC, under strong agitation (150 rpm), for 24 h. Each culture was performed in duplicates and samples were taken at regular intervals (0, 8 h and 24 h) to determine culturability (CFU/ml), pHi and membrane permeability.

2.4.1 Culturability determination

Culturability was determined by the classical plating method. Briefly, 100 µl of culture samples were spread 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.

2.4.2 Determination of intracellular pH (pHi) and membrane permeability

The pHi of *H. guilliermondii* cells was determined by the FRIM technique (Mortensen et al. 2006) using the pH sensitive probe 5(6)-carboxy fluorescein diacetate succinimidyl ester (CFDA-SE) and membrane permeability was assessed by staining cells with propidium iodide (PI), as described in Branco et al. (2015). Briefly, cells were double stained with the pH-sensitive probe CFDA-SE and with the membrane-impermeant dye PI and analysed in a fluorescent microscope (Zeiss Axioscop 50, Germany) equipped with a Zeiss Neofluar 40× objective (numerical aperture 0.75) and a HBO 50 W lamp to provide excitation of the probe used. To determine pHi, double-stained cells were excited for 3s at 488 and at 435 nm and emission (above 520 nm) was recorded with a cooled CCD-camera (CoolSnapfx; Photometrics, Birkerød, Denmark). To minimize photo bleaching of CFDA-SE stained cells, a 2.5% neutral-density filter was used in the excitation path. Membrane permeability was determined by exciting the double-stained cells for 3s at 540 nm and recording emission (above 610 nm) in the same CCD-camera. Afterwards, images were analyzed using RS Image software (Roper Scientific, version 1.9.2) and data was treated with the Image J 1.37v software program (http://rsb.info.nih.gov/ij). Both pHi and membrane permeability were determined by analyzing 50 cells in each sample from two independent assays.

The pHi of single cells was calculated as the ratio of the fluorescence intensity emitted by CFDA-SE stained-cells excited at 488 and at 435 nm (R488/435). To determine the relationship between the fluorescence emitted by cells stained with CFDA-SE and the respective pHi value a calibration curve was constructed (**Fig. 1**), as described in Branco et al. (2015). Briefly, ethanol-dead cells of *H. guilliermondii* were resuspended in buffer solutions at different pH values (ranging from 5.5-8) and then stained with CFDA-SE. The fluorescence intensity emitted by stained-cells excited at 488 and at 435 nm (R488/435) was analysed and recorded using the same set-up above-described. The background fluorescence intensity was subtracted from the fluorescence intensity of stained cells. To determine the minimum PI fluorescence intensity

emitted by dead cells, a cell suspension was incubated with 70% (v/v) of ethanol for 30 min at 25 °C and then cells were stained with 10 µl of PI solution (1 mg/ml). Afterwards cells were analysed by epifluorescence microscopy.

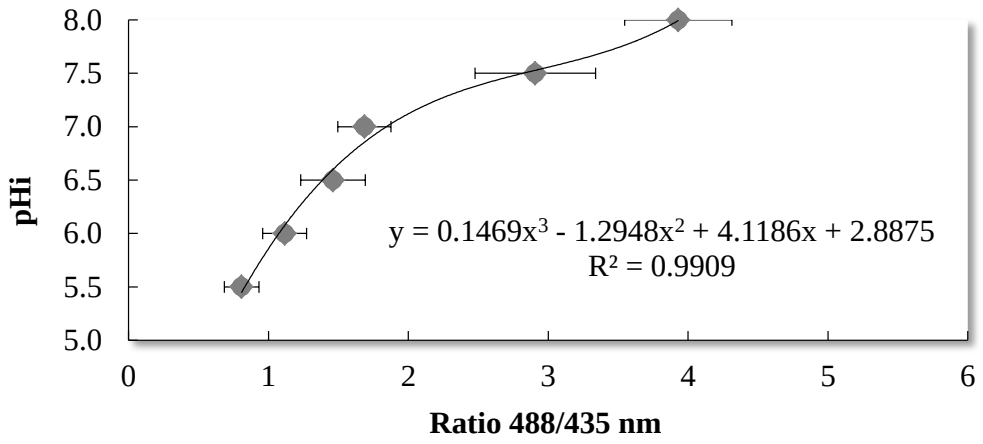

Fig. 1: Calibration curve of the fluorescence ratio (R488/435 nm) emitted by *H. guilliermondii* cells stained with the pH-sensitive probe CFDA-SE at different pHi values. Values represented correspond to means of 50 single cell measurements ±SD (error bars). Calibration points were fitted by a third-degree polynomial curve.

2.5. Effect of the synthetic analogues and of saccharomycin on the proton movements in *H. guilliermondii* cells

Movements of protons (i.e., H^+-influx and H^+-efflux) in *H. guilliermondii* were evaluated after cells had been exposed to both saccharomycin and the synthetic analogues.

First, *H. guilliermondii* cells were cultivated in 250 ml of YEPD medium (both at pH 3.5 and pH 6.0), at 30 ºC under strong agitation (150 rpm), until an optical density (OD 640) of approximately 1.0 (ca 16 h) was attained. Then, cells from each culture (i.e. from YEPD at pH 3.5 and YEPD at pH 6.0) were harvested (centrifuged at 12,000×g for 3 min at 4°C) and resuspended in 3 ml of YEPD with 30 g/l of ethanol at pH 3.5 and at pH 6.0, respectively, with 250 µg/ml of saccharomycin (Saccharomycin-assay). *H. guilliermondii* cells cultivated in YEPD at pH 6.0 (at the same conditions) were harvested and resuspended in 3 ml of YEPD with 30 g/l

of ethanol at pH 6.0 with the synthetic analogues AMP2/3+AMP1 (mixed at a 4:1 ratio) in a final concentration of 1000 µg/ml (Synthetic AMPs assay). Control assays were performed with *H. guilliermondii* cells resuspended in 3 ml of YEPD (with 30 g/l ethanol) at pH 3.5 and at pH 6.0, without neither saccharomycin nor the synthetic analogues. Cells in both assays (i.e. in accharomycin- and in Synthetic AMPs assays) were incubated at 30 ºC with shaking (150 rpm) for 4 h. The pH of cultures in all assays was measured immediately upon inoculation and after 4 h of incubation, using a standard pH meter (PHM62; Radiometer Copenhagen), showing that the pH of cultures (external pH) didn't change during the 4 h of incubation. All assays were performed in duplicates.

H^+ movements in *H. guilliermondii* cells were determined as described by Viana et al. (2012). Briefly, after incubation at the above-described conditions, cells were harvested (12,000 ×g, 3 min, 4°C) and washed twice with ice-cold water. Suspensions were kept on ice for at least 1 h before determination of H^+ movements. H^+ movements were measured by recording the pH of unbuffered cells suspension in a 2 ml reading-cell with magnetic stirring, using a standard pH meter (PHM62; Radiometer, Copenhagen, Denmark) connected to a potentiometer recorder (BBC-Goerz Metrawatt SE460) (Madeira et al., 2010).

For H^+-efflux measurements, 100 µl of the cell suspension was mixed with 800 µl of water and then pH was adjusted to 5. Afterwards, 20% glucose solution (100 µl) was added to initiate the H^+ extrusion, observed as the acidification of the unbuffered environment. The maximum rate of increase in the extracellular H^+ concentration, calibrated with 10 mM NaOH, was taken as a measure of H^+ extrusion activity (Madeira et al., 2010). For passive H^+-influx determination, 100 µl of the cell suspension was mixed with 900 µl of water. H^+ net influx corresponds to the H^+ entering the cells by passive diffusion and the H^+ being pumped out by the ATPase. To minimize ATPase activity, H^+-influx assays were performed in the presence of 2-deoxy-D-glucose (1 mM) (to decrease the level of ATP) and with antimycin (2 mg/ml) to inhibit the respiratory chain (Madeira et al., 2010). The rate of H^+-influx was calculated as the steady rate (for at least 10 min) of decrease in the concentration of extracellular H^+, recorded immediately after adjusting pH to 4.0. Calibrations were performed with 10 mM HCl. Dry weight biomass present in 100 µl of the cell suspensions used in each assay was determined after drying at 90 ºC, until constant weight, in pre-weighed aluminum foil cups. H^+-fluxes were calculated *per* g of dry weight biomass. All experiments were performed in triplicate. The

minimum significant difference was calculated to permit comparison of means as described by Fry (1993) and establish the basis for the rejection of the null hypothesis that means the differences are statistically significant ($P \leq 0.05$).

2.6. Sugar transport assays

H. guilliermondii cell cultures were grown in YEPD medium in the presence and in the absence of saccharomycin and the synthetic AMPs as described in section 2.5, and each assay was performed in triplicate. Initial D-[^{14}C] glucose uptake rates were measured as described previously by Leandro et al. (2011) with some minor modifications. Briefly, 250 ml of cell cultures were harvested at OD 640~1 and then cells were washed twice with ice-cold water and resuspended in 5 ml of water. Afterwards, 20 µl of this cell suspension was mixed with 20 µl of 100 mM Tris/ citrate buffer at pH 5 in a 15 ml conical glass tube and incubated at 25 ºC for 5 min. To initiate the transport assay 10 ml of D-[^{14}C] glucose solution was added, prepared by mixing labelled and non-labelled sugar in a final glucose concentration of 1 mM (with specific activity 10107 c.p.m. nmol^{-1}) and 25 mM (with specific activity 623 c.p.m. nmol^{-1}). To stop the reaction, 5 ml of ice-cold demineralized water was added after 5 s, and the suspension was filtered immediately through a moist Whatman GF/C filter. 5 ml ice-cold demineralized water was added to wash the filter and then transferred to a scintillation vial with 5 ml scintillation fluid (OptiPhase 'HiSafe' 2, Perkin Elmer). Radioactivity was measured in a Tri-Carb 1600 CA liquid scintillation analyser (Packard Instruments). Controls for each sugar concentration were prepared by adding 5 ml ice-cold demineralized water to the cell suspension and Tris/citrate buffer, and after that D-[14C] glucose (reaction time 0 s) was added. Inhibition assays were performed with 20 ml of a glucose solution, prepared in 100 mM Tris/ citrate buffer at pH 5. This solution was added to 10 ml of the labelled glucose in the glass tube and the reaction was started by adding 20 µl of the cell suspension. Dry weight biomass of the cell suspensions used was determined as described in section 2.5.

3. RESULTS AND DISCUSSION

3.1. Effect of synthetic analogues of the GAPDH-derived AMPs on culturability, intracellular pH and membrane permeability of *H. guilliermondii* cells

We previously showed that saccharomycin (i.e., the naturally-secreted GAPDH-derived AMPs) induce a loss of culturability, membrane permeabilization and loss of pH homeostasis on *H. guilliermondii* cells (Branco et al. 2015). Besides, in Branco et al. (2017a) we showed that synthetic analogues of saccharomycin (i.e. AMP2/3 and AMP1) also induce loss of culturability of *H. guilliermondii*, although the MIC is significantly higher (1000 µg/ml) than that of the natural biocide (250 µg/ml).

In the present work, *H. guilliermondii* was incubated in YEPD without (Control assay) and with the synthetic AMPs (Synthetic AMPs assays) and the culturability, the membrane permeability and the intracellular pH (pHi) of cells were assessed during the growth assays. Synthetic AMPs assays were performed in YEPD at pH 6.0, and not at the enological pH conditions (pH 3.5) previously used for Saccharomycin assays (Branco et al. 2017a), since these AMPs are anionic in nature (p*I*=4.35), and thus do not dissolve at that acidic pH.

The pHi of *H. guilliermondii* (both the average pHi and the individual cell pHi) was evaluated by the FRIM method during the control and Synthetic AMPs assays, and the respective profiles are represented in **Fig. 2**. During the control assay, the average pHi increased from 6.8 to 7.2 in the first 8 h, maintaining this value throughout the next 16 h (**Fig. 2A**). Conversely, the average pHi of *H. guilliermondii* cells during the Synthetic AMPs assay dropped from an initial value of 6.5 to a final value of 6.0 within the first 8 h, keeping that value for 24 h (**Fig. 2B**). These results demonstrate that the pH homeostasis of *H. guilliermondii* is not affected negatively by growth itself, since the average pHi slightly increased during the Control assay (**Fig. 2A**). Besides, these results also show that the synthetic AMPs, likewise as saccharomycin (Branco et al., 2015), affect negatively the pH homeostasis of *H. guilliermondii* (**Fig. 2B**).

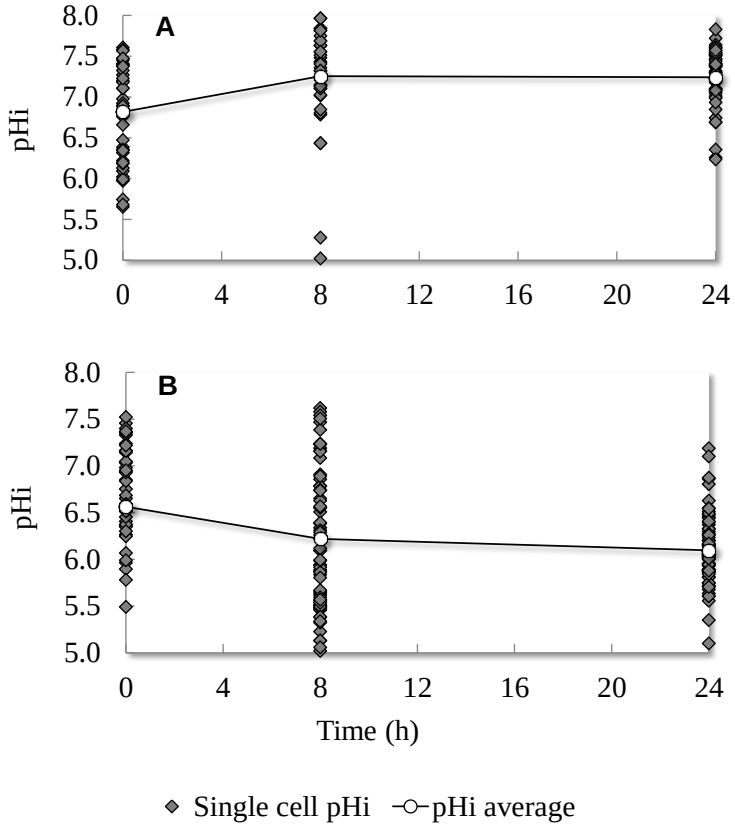

Fig. 2: Average and individual pHi values of *H. guilliermondii* cells (50 cells analysed *per* sample) during growth assays performed in YEPD at pH 6.0, without (Control) **(A)** and with the synthetic AMPs (Synthetic AMPs) **(B)**.

Since the FRIM method allows to assess the pHi of individual cells, we grouped *H. guilliermondii* cells into three subpopulations according to their pHi, i.e.: severely injured cells for pHi=5.5-6; sub-lethally injured cells for pHi= 6-7; healthy cells for pHi=7-8. Results showed that when *H. guilliermondii* was cultivated in the absence of the synthetic AMPs (**Fig. 3A**), culturability increased from 10^5 to 10^8 within 24 h and the percentage of healthy cells (i.e., cells with pHi values ranging from 7-8) was high (*ca* 81%) throughout the assay. Conversely, when *H. guilliermondii* cells were incubated in the presence of the synthetic AMPs (**Fig. 3B**), culturability declined from about 10^5 to 10^3 CFU/ml and the percentage of severely-injured cells (pH=5.5-6) increased from 9.1% to 48.7%, in the first 8 h. However, after 24 h, the percentage of *H. guilliermondii* cells exhibiting pHi values ranging from 5.5-6 decreased to 37.7% and

culturability increased to 6×10^4 CFU/ml, indicating that cells were able to recover even in the presence of the synthetic AMPs **(Fig. 3B**). Branco et al. (2015) also reported that *H. guilliermondii* cells exposed to saccharomycin were able to recover their culturability, but only after they had been transferred into fresh YEPD medium and incubated for 24 h. In the present work, we found that *H. guilliermondii* cells exposed to the synthetic AMPs initially decreased culturability (in the first 8 h) but, after 24 h, recovered their culturability and pHi values increased (**Fig. 3B**). These results confirm that the synthetic AMPs do not exert an antimicrobial effect as strong as saccharomycin, as previously reported by Branco et al. (2017a). In short, our results show that the synthetic AMPs, at the conditions used in the present study (i.e., in YEPD at pH 6.0), induced a transient disturbance in the pH homeostasis of *H. guilliermondii*, while saccharomycin (in YEPD at pH 3.5) induced a permanent loss of pH homeostasis (Branco et al., 2015).

Membrane permeability of *H. guilliermondii* cells exposed to saccharomycin was previously evaluated by Branco et al. (2015), and the percentage of cells with compromised membranes (PI-stained cells) was found to increase from 0% to 77.7% within 24 h. In the present work, we show that the chemically synthesized analogues of saccharomycin also affect the membrane permeability of *H. guilliermondii* cells. Indeed, the percentage of *H. guilliermondii* cells exhibiting compromised membranes (PI-stained cells) increased from 6% to 35% after 8 h of exposure to the synthetic AMPs (**Table 1**). However, after 24 h, the percentage of cells exhibiting compromised membranes (PI-stained cells) was virtually null (**Table 1**). These results probably mean that the sub-lethally injured cells found after 8 h of incubation were able to adapt to the synthetic AMPs and recovered its growth (**Fig. 3B**), thus increasing the percentage of viable cells within the total cell population.

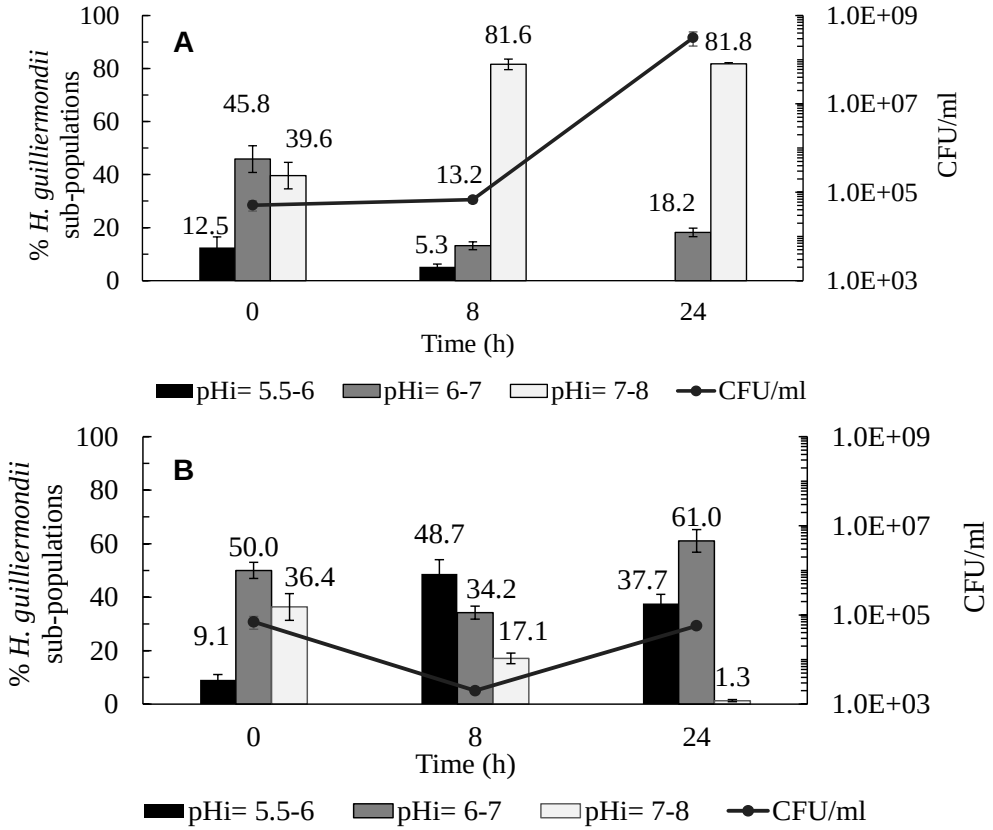

Fig. 3: Culturability of *H. guilliermondii* and percentage of cells within the subpopulations of severely injured (pHi=5.5-6), sub-lethally injured (pHi=6-7) and healthy cells (pHi=7-8), during growth assays performed in YEPD at pH 6.0, without (Control assay) **(A)** and with the synthetic AMPs (Synthetic AMPs assay) **(B)**. Each variable represented corresponds to means ± SD (error bars) of duplicate experiments.

Table 1: Percentage of *H. guilliermondii* cells with compromised membrane integrity (PI-stained cells) during the Control and Synthetic AMPs assay. Each variable represented corresponds to means ± SD (error bars) of duplicate experiments.

Time (h)	% PI-stained cells	
	Control assay	Synthetic AMPs assay
0	0.0±0.0	6.0±0.0
8	0.0±0.0	35.9±2.6
24	0.0±0.0	0.0±0.0

3.2. Effect of the synthetic analogues and saccharomycin on proton fluxes in H. guilliermondii cells

In harsh environments such as wine fermentations, with low external pH values (3.0-3.5), it is expected that passive H^+-influx increases due to the higher pH gradient established between intracellular and extracellular environment. In order to prevent acidification of the cytosol, influx of protons has to be balanced by proton extrusion via the plasma membrane ATPase. Cell membrane permeability to protons determines the rate of protons leakage inward cell, by passive diffusion through the membrane bilayer (Leão and van Uden, 1984).

In order to assess the causes underlying the drop of pHi in *H. guilliermondii* cells exposed to the synthetic AMPs and to saccharomycin, we determined proton fluxes by measuring membrane permeability of cells to H^+ cations (H^+-influx) and their ability to extrude H^+ protons via plasma membrane ATPase (H^+-efflux).

3.2.1. Proton influx

H^+-influx rates were measured in *H. guilliermondii* cells incubated in YEPD with the synthetic AMPs (1000 µg/ml) at pH 6.0, and also in YEPD with saccharomycin (250 µg/ml) at pH 6.0 and at pH 3.5. The reasons underlying the higher MIC of the synthetic AMPs (1000 µg/ml) by comparison with the MIC of saccharomycin (250 µg/ml) probably results from the

structure adopted by the GAPDH-derived peptides in the natural biocide which may involve the formation of aggregates of several molecules that probably enhances its activity, as discussed in Branco et al. (2017a). Control assays were performed with *H. guilliermondii* incubated in YEPD without the AMPs, either at pH 3.5 or at pH 6.0. Results (**Fig. 4A**) showed that the external pH *per si* has no effect on the permeability of cells to H^+ protons, since no statistical differences in H^+ influx rates were found between assays (i.e. control at pH 3.5 and control at pH 6.0). Conversely, H^+-influx increased significantly (66.5 %), by comparison with the Control assay, when *H. guilliermondii* cells were incubated with saccharomycin at pH 3.5 (**Fig. 4A**). Also, in cells incubated with both saccharomycin and the synthetic AMPs at pH 6.0, H^+-influx rates increased relatively to control (by 57.7% and 69.2%, respectively) (**Fig. 4A**). These results demonstrate that both saccharomycin and the synthetic AMPs significantly increase the permeability of *H. guilliermondii* cells to H^+ protons.

3.2.2. Proton efflux and glucose uptake

3.2.2.1 Proton efflux

Proton homeostasis is a key mechanism for good yeast performance. Environmental stress factors lead to the dissipation of the H^+-gradient across the plasma membrane and to intracellular acidification which also induces the stimulation of plasma membrane H^+-ATPase activity (Monteiro et al., 1994; Rosa and Sá-Correia, 1992). Plasma membrane H^+-ATPase pumps out H^+ to maintain pH homeostasis and to generate an electrochemical gradient essential to drive the transport of metabolites into the cell (Opekarova and Sigler, 1982). Thus, proton efflux rates can be correlated with the activity of plasma membrane H^+-ATPase (Opekarova and Sigler, 1982), which is an important factor in maintaining viability during a stress challenge.

In the present work, we found that both saccharomycin and the synthetic AMPs disturb the pH homeostasis of *H. guilliermondii* cells, what can result from destabilization of plasma membrane H^+-ATPase activity, leading to decreased H^+-efflux rates. To check this hypothesis, we measured H^+-efflux rates in *H. guilliermondii* cells exposed to both the synthetic AMPs (at pH 6.0) and to saccharomycin (at pH 3.5 and 6.0). In the presence of saccharomycin at pH 3.5, the H^+-efflux in *H. guilliermondii* cells significantly decreased relatively to Control assay (from 0.35 to 0.10 mmol (g dry biomass)$^{-1}$ h^{-1}) (**Fig. 4B**). This reduction in H^+-efflux (by 75.6%) can be

due to a reduced plasma membrane H^+-ATPase activity. However, in assays performed at pH 6.0, although a reduction of H^+-efflux rates was also observed in cells exposed to both saccharomycin and synthetic AMPs, values were not statistically different from those in control assays (**Fig. 4B**).

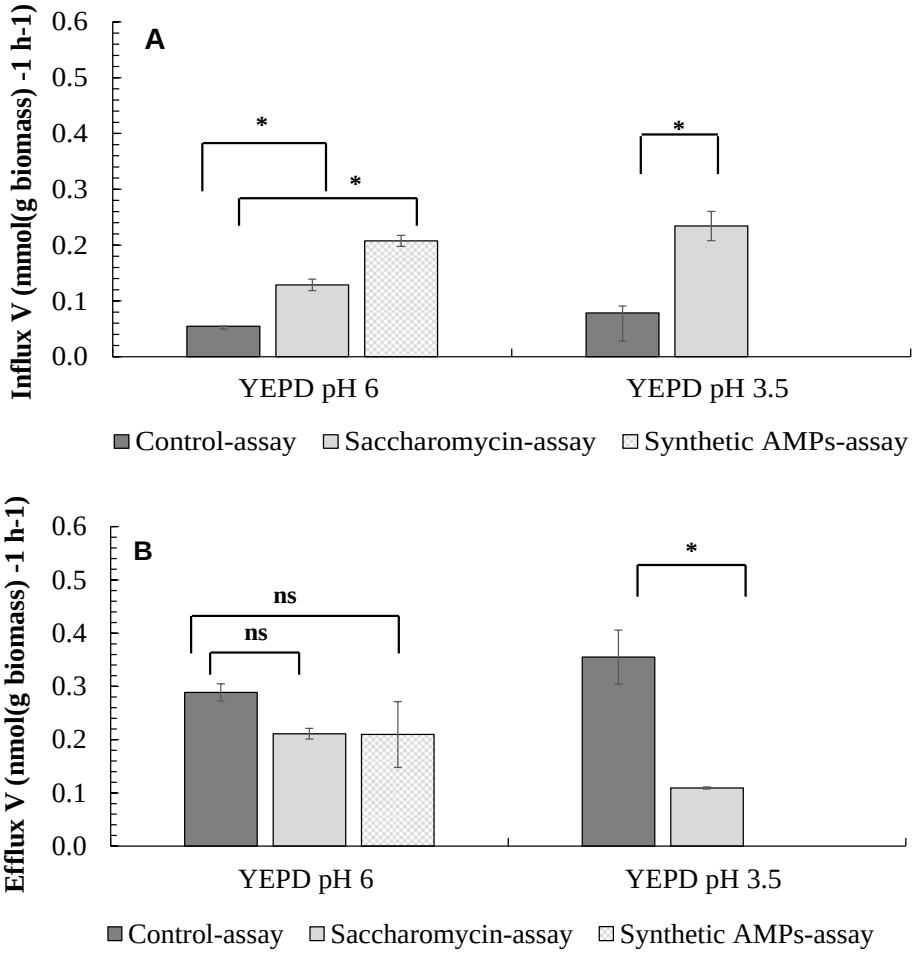

Fig. 4: Rates of net H^+-influx **(A)** and H^+-efflux **(B)** in *H. guilliermondii* cells incubated (for 4 h) in YEPD without (Control assay) and with saccharomycin (Saccharomycin assay) at pH 6.0 and 3.5, and with the synthetic AMPs (Synthetic AMPs assay) at pH 6.0. Values represented correspond to means ± SD (error bars) of triplicate experiments. *, statistically different values (*P<0.05*)*;* **ns**, non-statistically different values (*P>0.05*).

3.2.2.2 Glucose uptake

An important factor for cell survival is the existence of a viable energy source, with glucose being preferred over other sugars for most of the yeasts and having a profound effect on many cellular functions. As a response to stressful conditions, cells induce the expression of a plethora of genes that affect glucose metabolism, including glucose transporters (Gasch, 2003).

In *H. guilliermondii* glucose is transported by facilitated diffusion through Hxt-like transporters and the absence of a glucose-H^+ symport was firstly reported by Loureiro-Dias (1988). In order to confirm this result, we grew *H. guilliermondii* cells up to early stationary phase, starved cells for glucose and registered the pH variation upon a pulse of glucose. As expected, no alkalization was observed in all the assays, although a clear acidification was observed, showing that glucose entered the cells and was metabolized.

Plasma-membrane H^+-ATPase hydrolyses ATP to transport protons from the cytosol to the extracellular medium. H^+-ATPase activity is regulated in response to growth conditions (Eraso and Gancedo, 1987; Rosa and Sá-correia, 1991) in which glucose has an important role, regulating the expression and catalytic activity of this pump. Glucose metabolism increases ATPase gene (*PMA1*) expression (Portillo et al., 1989). In addition, glucose induces a modification of the enzyme's kinetic properties (Serrano, 1983), resulting in a global stimulation of ATPase activity.

Our results (**Fig. 4B**) seem to indicate that plasma membrane H^+-ATPase activity is significantly impaired by the presence of the GAPDH-derived AMPs. Thus, we measured the glucose uptake rate in the presence of both saccharomycin and the synthetic AMPs to discard a putative effect of AMPs on glucose transporters that could lead to a reduction of ATP, and consequently to a decrease in the net H^+-efflux rate. With that purpose, cells were incubated in YEPD at pH 6.0 and 3.5, in the absence/presence of both the natural and the synthetic AMPs, and D-[^{14}C] glucose uptake rates were determined with two concentrations of radio-labeled glucose (25 mM and 1 mM). To our surprise, glucose transport rate across the plasma membrane of *H. guilliermondii* cells exposed to both saccharomycin and the synthetic AMPs was stimulated in the presence of the AMPs (**Fig. 5**). Thus, our results showed that glucose transport is not negatively affected by AMPs, discarding the hypothesis of lower ATPase activity due to lack of glucose.

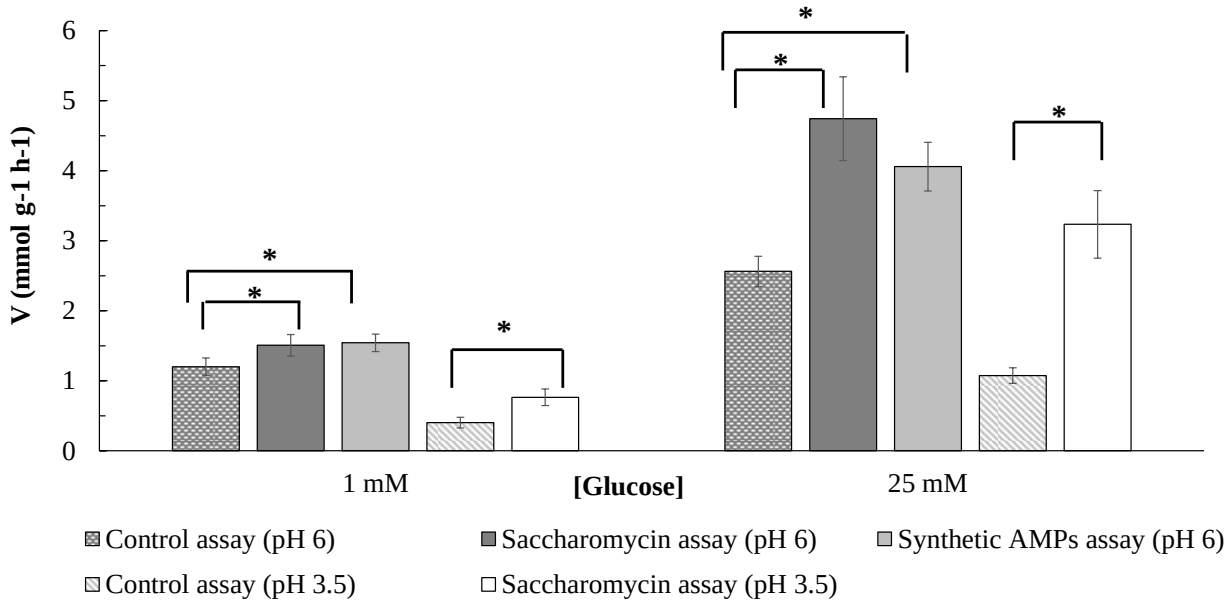

Fig. 5: Glucose uptake rates in *H. guilliermondii* cells incubated (for 4 h) in YEPD without (Control assay) and with saccharomycin (Saccharomycin assay) at pH 6.0 and 3.5, and with the synthetic AMPs (Synthetic AMPs assay) at pH 6.0. Values represented correspond to means ± SD (error bars) of triplicate experiments. *, statistically different values ($P<0.05$)

In conclusion, our data show that both saccharomycin and its synthetic analogues induce a perturbation in the plasma membrane that increases the proton influx and inhibits the proton efflux, leading to a pHi drop and loss of culturability in *H. guilliermondii*. Thus, it may be speculated that saccharomycin and/or its synthetic analogues can be used as biopreservatives in wine, thereby allowing a reduction of the SO_2 levels usually needed to stabilize wines, in accordance with the most recent recommendations (Ribéreau-Gayon et al., 2006).

Acknowledgements

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SUPPLEMENTARY DATA

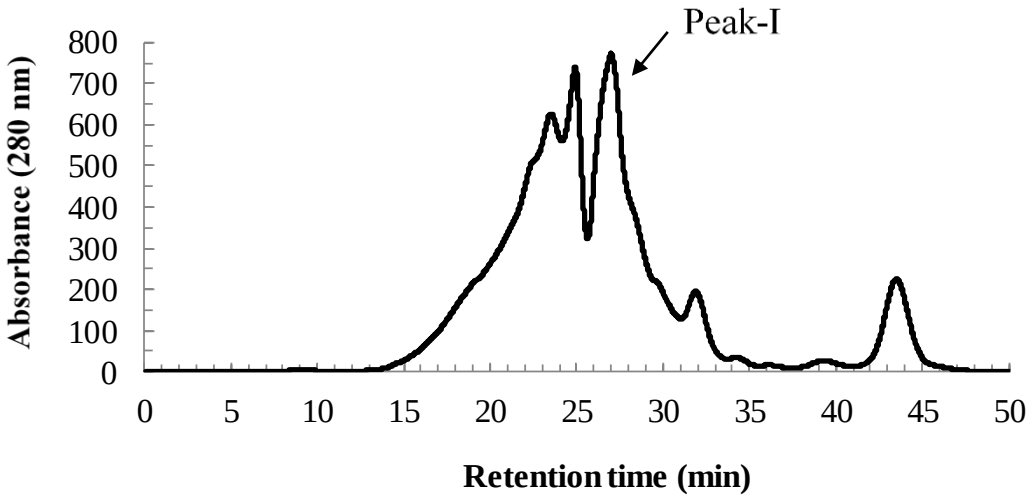

Fig. S1: Gel-filtration chromatographic profile of the concentrated (10-fold) peptidic fraction (2-10 kDa) obtained from the supernatant of synthetic grape juice fermentation carried out by *S. cerevisiae* CCMI 885. Fraction-I indicated in figure contains the GAPDH-derived AMPs that compose saccharomycin.

The results presented in this Chapter are published in:

Peer-Reviewed Journal

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Poster presentation

Branco P, Albergaria H, Arneborg N, Prista C (2016) GAPDH-derived AMPs affect the pH homeostasis of sensitive yeast cells by decreasing the H^+-ATPase membrane activity. PYFF6 - 6th Conference on Physiology of Yeast and Filamentous Fungi (PYFF), *Instituto Superior Técnico*, University of Lisbon, Lisbon, 2016 July 11-14.

Chapter VII

Expression of GAPDH-derived AMPs in *Saccharomyces cerevisiae* and evaluation of their biopreservative potential in wine fermentations

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2017 (to be submitted)

ABSTRACT

Wine spoilage is mostly caused by *Dekkera bruxellensis* and is generally controlled by the addition of sulphur dioxide (SO_2). Reduction of SO_2 levels in wines is a goal chased by producers due to its negative impact on wine aroma and to consumer's health concerns. We recently found that *Saccharomyces cerevisiae* produces antimicrobial peptides (AMPs) derived from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) which have inhibitory effect on *D. bruxellensis.* Therefore, *S. cerevisiae* strains were constructed to over-express the corresponding sequences of the genes that encode these AMPs. Relative expression levels of these AMPs in transformed strains of *S. cerevisiae* showed that they could express the desired sequences during wine fermentation. Furthermore, modified strains secreted higher amounts of the AMPs and showed a higher antagonistic effect against *D. bruxellensis* than the respective wild type strain. The use of these AMPs as natural biopreservatives in winemaking, to complement the protective effect of SO_2, could be a very interesting solution to reduce the required amount of SO_2. Therefore, we evaluated the conjugated effect of SO_2 with the AMPs on the survival rate of *D. bruxellensis* in simulated wines with different levels of ethanol (10%, 12%, 13% and 14% v/v) that were artificially contaminated with 5×10^3 CFU/ml of *D. bruxellensis*. Results showed that with 1 mg/ml of these AMPs, the concentration of SO_2 required to achieve total death of *D. bruxellensis* within 48 h was only 14.25 mg/l of total SO_2, a value much lower than that usually used in winemaking (100-150 mg/l of SO_2).

Keywords: Antimicrobial peptides, Spoilage wine yeasts, Wine preservatives, Genetically-modified wine yeasts

1. INTRODUCTION

The indigenous microbiota of grape musts includes an immense variety of yeast species belonging to different genera (Fleet, 1993) that are able to grow and ferment sugars. However, nowadays, the majority of wine fermentations are initiated by yeast starter cultures, most commonly composed by *Saccharomyces cerevisiae* strains (Barnett and Lichtenthaler, 2001; Steensels et al., 2014) due to their ability to produce high levels of ethanol and to survive in the harsh environmental conditions of wine (Pretorius, 2000; Bauer and Pretorius, 2000, Sabate et al., 2002). Besides, *S. cerevisiae*'s dominance during alcoholic fermentation is also due to microbial interactions, mediated both by a cell-to-cell contact mechanism (Nissen et al., 2003; Nissen and Arneborg 2003; Renault et al., 2013) and the secretion of antimicrobial peptides (AMPs) that induce death of several wine-related yeasts and bacteria (Albergaria et al., 2010; Branco et al., 2014; Albergaria and Arnerborg, 2016). Recently, we found that these two phenomena, i.e. cell-to-cell contact and secretion of AMPs, play a combined role in the early death of the wine yeast *L. thermotolerans* during mixed-culture fermentation with *S. cerevisiae* (Kemsawasd et al., 2015).

Microbial spoilage is a serious problem for the wine industry since it renders the product unacceptable and can lead to large economic losses. The main wine spoilage microorganisms are lactic acid and acetic acid bacteria, as well as some yeasts such as those belonging to the genera *Dekkera*/*Brettanomyces, Candida, Hanseniaspora, Pichia, Metschnikowia, Saccharomycodes, Schizosaccharomyces* and *Zygosaccharomyces* (Fleet, 2003; Enrique et al., 2007). Among wine spoilage yeasts, *Dekkera bruxellensis* is considered a major cause of wine spoilage worldwide (Fugelsang, 1997; Loureiro and Malfeito-Ferreira, 2003). This species confers phenolic off-odours to red wine described as ''barnyard-like'' or ''horsey" (Fugelsang, 1997) and produces biogenic amines (Caruso et al., 2002**)**.

In the last years, several research groups focused their attention on *D. bruxellensis* with the aim of understanding their spoilage ability and to avoid their proliferation in wine (Suárez et al., 2007; Chandra et al., 2016). Control of *D. bruxellensis* contamination in the wine industry is typically carried on by filtration of wine and barrel sanitization. However, these control measures proved to have limited efficiency and are not able to prevent subsequent recontamination (Millet and Lonvaud-Funel, 2000; Peri et al., 1988). The use of chemical preservatives such as benzoic

acid, sorbic acid and dimethyl dicarbonate (DMDC) is able to inhibit *D. bruxellensis* in wine but only in concentrations above their legal limits (Benito et al., 2009).

In wineries, the most common preservative practice is the addition of sulphur dioxide (Ribéreau-Gayon et al., 2006), which is highly toxic to most of the non-*Saccharomyces* yeasts but not to most of the *Saccharomyces* strains (Fleet 1992; Romano and Suzzi, 1993). Numerous finished and bottled wines that aged for long periods in oak barrels with low sulphur dioxide concentrations and less filtration prior to bottling are also known to host *Brettanomyces/Dekkera* populations (Herezstyn, 1986; Arvik et al., 2002). In addition, sulphur dioxide has a negative impact on wine aroma and can cause health problems; consequently, excessive doses must be avoided. Thus, the possibility of further reducing the authorized concentrations in different kinds of wines is sought after (Ribéreau-Gayon et al., 2006).

Oenological research has always tried to pursuit other substances able to enhance sulphur dioxide antimicrobial properties or to perform a similar role without its disadvantages (Ribéreau-Gayon et al., 2006). Our recent finding that *S. cerevisiae* strains produce AMPs derived from the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that induce death of several wine yeasts (Branco et al., 2014, 2017), opens interesting prospects regarding their use as alternative biopreservatives in winemaking. Nevertheless, the concentration of AMPs naturally-secreted by *S. cerevisiae* not only is strain-dependent (Branco et al., 2017) and also might not assure complete death of undesirable wine contaminants, namely of *D. bruxellensis* strains.

Thus, the main goal of this work was to construct and characterize a genetically modified *S. cerevisiae* strain able to produce higher amounts of the GAPDH-derived AMPs. We also investigated if the addition of these AMPs to finished wines allows reducing sulphur dioxide levels, usually used in winemaking for preservation purposes.

2. MATERIALS AND METHODS

2.1 Strain, plasmid and growth conditions

The following yeasts were used in the present work: *Saccharomyces cerevisiae* CCMI 885 (Culture Collection of Industrial Microorganisms of ex-INETI, Lisbon, Portugal) and *S. cerevisiae* PYCC 5484 (CEN.PK113-5D, *MATa ura3-52 HIS3, LEU2 TRP1 MAL2-8c SUC2*) (Portuguese Yeast Culture Collection, FCT/UNL, Caparica, Portugal); *Pichia pastoris* GS115

(Invitrogen, California, EUA); *Dekkera bruxellensis* ISA 2211 (*Instituto Superior de Agronomia*, Lisbon, Portugal). All yeasts except *D. bruxellensis* were maintained in YEPD medium (5 g/l yeast extract, 10 g/l peptone, 20 g/l glucose and 20 g/l agar) and stored at 4 ºC. *D. bruxellensis* was maintained in YEPD medium with 5 g/l of calcium carbonate (Merck, Darmstadt, Germany) and stored at 4º C.

Inoculums were prepared by transferring biomass from a YEPD-agar slant (pre-grown at 30 ºC for 48 h) into 250 ml-flasks with 100 ml of YEPD and incubating flasks at 30 ºC and 150 rpm, for 16 h. All media were autoclaved at 120ºC for 20 min.

The centromeric plasmid p416 TEF was used for cloning, conferring *TEF* promoter and *CYC1* terminator. For propagation of these plasmids, *Escherichia coli* DH5α strain was used as host (Hanahan, 1983). *E. coli* transformants were grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 µg/ml), at 37 ºC. *S. cerevisiae* PYCC 5484 was used as host strain for heterologous expression of partial sequence of the *TDH1* gene (located between 925 and 963bp of *TDH1* codifying region) and the *TDH2* gene (located between 925 and 963bp of *TDH2* codifying region), previously inserted in p416 TEF. These modified strains are, from now on, called *S. cerevisiae pTDH1* and *S. cerevisiae pTDH2*, respectively. *S. cerevisiae* PYCC 5484 transformed with empty p416 TEF, from now on called *S. cerevisiae* K1, was used as negative control. Transformed yeast strains were grown and maintained in YNB medium without amino acids (DIFCO) with 2% (w/v) glucose.

2.2 Production and purification of the naturally-secreted GAPDH-derived AMPs

The GAPDH-derived AMPs were partially purified from the cell-free supernatant (7 day-old) of a synthetic grape juice (SGJ) performed by *S. cerevisiae* CCMI 885. The SGJ, containing 110 g/l of glucose plus 110 g/l of fructose, and pH of 3.5, was prepared as described in Pérez-Nevado et al. (2006). The fermentation supernatant was ultrafiltrated by centrifugal filter units (Vivaspin 15R, Sartorius, Germany) equipped with 10 kDa cut-off membranes and permeate (<10 kDa) was concentrated (10-fold) using 2 kDa centrifugal filter units. The peptidic fraction (2-10 kDa) was 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 an UV detector (Merck Hitachi, Darmstadt, Germany). The peptidic supernatant fraction (2-10 kDa) was eluted with 0.1 M ammonium acetate at a flow rate

of 0.7 ml/min. The bioactive fraction containing the GAPDH-derived peptides (retention time between 25-27 min) was collected and lyophilized, as described in Branco et al. (2014).

2.3 Sensitivity of *Pichia pastoris, S. cerevisiae* CCMI 885 and *S. cerevisiae* PYCC 5484 to the GAPDH-derived AMPs

To determine the sensitivity of *Pichia pastoris, S. cerevisiae* CCMI 885 and *S. cerevisiae* PYCC 5484 to the GAPDH-derived AMPs, yeast strains were grown in the presence of different concentrations of the bioactive fraction (purified as described in section 2.2) that contains the AMPs. Growth inhibitory assays were performed in 96 well-microplates in triplicate independent assays, with each well containing 100 µl of YEPD medium without the AMPs (negative control) and with the AMPs at final protein concentrations of: 0.125, 0.25, 0.5 and 1 mg/ml. Media were inoculated with 10^5 cells/ml of each of the above-mentioned yeast strains and the microplate was incubated in a Thermo-Shaker (Infors HT, Bottmingen, Switzerland) at 30 °C under strong shaking (700 rpm). Cell growth was followed during 48 h by absorbance measurements (at 590 nm) in a Microplate Reader (Dinex Technologies Inc., Chantilly, USA).

2.4 Plasmid DNA manipulations and cloning of the *TDH1* and *TDH2* nucleotide sequences that codify to the GAPDH-derived AMPs in *S. cerevisiae* strain K1

Plasmid DNA from *E. coli* DH5α was isolated using GenEluteTM Plasmid Miniprep Kit (Sigma-Aldrich). The nucleotide sequence of *TDH1* (925-963 pb) encoding to AMP1 (ISWYDNEYGYSAR) and the nucleotide sequence of *TDH2* (925-963 pb) encoding to AMP2/3 (VSWYDNEYGYSTR) were used to design primers in order to obtain PCR products containing AMP1 and AMP2/3 sequences, resulting from primer dimerization. Forward and reverse primers were design with the restriction sites for XbaI and SalI, respectively (**Table 1**). PCR amplification of the primer-dimer corresponding to the DNA sequence of AMP1 and AMP2/3 was carried out in an Eppendorff thermocycler with High-fidelity DNA polymerase Phusion F-530 (Finnzymes, Thermo Fisher Scientific, Massachusetts, EUA). The amplified products were digested with XbaI and SalI, purified using the purification kit ‘‘GFX PCR DNA and Gel Band Purification’’ (GE Healthcare, Little Chalfont, UK) and cloned into the corresponding restriction sites of p416 TEF, digested by the same restriction enzymes.

Cloning was performed according to standard protocols described in Sambrook et al. (1989). The plasmids were cloned into *E. coli* DH5α strain, propagated, subjected to extraction and restriction analysis. *E. coli* plasmid isolation was performed by alkaline extraction as described in Birnboim and Doly (1979) and modified as described in Sambrook et al. (1989). The correct insertion of *TDH1* (925-963 pb) and *TDH2* (925-963 pb) in the plasmid was checked by restriction digestion. Transformation of *S. cerevisiae* PYCC 5484 strain with the plasmids containing the partial sequence of *TDH1* (925-963 pb) (*S. cerevisiae* p*TDH1*) and *TDH2* (925-963 pb) (*S. cerevisiae* p*TDH2*) was performed by the lithium acetate method described in Geitz and Schiestl (1995). Transformants were selected on YNB medium without uracil. Plasmid isolation from yeasts was performed as described in Tillotson et al. (2013) with some modifications. Briefly, one *S. cerevisiae* colony freshly grown on an YNB plate was transferred into 30 µl of SDS 1% (w/v) in deionized water. Afterward, cells were vortexed for 1min and then frozen at -80ºC for 2 min and subsequently heated at 95ºC for 2 min; the freeze/thaw was repeated once more to ensure cell lyses. Cells were centrifuged at maximal speed for 1 min and the supernatant was used as a template for PCR reaction. PCR products were loaded in an agarose gel for electrophoresis, band was extracted from the gel and then purified using the purification kit ‘‘GFX PCR DNA and Gel Band Purification’’ (GE Healthcare, Little Chalfont, UK). The purified PCR products were sequenced by external services (STAB VIDA, Monte da Caparica, Portugal) in order to confirm the insertion of the sequences of interest.

2.5 RNA extraction and cDNA preparation

The modified *S. cerevisiae* strains, p*TDH1* and p*TDH2* were pre-grown for 24 h in YNB without uracil (approximately 10^7 cells/ ml). RNA extraction from cells was performed with Trizol Reagent (Invitrogen, California, USA). Small RNAs from the above-mentioned *S. cerevisiae* strains were separated and purified from total RNAs with *mir*Vana™ miRNA Isolation Kit (Applied Biosystems, California, USA) according to the manufacturer’s protocol. All RNA samples were treated with RNase free DNase I (Qiagen, Hilden, Germany) and quantified using absorption of UV light at 260 nm. Small cDNAs from modified *S. cerevisiae* strains were synthesized from small mRNAs using TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, California, USA). RT primers designed by the manufacturer and specific for each small mRNA sequence were used for small cDNAs synthesis (Custom

TaqMan® Small RNA Assays, Applied Biosystems, California, USA). Total cDNAs were synthesized from total mRNA of *S. cerevisiae* p*TDH1*, *S. cerevisiae* p*TDH2* using TaqMan® MicroRNA Reverse Transcription Kit and oligod(T) primer (STAB VIDA, Monte da Caparica, Portugal). All cDNAs synthesis were performed following manufacturer's instructions (Applied Biosystems, California, USA), and were used for Real Time PCR reaction using the primers described in **Table 1**.

Table 1: Primers used in this study. XbaI and SalI restriction sites are underlined.

PCR primer	**partial *TDH1***
Forward	5´-TGCTCTAGAGCAATGATTTCCTGGTACGATAACGAATACGGTTACTCC-3´
Reverse	5´-ATACGCGTCGACTAATCTGGCGGAGTAACCGTATTCGTTATCGTA-3´
PCR primer	**partial *TDH2***
Forward	5´-TGCTCTAGAGCAATGTTTCCTGGTACGACAACGAATACGGTTACTCTA-3´
Reverse	5´-ATACGCGTCGACTAATCTGGCGGAGTAACCGTATTCGTTATCGTA-3´
RT-PCR primer	***TDH1***
Forward	5´-CAAGAAGGCTGTTAAGGCTG-3´
Reverse	5´-CGGAGGCATCGAAGATGGAA -3´
RT-PCR primer	***TDH2***
Forward	5´-TCACTGCTCCATCTTCCACC-3´
Reverse	5´-TTTGGGTGGCGGTCATGGA-3´

2.6 Quantitative reverse transcription PCR (RT-qPCR)

Small and total cDNAs obtained as described in section 2.5 were used as template for RT-qPCR reactions. To determine the best amount of cDNA to be used as template, five dilutions of the cDNA sample were tested. Each dilution was analysed in triplicate.

The specific primers to amplify the smalls cDNAs *TDH1* (925-963 pb) and *TDH2* (925-963 pb) were obtained from Custom TaqMan® Small RNA Assays (Applied Biosystems, California, USA). The same primers were used to amplify the genomic sequence corresponding to the partial sequence of *TDH1* (925-963 pb) and *TDH2* (925-963 pb) gene of the modified *S. cerevisiae* strains. RT-qPCR amplifications were obtained by using Luminaris Probe qPCR Master Mix (Thermo Fisher Scientific, Massachusetts, USA). The RT-qPCR reactions for small cDNAs (2 min, 50ºC; 95ºC 10 min; 40 cycles: 15 s 95ºC, 30 s 60ºC, 30 s 72ºC) and for total cDNA (95ºC 10 min; 40 cycles: 15 s 95ºC, 30 s 60ºC, 30 s 72ºC) were performed in multiplate PCR 96-well clear plates (BIO-RAD, California, EUA) in an iQTM Multicolor Real-time device (BIO-RAD, California, EUA). RT-qCR reactions were performed in triplicate. Each run was completed with a melting curve analysis to confirm the specificity of amplification and the lack of primer dimers. Additionally, PCR products were resolved on 2% (w/v) agarose gels, run at 4 V/cm in Tris-acetate-EDTA buffer (TAE), along with a 50-bp DNA-standard ladder (Invitrogen GmbH, Karlsruhe, Germany) to confirm the existence of a single product of the desired length. The comparative Cq method was used to quantify gene expression (Livak and Schmittgen, 2001). Gene expression of the inserted sequence from *TDH1* (925-963 pb) gene and *TDH2* (925-963 pb) gene was normalized with respect to the expression of the genomic sequence corresponding to the *TDH1* (925-963 pb) and *TDH2* (925-963 pb) (as the reference genes).

2.7 AMPs identification by indirect Enzyme-Linked Immunosorbent Assay (ELISA)

Polyclonal rabbit antiserum raised against synthetic GAPDH-1 (309-321) was obtained by multiple intradermic injections into rabbits. The experiments on rabbits were carried out at GenScript Inc. Company (GenScript HK Limited, Hong Kong).

1 mg/ml of synthetic GAPDH-1 (309-321) synthetized by GenScript Inc. Company (GenScript HK Limited, Hong Kong) was used to construct a calibration curve by diluting to 1:32, 1:64, 1:128, 1:256, 1:512 in a phosphate-buffered saline solution (PBS) at pH 7.2. Then, three replicates of 100 µl were taken from each diluted sample and transferred to a 96-well

microplate MICROLON® high binding (Greiner Bio-One, Germany). 100 µL of each sample was used for coating each well of the 96-well microplate. The samples used were supernatants from 12 h, 24 h, 48 h and 192 h collected from single fermentations of *S. cerevisiae* K1, p*TDH1*, *S. cerevisiae* p*TDH2* (data not shown). Then the 96-well microplate was incubated overnight at 4º C. Afterwards 100 µl of 6 M of urea was added to samples in order to denature the proteins and improve their detection by indirect ELISA as previously described by Hnasko et al. (2011). The plate was thereafter washed 4 times using a PBS-Tween washing solution (0.05% Tween 20 in 0.01 M PBS). The samples were blocked during 2 h at room temperature by adding 200 µl of blocking solution containing bovine serum albumin (BSA 1% w/v) in PBS and washed 4 times with washing solution. Next, 100 µl of the primary polyclonal rabbit (GenScript HK Limited, Hong Kong) antibody specific to GAPDH-derived AMPs in a final concentration of 10 µg/ml diluted in 1% BSA was added to each well and incubated for 2 h at 37ºC. The unbound material was removed by washing the microplate 4 times with PBS-Tween solution, thereafter, secondary antibody (anti-rabbit IgG-fab specific, alkaline phosphatase conjugate, Sigma- Aldrich, USA) was diluted (1.0 µg/ml in 1% BSA) and 100 µl was added to each well followed by 2 h incubation at 37 ºC. Subsequently, the microplate was washed 4 times with PBS-Tween solution, followed by the addition of 100 µl/well of alkaline phosphatase substract (100 mM Tris-HCL, 100 mM Nacl, 5 mM $MgCl_2$, 1 mg/ml para-Nitrophenyl phosphate (PnPP) to the microplate and incubation for 10 to 30 min at room temperature in the dark. The enzyme–substrate reaction was stopped by adding 100 µl of 3N NaOH to each well. The optical density (OD) was measured at 405 nm using a microplate reader (Bio-Rad, Benchmark, USA).

To establish the relationship between the absorbance and concentration, a standard curve was constructed for AMPs. The concentration (µg/ml) of AMP1 and of AMP2/3 present in the supernatants of all *S. cerevisiae* strains was calculated according to the linear regression equation. All samples were analysed in triplicate.

2.8 Single and mixed-culture alcoholic fermentations performed with *S. cerevisiae* wild-type (K1) and modified strains (p*TDH*1 and p*TDH*2)

Alcoholic fermentations were performed in 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) and supplemented with 120 mg/l of uracil in the fermentations performed with *S. cerevisiae* K1. Mixed-cultures alcoholic fermentations were performed with two different initial cell densities of

D. bruxellensis ISA 2211 (i.e. 5×10^3 and 1×10^5 cells/ml) together with 1×10^5 cells/ml of each of the *S. cerevisiae* modified strains (*S. cerevisiae* p*TDH1* and *S. cerevisiae* p*TDH2*) and with the wild type strain *S. cerevisiae* K1. Two single-culture alcoholic fermentations of *D. bruxellensis* (with initial cell density of 5×10^3 cells/ml and 1×10^5 cells/ml) were performed and used as negative control for the antagonism exerted by the *S. cerevisiae* strains (data not shown). Single-culture alcoholic fermentation of *S. cerevisiae* p*TDH1*, *S. cerevisiae* p*TDH2* and *S. cerevisiae* K1 (data not shown) were performed. Cells collected from fermentation samples at 12 h, 24 h, 48 h and 196 h, were used for analyses of the partial *TDH1* and *TDH2* gene expression by RT-qPCR. Supernatants from those samples were ultrafiltrated by centrifugal filter units (Vivaspin 15R, Sartorius, Germany) equipped with 10 kDa cut-off membranes and the peptidic fraction was analysed by indirect ELISA to identified the GAPDH-derived AMPs. All alcoholic fermentations were carried out in 500 ml flasks containing 250 ml of SGJ and incubated at 25 ºC, under gentle agitation (80 rpm). Alcoholic fermentations were carried out in duplicates and samples were taken daily to determine culturability, sugars consumption and ethanol production. Culturability (CFU/ml) of *S. cerevisiae* and *D. bruxellensis* during the fermentations was determined by the classical plating method. Briefly, to specifically differentiate CFU counts of *D. bruxellensis* and *S. cerevisiae*, we used YEPD agar medium supplemented with 0.01% of cycloheximide, as described in Branco et al. (2014) for *H. guilliermondii*. Sugars and ethanol concentrations in alcoholic fermentations were analysed using a High-Performance Liquid Chromatography (HPLC) system (Merck Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany). Fermentation samples were first filtrated by 0.45 μm Millipore membranes (Merck Millipore, Algés, Portugal) and then injected on a Sugar-Pak column (Waters Hitachi, Milford, USA) and eluted with a degassed aqueous mobile phase of CaEDTA (50 mg/l) at 90 °C using a flow rate of 0.5 ml/min. All samples were analysed in duplicate

2.9 Biopreservative potential of the AMPs in conjugation with sulphur dioxide and ethanol

To determine the biopreservative potential of the AMPs, simulated wines were prepared using a modified SGJ (pH 3.5), containing 4.5 g/l of residual sugars (glucose plus fructose) and ethanol at 10%, 12%, 13% and 14 % (v/v). Each simulated wine was artificially contaminated with *D. bruxellensis* at an initial cell density of 5×10^3 cell/ml. First, we evaluated the inhibitory effect of sulphur dioxide against *D. bruxellensis* by adding 25, 50, 100 and 150 mg/l of

potassium metabisulphite (PMB) (Sigma-Aldrich, Missouri, EUA) to a final volume of 300 µl of each simulated wine (i.e. modified SGJ with 10%, 12%, 13% and 14% (v/v) of ethanol).

The assay to define the inhibitory effect of ethanol in *D. bruxellensis* was performed in the same conditions above-described, using 10 %, 12 %, 13 % and 14 % (v/v) of ethanol without PMB. Control assays were performed using SGJ at pH 3.5 without the inhibitory factor (PMB or ethanol). The conjugated effect of PMB and ethanol was also assessed by testing the combinations of 10 %, 12 % 13 % or 14 % (v/v) of ethanol with 25, 50, 100 and 150 mg/l of PMB in a final volume of 300 µl of SGJ at pH 3.5. *D. bruxellensis* was inoculated at an initial cell density of 5×10^3 cell/ml in each assay.

The synergistic effect of AMPs (obtained as described in section 2.2), PMB and ethanol was evaluated by performing inhibitory-assays using mixtures of different concentrations of ethanol (10 %, 12 %, 13 % and 14 % (v/v)), AMPs (0.25, 0.5 and 1 mg/ml) and PMB (50 and 25 mg/l), under the conditions described before. Control-assays were performed in 300 µl of SGJ at pH 3.5, containing each of the above-mentioned ethanol concentrations but without AMPs and PMB. All inhibitory-assays were performed in triplicates in 96 well-microplates and 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. 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.

3. RESULTS AND DISCUSSION

3.1 Sensitivity of *P. pastoris* and *S. cerevisiae* strains to the GAPDH-derived AMPs

Recently, AMPs have received increasing attention as potential novel pharmaceutical agents. As a result, production of large quantities of AMPs in an economically viable process is required (Ingham and Moore, 2007; Li, 2009).

AMPs can be reliable prepared by chemical synthesis, but this is extremely expensive. Isolation from natural sources rarely meets the requirements for quantity and cost-efficiency and is typically a complex and time-consuming process; therefore, it is not an efficient way for obtaining AMPs in large amounts (Park, 1998; Pyo et al., 2004; Xu et al., 2007; Li, 2009). The

recombinant approach is relatively low cost and easy to scale up, being a more attractive methodology for large-scale production of AMPs (Li, 2009). *E. coli* and yeast are the two major systems used to produce recombinant AMPs. The first yeast employed to produce recombinant proteins was *S. cerevisiae*, since vast genetic techniques are available for this species (Li et al., 2005; Xu et al., 2007).

In order to select the most adequate host for cloning and expressing the GAPDH-derived AMPs, *S. cerevisiae* CCMI 885, *P. pastoris* GS115 and *S. cerevisiae* PYCC 5484 were previously tested for their sensitivity to these AMPs. *S. cerevisiae* strains CCMI 885 and *S. cerevisiae* PYCC 5484 were tested, since they both are wine yeasts and are able to produce high amounts of AMPs (CCMI 885) (Branco et al., 2017). As for *P. pastoris* GS115, it is a commonly used strain for high expression levels of recombinant proteins. We tested sensitivity to four concentrations of the purified AMPs (0.125, 0.25, 0.5 and 1 mg/ml) by following growth during 48 h in the presence and in the absence of the AMPs (control assay). Results presented in **Table 2** show that *S. cerevisiae* PYCC 5484 was the most resistant strain to the AMPs, with no growth inhibition observed as compared to the control assay. All the other yeasts showed sensitivity to the different concentrations of the AMPs tested (**Table 2**). Given these results, *S. cerevisiae* PYCC 5484 was chosen as the host strain for heterologous expression of partial sequence of *TDH1* (925-963 pb) gene (corresponding to AMP1) and *TDH2* (925-963 pb) gene (corresponding to AMP2/3).

Table 2: Sensitivity of *P. pastoris* GS115, *S. cerevisiae* CCMI 885 and *S. cerevisiae* PYCC 5484 to different concentrations of the AMPs. Values are represented in percentage of growth inhibition (measured by absorbance, 590 nm) relative to the control assay (without AMPs).

Yeast strains	**Growth inhibition (%)**			
	AMPs (mg/ml)			
	1	**0.5**	**0.25**	**0.125**
P. pastoris GS115	32.4	13.0	11.1	9.0
S. cerevisiae CCMI 885	42.8	33.0	13.9	12.0
S. cerevisiae PYCC 5484	0	0	0	0

3.2 Expression levels of the nucleotide sequences inserted in *S. cerevisiae* strain K1 and concentration of the AMPs secreted during fermentation

Evaluation of the expression levels of the inserted nucleotide sequences in the transformed *S. cerevisiae* strains, p*TDH1* and p*TDH2*, by RT-qPCR

Single alcoholic fermentations of *S. cerevisiae* K1 (empty plasmid), *S. cerevisiae* p*TDH1* and *S. cerevisiae* p*TDH2* were performed (data not shown). The relative expression levels of the partial *TDH1* (925-963 pb) gene and of the partial *TDH2* (925-963 pb) gene were evaluated by RT-q PCR. Relative expression levels of the nucleotide sequences inserted in *S. cerevisiae* strains, which codify for the AMP1 and AMP2/3, were normalized with the expression levels of the corresponding genomic sequences. Changes in levels of AMPs mRNAs of interest were detected in both strains. *S. cerevisiae* p*TDH1* and *S. cerevisiae* p*TDH2* strains express higher levels of the partial *TDH1* (1.5 fold) and partial *TDH2* (0.8 fold), relative to the genomic expression of partial *TDH1* and partial *TDH2*, respectively (**Fig. 1**). As expected, the quantification cycle (Cq) of both plasmidic partial sequences of *TDH1* and *TDH2* did not change significantly along the fermentation time (data not shown). Consequently, changes in relative expression of these peptides reflect the different levels of expression of both genomic partial sequences of *TDH1* and *TDH2* along the fermentation (**Fig. 1**). At 24 h, when Tdh1 is mostly synthesised (beginning of stationary phase), the relative expression level of the plasmidic partial sequence *TDH1* in *S. cerevisiae* p*TDH1* is lower than the expression level at 12 h, when *TDH1* gene is less expressed (Boucherie et al., 1995). As for partial sequence *TDH2*, the relative expression levels increased along the fermentation time (**Fig. 1**), reflecting the decrease of genomic expression of *TDH2*. These results are in agreement with the study of Varela et al. (2005) under winemaking conditions, showing that the expression levels of *TDH1* in *S. cerevisiae* during the exponential phase was lower than the expression levels detected in the early-stationary and late-stationary phase, the opposite being observed for *TDH2* expression levels.

Determination of AMPs levels secreted by *S. cerevisiae* strains by indirect ELISA

The total production of AMPs by each modified *S. cerevisiae* strain was quantified by indirect ELISA. Samples used were the < 10 kDa peptidic fraction obtained from supernatants

collected after 12 h, 24 h, 48 h and 196 h of single fermentations. Comparatively with the wild type strain *S. cerevisiae* K1, both modified yeasts produced higher concentrations of the AMPs (**Fig. 2**). At 12 h, *S. cerevisiae pTDH1* produced 44% more and *S. cerevisiae* p*TDH2* 18% more AMPs than *S. cerevisiae* K1 strain **(Fig. 2**). As expected from the above results, at the beginning of the stationary phase (24 h-48 h) *S. cerevisiae* p*TDH1* and *S. cerevisiae* p*TDH2* reached the maximum production of AMPs. *S. cerevisiae* p*TDH1* produced 64% (24 h) and 65% (48 h) more AMPs and *S. cerevisiae* p*TDH2* produced 24% (24 h) and 26% (48 h) more AMPs than *S. cerevisiae* K1, respectively (**Fig. 2**). These results are in agreement with higher extracellular accumulation of AMPs observed at the beginning of the stationary phase of growth (24 h-48 h) in alcoholic fermentations (Albergaria et al., 2010).

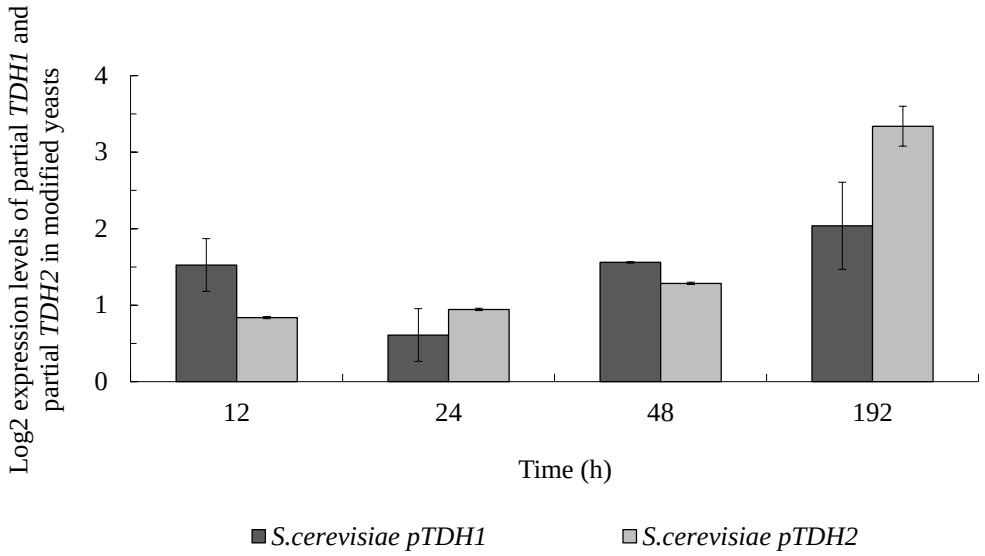

Fig. 1: Analyses by RT-qPCR of inserted nucleotide sequence *TDH1* (925-963 pb) and *TDH2* (925-963 pb) in *S. cerevisiae* p*TDH1 S. cerevisiae* p*TDH2* at 12 h, 24 h, 48 h and 192 h of fermentation in SGJ medium. The relative expression of the inserted partial sequence of *TDH1* and *TDH2* was normalized against the respective genomic partial sequence of *TDH1* and *TDH2*. Values represented are means ± SD (error bars) of the Log2 expression values of two independent biological experiments analysed in triplicate by RT-qPCR. The cut-off value was set to 1.5.

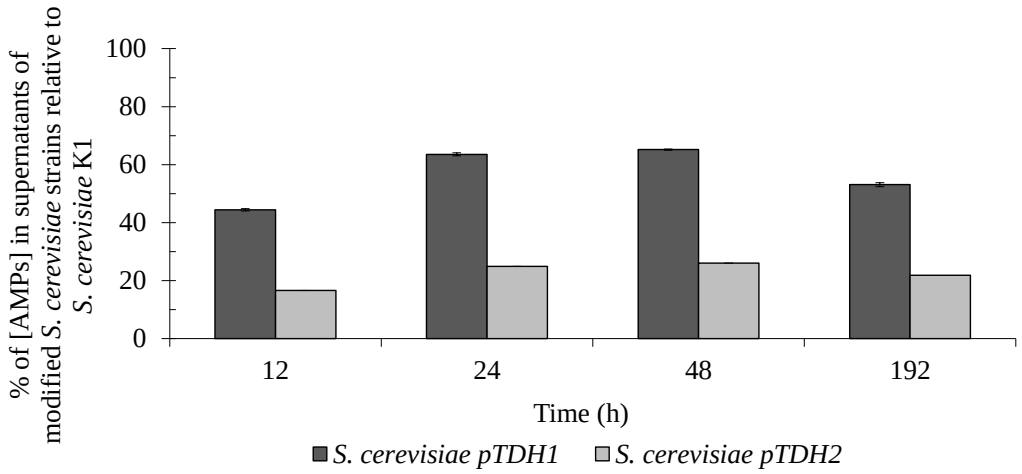

Fig. 2- Total AMPs production by *S. cerevisiae pTDH1* and by *S. cerevisiae pTDH2* relative to the production by *S. cerevisiae* K1 analysed by indirect ELISA. The bioactive peptidic fraction (< 10kDa) containing the AMPs was obtained from time 12 h, 24h, 48 h, and 192 h of *S. cerevisiae* strains alcoholic fermentations. Values represented are means ± SD (error bars) of two independent biological experiments analysed in triplicate by indirect ELISA.

3.4 Mixed-cultures alcoholic fermentations performed with *S. cerevisiae* K1, and with the modified strains *S. cerevisiae pTDH1* and *S. cerevisiae pTDH2* with *D. bruxellensis*

In guided fermentations, the actively growing starter culture dominates the native yeast species present in grape must and dominate the fermentation process (Pretorius, 2000). The initial viable population of yeasts in the grape musts, usually ranging 10^4-10^6 CFU/ml, is often decreased by the use of pesticides in the vineyards and antiseptics in the musts (Henick-Kling et al., 1998; Guerra et al., 1999). To simulate wine fermentations with addition of *S. cerevisiae* starter cultures, mixed fermentations of *S. cerevisiae* strains with *D. bruxellensis* were performed in SGJ medium using an initial higher cell density of *S. cerevisiae* strains (10^5 cells/ml) than of *D. bruxellensis* cells (5×10^3 cells/ml) (**Fig.3**). Mixed fermentations with 10^5 cells/ml of both species were performed as control for initial cell viability dependent behaviour (**Fig.4**). Single fermentation of *D. bruxellensis* inoculated with 5×10^3 cells/ml and 1×10^5 cells/ml, were performed as control for the antagonistic effect of *S. cerevisiae* (data not shown).

In both single fermentations (control for the antagonistic effect of *S. cerevisiae*), the cell density of *D. bruxellensis* reached approximately 5×10^7 CFU/mL in 48 h, and this viability was maintained until the end of fermentation (data not shown). Cell growth profiles in all mixed-culture alcoholic fermentations showed that both yeasts grew together in the first day of fermentation, then *D. bruxellensis* viability began to decrease, independently of the initial cell density (**Fig. 3-A, B, C** and **Fig. 4-A, B, C**). Comparing the profiles of *D. bruxellensis* with both initial cell densities, during the mixed-culture alcoholic fermentations with *S. cerevisiae* K1 (**Fig. 3-A** and **Fig. 4-A**) and with the modified *S. cerevisiae* strains *pTDH1* and *pTDH2* (**Fig. 3-B, C, Fig. 4-B, C**), it is clear that both modified *S. cerevisiae* strains have a stronger negative effect on *D. bruxellensis* than the wild type strain *S. cerevisiae* K1. The cell viability of *D. bruxellensis* was entirely lost within the first 96 h (for both initial cell densities) in mixed fermentation with *S. cerevisiae pTDH1* (**Fig. 3-B** and **Fig. 4-B)** and within 144 h and 192 h for mixed-culture fermentations performed with *S. cerevisiae pTDH2* with 5×10^3 and 1×10^5 initial cell densities, respectively (**Fig. 3-C** and **Fig. 4-C)**. In contrast, in mixed-culture fermentations of *S. cerevisiae* K1 with *D. bruxellensis*, the viability of *D. bruxellensis* was never completely lost within 192 h, even when the initial cell density of *D. bruxellensis* was lower than the initial cell density of *S. cerevisiae* K1, decreasing approximately four orders of magnitude in both cases (**Fig. 3-A** and **Fig. 4-A**). These results show that the modified *S. cerevisiae* strains exert higher antagonist effect than the wild type strain. Additionally, we also observed that the viability of *D. bruxellensis* in mixed-cultures fermentations with *S. cerevisiae* strains was equally affected independently of its initial cell density (**Fig. 3** and **4**).

Several harsh conditions are imposed to yeasts in wine environment such as osmotic stress caused by high sugar concentrations, low pH values, low oxygen availability, low nitrogen concentrations and high levels of organic acids and ethanol accumulation, with deleterious effects on yeast metabolism and growth (Ingram and Buttke, 1984; Bauer and Pretorius, 2000; Pretorius, 2000). *S. cerevisiae* dominance over other microbial competitors has always been attributed to their higher capacity to withstand the increasingly adverse conditions occurring as the fermentation proceeds, namely the high levels of ethanol present in the medium.

Regarding ethanol effect, we also evaluated the relationship between ethanol concentration and *D. bruxellensis* viability along mixed fermentations. In mixed-cultures fermentation, viability of *D. bruxellensis* decreased independently of ethanol accumulation in the

medium. In the presence of *S. cerevisiae* K1, when ethanol concentration reached 57 g/l (96 h) (**Fig. 3-D**), *D. bruxellensis* viability was 1.8×10^5 CFU/ml (**Fig. 3-A**). Conversely, in the presence of *S. cerevisiae pTDH1* for a similar level of ethanol (58 g/l at 48 h) (**Fig. 3-E**), *D. bruxellensis* viability was merely 4×10^3 CFU/ml (**Fig. 3-B**). This effect was also observed in mixed-culture fermentation of *S. cerevisiae pTDH2/D. bruxellensis* **(Fig. 3-C**), although less severe than for *S. cerevisiae pTDH1*. In this condition, the ethanol concentration was 48 g/l at 48 h (**Fig. 3-F**), and the viability of *D. bruxellensis* (**Fig. 3-C**) was one order of magnitude lower (1.5×10^4 CFU/ml) than its viability in the presence of *S. cerevisiae* K1 (1.77×10^5 CFU/ml) (**Fig. 3-A**), even at higher concentration of ethanol (58 g/l) (**Fig. 3-D**). A similar result was observed in the mixed-culture fermentations performed with higher cell density of *D. bruxellensis* (**Fig. 4**).

These results clearly showed that ethanol concentration is not responsible for the differences detected in the loss of viability of *D. bruxellensis* on those fermentations (**Fig. 3** and **Fig.4**). *S. cerevisiae pTDH1* produces greater amounts of AMPs (**Fig. 2**) and higher amounts of AMP1 and show a higher antagonistic effect in mixed-culture alcoholic fermentation on *D. bruxellensis* cell health than the *S. cerevisiae* K1 strain or the *S. cerevisiae pTDH2* (**Fig. 3** and **4**). The effect of *S. cerevisiae pTDH1* on *D. bruxellensis* could be enhanced by the fact that AMP1 exhibited a much stronger antimicrobial effect than the AMP2/3 (Branco et al., 2017).

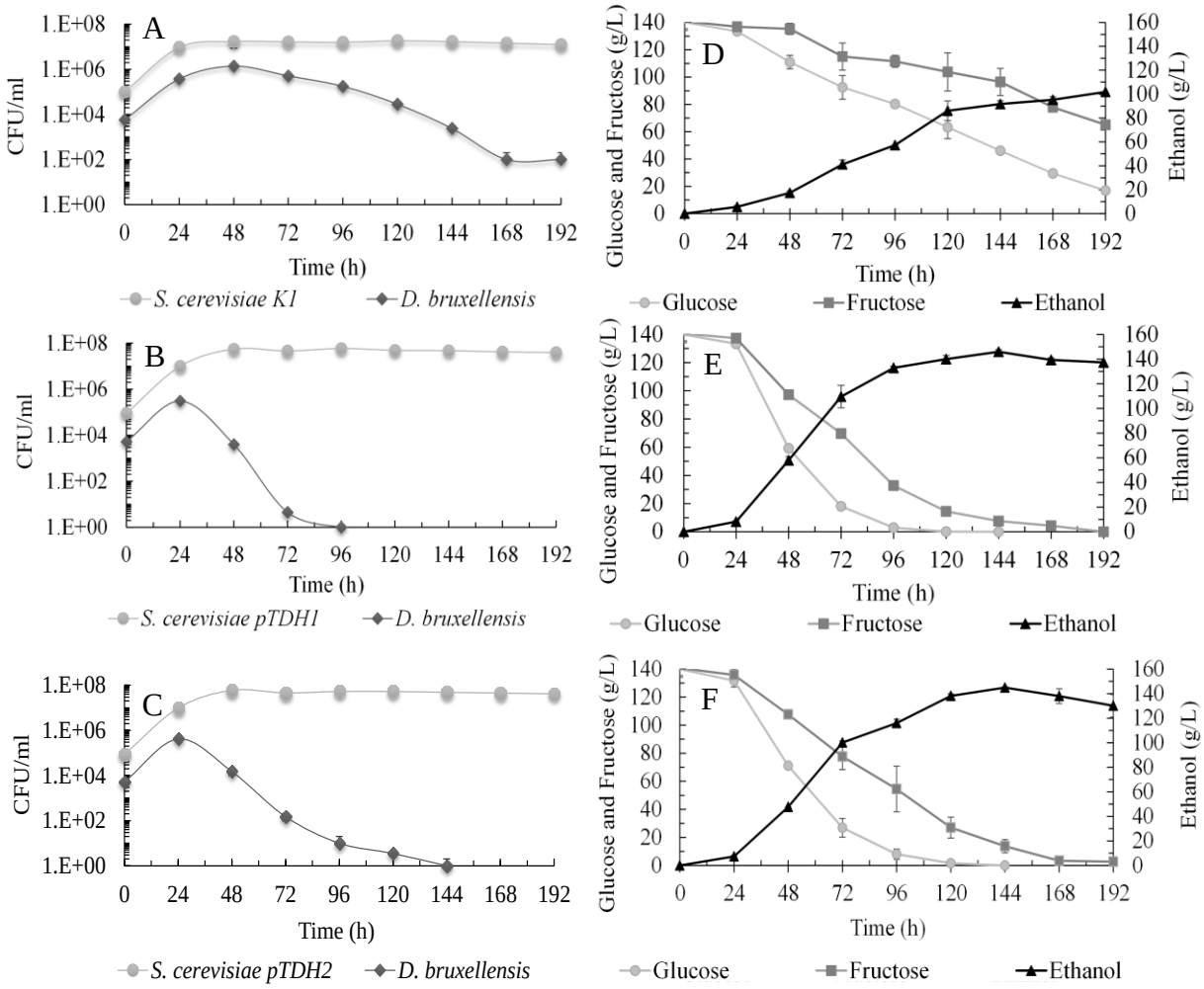

Fig. 3: Cell growth profiles of *S. cerevisiae* (initial cell density 10^5 cell/ml) and *D. bruxellensis* (initial cell density 5×10^3 cell/ml) (**A,B,C**), sugar consumption and ethanol production (**D,E,F**) during alcoholic fermentations performed with mixed cultures of *D. bruxellensis* with *S. cerevisiae* wild type strain K1 **(A)**, *S. cerevisiae pTDH1* **(B)** and *S. cerevisiae pTDH2* **(C)**. Data represented correspond to means ± SD (error bars) of duplicate independent assays.

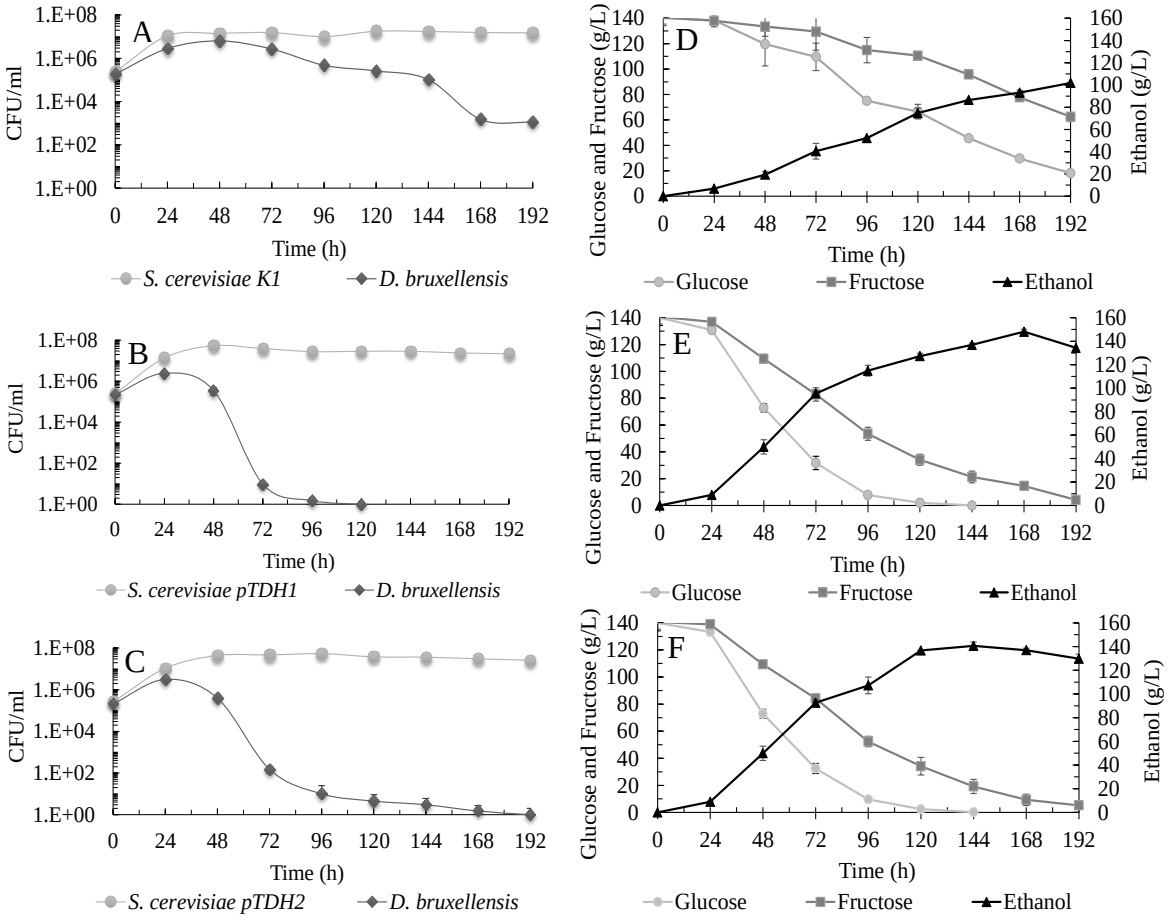

Fig. 4: Cell growth profiles of *S. cerevisiae* (initial cell density 10^5 cell/ml) and *D. bruxellensis* (initial cell density 10^5 cell/ml) **(A, B, C)**, and sugar consumption and ethanol production (**D, E, F**) during alcoholic fermentations performed with mixed cultures of *D. bruxellensis* with the *S. cerevisiae* wild-type strain K1 (**A**), *S. cerevisiae pTDH1* (**B**) and *S. cerevisiae pTDH2* (**C**). Data represented correspond to means ± SD (error bars) of duplicate independent assays.

3.5. Biopreservative potential of the AMPs in conjugation with potassium metabisulphite

The metabolic products of *D. bruxellensis* in wines are tetrahydropyridines, acetic acid, and volatile phenols, such as 4-ethylphenol (Loureiro and Malfeito, 2003). *D. bruxellensis* is the only responsible for the presence of 4-ethylphenol in wine that confer phenolic off-odours described as ‘‘barnyard-like’’ or ‘‘horsey’ (Fugelsang 1997; Dias et al., 2003). In order to avoid the development of undesirable microorganisms during winemaking, such as *D. bruxellensis*, the most conventional preservative is sulphur dioxide (Ribéreau-Gayon et al., 2006). In winery practice, sulphur dioxide may be added directly or as potassium metabisulphite (PMB) (Ribéreau-Gayon et al., 2006).

The use of adjuvants, complementing the preservative effect of sulphur dioxide, could be a solution to reduce the amount of this substance added to the wine. Therefore, the effect of these AMPs in conjugation with the major preservative in wine, sulphur dioxide, in the growth of *D. bruxellensis* ISA 2211 (a strain isolated from Douro red wines) was evaluated.

First, we tested the capacity of our *D. bruxellensis* strain to withstand different concentrations of PMB and/or ethanol. Results showed that *D. bruxellensis* was able to reach a cell density of approximately 3×10^8 CFU/ml, after 72 h, in the presence of all the ethanol concentrations tested, 10%, 12 %, 13 %, and 14 %, (**Table 3**). Likewise, when exposed to different concentrations of PMB (25, 50, 100 and 150 mg/l), *D. bruxellensis* reached the same cell density in 72 h (**Table 3**).

Table 3: Culturability (CFU/ml) of *D. bruxellensis* inoculated in a modified SGJ, without ethanol and without potassium metabisulphite (PMB) (control), with 10%, 12%, 13% and 14% (v/v) of ethanol (without PMB), and with 25, 50, 100 and 150 mg/l of PMB (without ethanol). Values presented correspond to means (± SD) of duplicate measurements of three independent biological experiments.

Time (h)	**Culturability of *D. bruxellensis* (CFU/mL)**								
	Control	**Ethanol (% v/v)**				**PMB (mg/l)**			
		10	12	13	14	25	50	100	150
0	$(5.0\pm0.2)\times10^{3}$	$(5.0\pm0.2)\times10^{3}$	$(5.0\pm0.2)\times10^{3}$	$(5.0\pm0.2)\times10^{3}$	$(5.0\pm0.2)\times10^{3}$	$(5.0\pm0.2)\times10^{3}$	$(5.0\pm0.2)\times10^{3}$	$(5.0\pm0.2)\times10^{3}$	$(5.0\pm0.2)\times10^{3}$
24	$(1.8\pm0.2)\times10^{8}$	$(3.3\pm0.1)\times10^{6}$	$(2.7\pm0.5)\times10^{6}$	$(2.4\pm0.1)\times10^{6}$	$(1.6\pm0.1)\times10^{6}$	$(4.0\pm0.1)\times10^{5}$	$(3.7\pm0.1)\times10^{5}$	$(3.0\pm0.1)\times10^{4}$	$(7.0\pm1.2)\times10^{3}$
48	$(1.9\pm0.2)\times10^{8}$	$(3.4\pm0.2)\times10^{8}$	$(3.0\pm0.1)\times10^{8}$	$(2.6\pm0.1)\times10^{8}$	$(1.9\pm0.2)\times10^{8}$	$(3.5\pm0.1)\times10^{7}$	$(3.9\pm0.1)\times10^{7}$	$(2.9\pm0.5)\times10^{7}$	$(1.6\pm0.6)\times10^{7}$
72	$(3.1\pm0.1)\times10^{8}$	$(3.2\pm0.2)\times10^{8}$	$(3.3\pm0.1)\times10^{8}$	$(3.3\pm0.3)\times10^{8}$	$(2.8\pm0.2)\times10^{8}$	$(3.5\pm0.3)\times10^{8}$	$(3.2\pm0.1)\times10^{8}$	$(2.8\pm0.5)\times10^{8}$	$(2.8\pm0.1)\times10^{8}$

Combination of ethanol with PMB was also tested for 10 %, 12 %, 13 %, and 14 % of ethanol conjugated with 25, 50, 100 and 150 mg/l of PMB. Results showed that 10% and 12% ethanol were only lethal when conjugated with PMB above 100 mg/l (**Fig. 5-A, B**) while for 13% and 14% ethanol the maximum PMB concentration that allowed growth was 25 mg/l (**Fig. 5-C, D**).

In our previous work (Branco et al., 2017), we showed that the MIC of GAPDH-derived AMPs for *D. bruxellensis* is 1 mg/ml. The biopreservative potential of AMPs in conjugation with PMB was tested for AMPs at 0.25, 0.5 and 1.0 mg/ml combined with the PMB and ethanol concentrations that allowed *D. bruxellensis* growth (**Fig. 6**). All assays were performed in modified SGJ medium with 4.5 g/l of total sugars (glucose + fructose) at pH 3.5 in order to simulate the conditions at the end of wine fermentation. Conjugated effect of the lower concentrations of ethanol tested (10% and 12%) with non-lethal PMB concentrations and 0.5 or 0.25 mg/ml of AMPs allowed similar growth of *D. bruxellensis* as compared to control without AMPs (**Fig. 6-A**, **B**). On the contrary, 1 mg/ml of AMPs combined with 10% or 12% of ethanol plus 50 mg/l or 25 mg/l PMB inhibited the growth of *D. bruxellensis* for 72 h (**Fig. 6-A**, **B**). A much stronger effect was observed for the same AMPs concentration, in the presence of 13 % or 14% ethanol in which 25 mg/l of PMB completely abolish the culturability after 48 h (**Fig. 6-C**, **D**). Even though we cannot discard the presence of viable but non-culturable (VBNC) cells, from the work of Chandra et al. (2016), we can predict that *D. bruxellensis* VBNC cells won't be able to produce considerable levels of 4-ethylphenol.

Barata et al. (2008) tested PMB in a range of 40 to 210 mg/l against 17 strains of *D. bruxellensis* in red wine, showing that most of *D. bruxellensis* strains tested, including the strain tested in the present study, were not able to grow in the range of 100-150 mg/l of PMB. Interestingly, our results showed that the required PMB concentration to induce total death of *D. bruxellensis* in wines at typical ethanol concentrations (13 to 14%) was reduced to 25 mg/l, when combined with 1 mg/ml of GAPDH-derived AMPs (**Fig. 6 C**, **D**). Nevertheless, more studies using other *D. bruxellensis* wine strains should be performed to accurately estimate the adequate sulphur dioxide concentration to be used.

According to the European regulation (EC) nº 606/2009, a maximum sulphur dioxide of 150 mg/l and of 200 mg/l can be added to red and white wines, respectively, with residual sugar lower than 5 g/l (glucose+fructose) and with 10%-14% of ethanol. Addition of PMB to wines

corresponds to about 57% of total sulphur dioxide (Ribéreau-Gayon et al., 2006), which means that 25 mg/l of PMB correspond to 14.25 mg/l of sulphur dioxide. Our results show that the addition of 25 mg/ml of PMB is enough to prevent spoilage by *D. bruxellensis* in wines with 13% or 14% of ethanol if 1 mg/ml of AMPs were added, which is a significant decrease in comparison to the maximum legal amount.

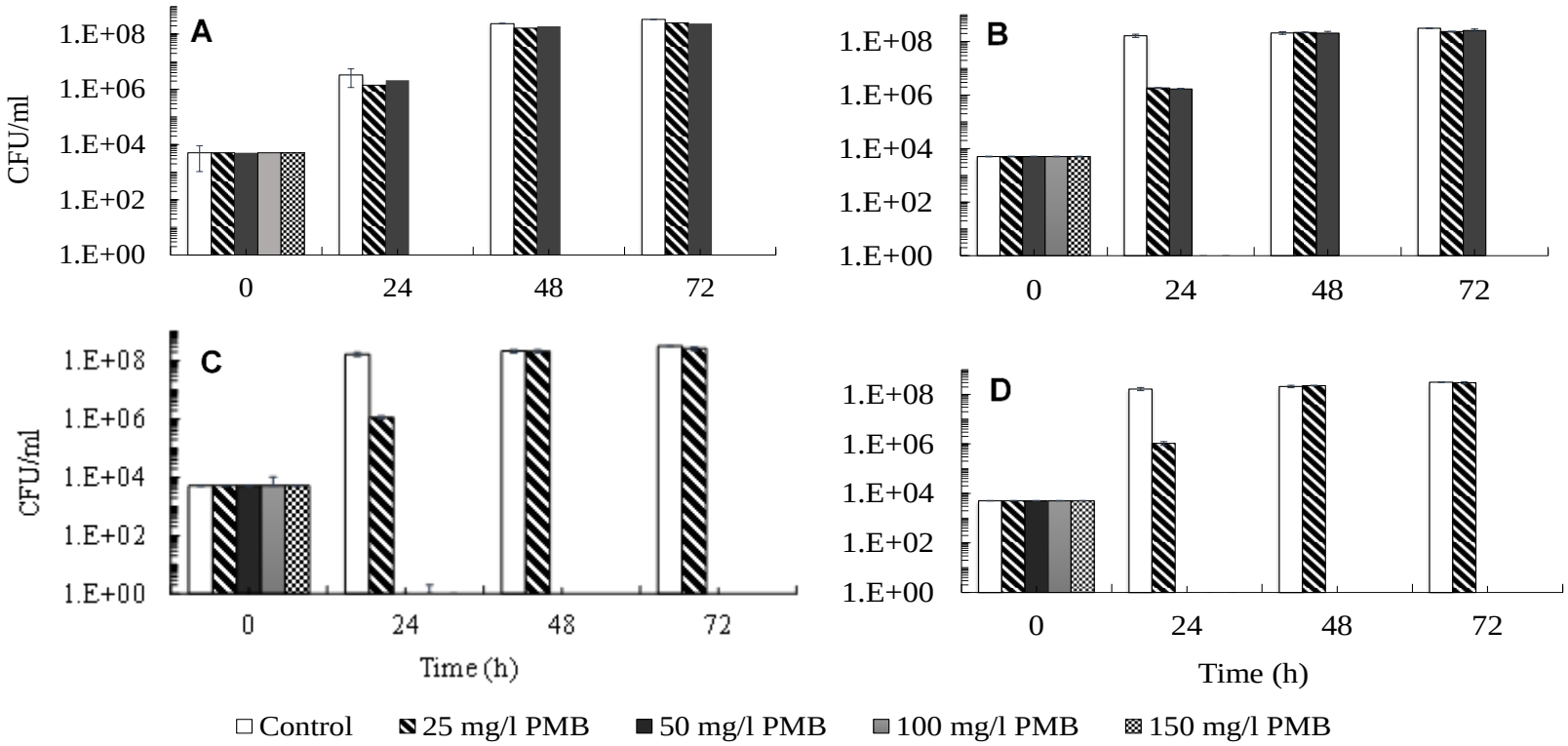

Fig. 5: Effect of potassium metabisulphite (PMB) at 25, 50, 100 and 150 mg/l on the cell viability (CFU/ml) of *D. bruxellensis* in simulated wines (SGJ with 4.5 g/l of residual sugars) with ethanol at 10% (v/v) **(A)**, 12% (v/v) **(B)**, 13% (v/v) **(C)** and 14% (v/v) **(D)**. Simulated wines were artificially contaminated with *D. bruxellensis* at an initial cell density of 5×10^3 CFU/ml. Control assays were performed in the same conditions without PMB. Data represented are means ± SD (error bars) of duplicate measurements of three independent biological experiments.

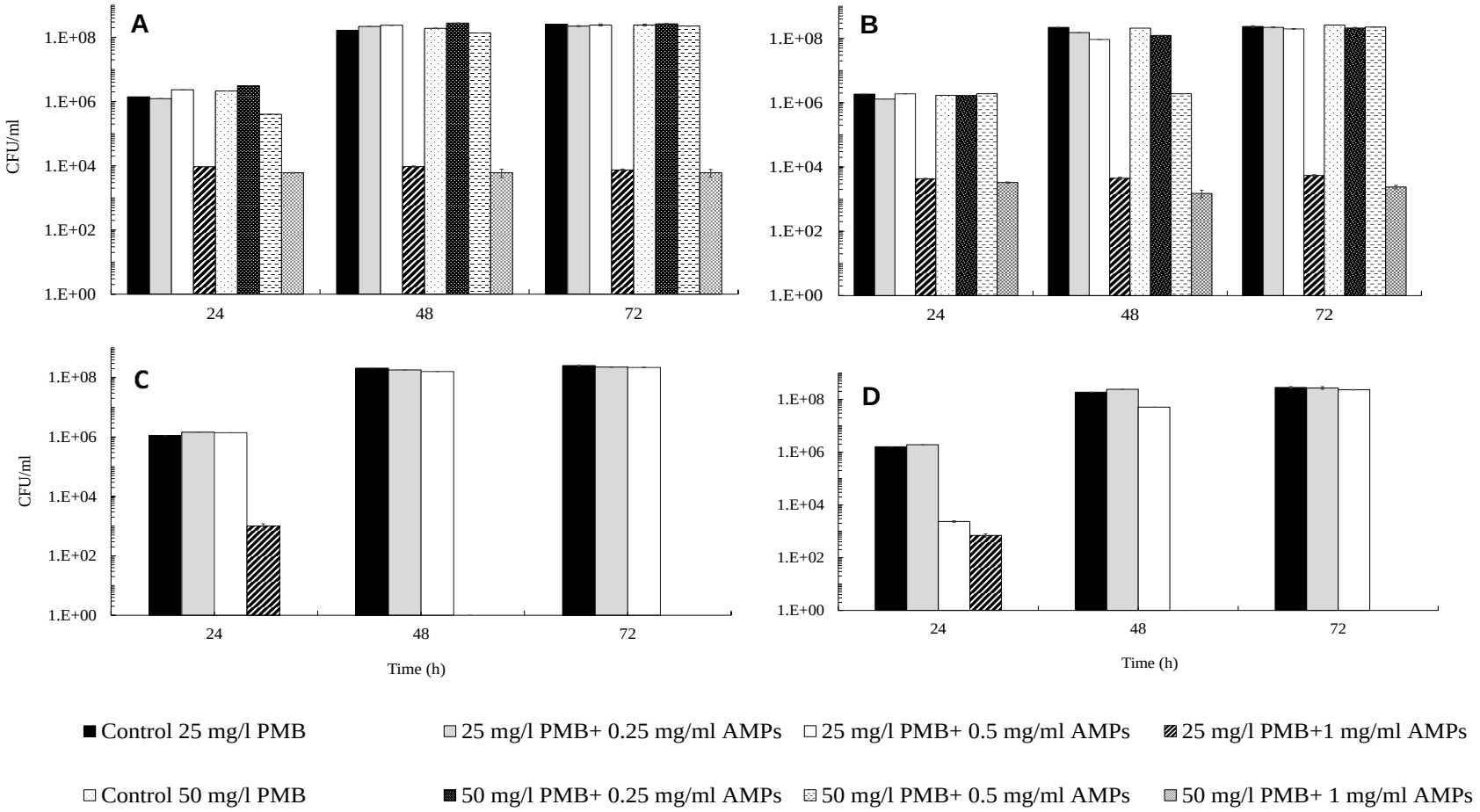

Fig. 6: Synergistic effect of the AMPs (at 0.25, 0.5 and 1.0 mg/ml) with potassium metabisulphite (PMB) at 50 mg/l (**A,B**) and 25 mg/l (**A,B,C,D**) on the cell viability (CFU/ml) of *D. bruxellensis* in simulated wines (SGJ with 4.5 g/l of residual sugars) containing 10% **(A)**, 12% **(B)**, 13% **(C)** and 14% (v/v) **(D)** of ethanol. Simulated wines were artificially contaminated with *D. bruxellensis* at an initial cell density of $5x10^3$ CFU/ml. Control assays were performed in the same conditions without the AMPs. Data represented are means ± SD (error bars) of duplicate measurements of three independent biological experiments.

The main goal of this study was to produce modified *S. cerevisiae* strains able to produce higher amounts of the GAPDH-derived peptides than the wild type strains. The modified *S. cerevisiae* strains were able to express higher levels of the sequences *TDH1* and *TDH2* coding AMP1 and AMP2/3, resulting in enhanced production of these AMPs than the respective wild type strain. Additionally, the modified *S. cerevisiae* strains exhibited a higher antagonistic effect on *D. bruxellensis* than the respective wild type strain. Our results also showed that addition of these AMPs significantly reduced the required concentration of PMB to inhibit the growth of *D. bruxellensis*, the major contaminant of wine industry. Further work in order to scale up the production of these AMPs is required. However, the possibility of using these AMPs as natural alternative biopreservative in alcoholic fermentations, wine and/or other food products looks promising to prevent economic losses due to microbial contaminations both wine industry and industrial fuel-ethanol production.

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Chapter VIII

Concluding remarks and future perspectives

Concluding remarks and future perspectives

Saccharomyces cerevisiae is, unquestionably, the most well-adapted yeast species to the wine environment, and consequently this species dominates wine fermentation (Bauer & Pretorius 2000; Bisson 1999; Hansen et al. 2001). Until recently, the studies on the yeasts population dynamics during wine fermentations ascribed the early death of non-*Saccharomyces* species (1-2 days of fermentation, 4-5 % v/v of ethanol) to their low fermentation capacity, as well as to their inhnability to survive under the harsh wine growth conditions, such as low: oxygen availability, depletion of nitrogen, low pH values, high levels of ethanol and organic acids (Bauer & Pretorius 2000; Bisson 1999). However, these factors do not entirely explain the succession of yeasts species throughout the fermentation process. Indeed, throughout the last decade, several authors (Albergaria et al. 2010; Comitini et al. 2005; Nehme et al. 2010; Nissen et al. 2003; Nissen & Arneborg 2003; Osborne and Edwards 2007; Renault et al. 2013) have raised other hypothesis to explain the early death of non-*Saccharomyces* yeasts during wine fermentations. Those hypotheses include the production of killer-like toxins such as antimicrobial peptides (AMPs) (Albergaria et al. 2010; Comitini et al. 2005; Nehme et al. 2010; Osborne & Edwards 2007) and death mediated by a cell-to-cell contact mechanism (Nissen et al. 2003; Nissen & Arneborg 2003; Renault et al. 2013). Specifically, Albergaria et al. (2010) discovered that the *S. cerevisiae* wine strain CCMI 885 excretes AMPs during alcoholic fermentations that are active against *Hanseniaspora uvarum, Hanseniaspora guilliermondii, Kluyveromyces marxianus, Lachancea thermotolerans* and *Torulaspora delbrueckii*.

In the present thesis we purified, sequenced and characterized those AMPs (Branco et al. 2014). We found that the naturally-secreted biocide, which we named saccharomycin, is composed of peptides derived from the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Branco et al. 2014). In order to investigate if secretion of saccharomycin was strain specific (strain CCMI 885), or a more general phenomenon of this species, we screened several *S. cerevisiae* strains for the production of these AMPs. Our study revealed that the GAPDH-derived AMPs are secreted by several *S. cerevisiae* strains (9 strains were screened), although at different levels. Thus, our results strongly suggest that this is a species-specific phenomenon of *S. cerevisiae* (Branco et al. 2017a). Besides, our work also

showed that saccharomycin has a large spectrum of action, being active against several non-*Saccharomyces* wine-related yeasts, such as *H. guilliermondii*, *Lachancea thermotolerans*, *Klyuveromyces marxianus*, *Torulaspora delbrueckii* and *Dekkera bruxellensis*, and bacteria such as *Oenococcus oeni* (Branco et al. 2014, 2017a). Taken together, our data demonstrates that secretion of GAPDH-derived AMPs by *S. cerevisiae* strains plays a decisive role in the yeasts population dynamics during wine fermentations. In summary, our work contributed to a better understanding of the factors underlying the yeasts growth pattern during wine fermentations, demonstrating for the first time that the competitive advantage of *S. cerevisiae* within the wine environment is not only due to its exceptional aptitude for alcoholic fermentation, but also due to a defensive strategy mediated by the secretion/excretion of GAPDH-derived AMPs.

Most AMPs are cationic in nature and interact with the anionic components of target cell membranes (Brogden 2005). Consequently, anionic antimicrobial peptides (AAMPs) are less common and exhibit a different mechanism of action and a weaker antimicrobial activity than cationic AMPs (Lai et al. 2002; Li 2009; Malkoski et al. 2001). Our work revealed that the natural biocide (saccharomycin) excreted by *S. cerevisiae* during wine fermentation is composed by two main anionic peptides (p*I*=4.35): AMP2/3 (peptide derived from the isoenzyme GAPDH2/3) and AMP1 (peptide from isoenzyme GAPDH 1) (Branco et al. 2014). Besides, we also found that the activity of the natural biocide is much higher than that of synthetic analogues, and seems to depend on the conjugated action of those two GAPDH-derived AMPs (i.e. AMP1 and AMP2/3) and of their relative proportion (Branco et al. 2017a, 2017c). In addition, it was not possible to test the antimicrobial activity of the synthetic AMPs at the acidic conditions used for saccharomycin (i.e. YEPD at pH 3.5), since the synthetic peptides were not able to dissolve (Branco et al. 2017a). These findings led us to suggest that the solubility and bioactivity of the natural biocide at the acidic conditions of wine (pH ranging 3.0-3.5) may result from its molecular structure that might involve some cationic metal and/or aggregates of several peptides. In fact, the activity of anionic AMPs can be enhanced by several factors, namely 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). Moreover, Dashper et al. (2005) showed that the antibacterial effect of an anionic AMP (kappacin) increases in the presence of the divalent metal cations Zn^{2+} and Ca^{2+}. In fact, when we investigated the internalization ability of the AMPs in non-*Saccharomyces* wine yeasts (*H. guilliermondii* and *D. bruxellensis*) we found that the

percentage of cells that internalised the AMPs was significantly higher when cells were incubated in YEPD (ca. 30%) instead of water (less than 10%) (Branco et al. 2017a). These results suggest that some component of the YEPD medium, possibly a metal cation, may enhance the activity of these AMPs However, to definitively confirm the involvement of divalent metal cations on the molecular structure of the native biocide it will be necessary to performed additional assays such as a structural analysis of the AMPs by nuclear magnetic resonance (NMR) after being incubated and bonded to divalent metal cations (Dashper et al. 2005).

The mode of action of AMPs on target cells has been extensly studied and includes a diverse range of antimicrobial mechanisms such as membrane permeabilization, which was first observed by Zasloff (1987) on protozoa exposed to magainin. Inhibition of ATPase activity and H^+ translocation was also described as a mechanism of action of lactoferrin on *Lactococcus lactis* which leads to a lethal perturbation of the intracellular pH and proton gradient (Andrés & Fierro 2010). Induction of apoptosis was also described as a mechanism of action of cecropin, an AMP of *Musca domesticain* on human hepatocellular carcinoma cell line BEL-7402 (Jin et al. 2010). This phenomenon was equally described for the killer toxins K1, K28 and zygocin produced by certain strains if *S. cerevisiae* that induce apoptosis in sensitive strains (Reiter et al. 2005). Therefore, to study the mechanisms involved in death induced by the GAPDH-derived AMPs identified in the present work, we evaluated membrane permeabilization, intracellular pH (pHi), proton influx/efflux rates and molecular markers typical of death by apoptosis on sensitive cells exposed to the AMPs. Results showed that the main target of our AMPs is the cell membrane since its permeabilization was observed in all the sensitive yeasts evaluated (Branco et al. 2015, 2017a). The drop of pHi was also detected in sensitive cells exposed to the GAPDH-derived AMPs (Branco et al. 2015), which could be a consequence of cell membrane permeabilization and/or due to destabilization of the plasma membrane H^+-ATPase activity. Indeed, a decrease of H^+-efflux rate was detected (Branco et al. 2017c), which can be correlated with the activity of plasma membrane H^+-ATPase (Opekarova & Sigler 1982). Besides, we also checked the effect of these AMPs on the glucose transport of sensitive yeasts since the activity of the plasma membrane enzyme H^+-ATPase is energy-dependent, thus depending on the availability of ATP (Rosa & Sá-correia, 1991; Serrano 1983). To check if the GAPDH-derived AMPs would affect glucose transporters and, in this way, compromise ATP availability, we determined the glucose uptake rates of *H. guilliermondii* in the presence/absence of the AMPs.

Our results showed that the AMPs do not negatively affect glucose transporters, thus, discarding the hypothesis of lower ATPase activity due to lack of ATP. However, to definitely confirm that the activity of plasma membrane H^+-ATPase is directly affected by these AMPs, it would be necessary to extract the plasma membrane of non-*Saccharomyces* yeasts after being exposed to the AMPs and then to determine the H^+-ATPase activity through colorimetric ATPase assays such as inorganic phosphate concentration measurements (ATP hydrolysis yields inorganic phosphate) (Andrés & Fierro, 2010). Apoptotic cell death induced by AMPs has been reported before by several authors (Jin et al. 2010, Reiter et al. 2005). Thus, we investigated molecular markers typical of death by apoptosis in sensitive yeast cells (*H. guilliermondii)* after being exposed to the AMPs. Typical molecular markers such as 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), were analysed in *H. guilliermondii* cells after being exposed to AMPs. Results showed that *H. guilliermondii* cells exhibited cellular markers characteristic of death by apoptosis such as DNA fragmentation, a typical late apoptosis phenomenon. This part of the work leads us to conclude that the physiological alterations induced by the GAPDH-derived AMPs on sensitive cells implicates membrane permeabilization, drop of intracellular pH (pHi), decrease of proton efflux, increase of proton influx and induction of apoptosis on target cells (Branco et al. 2015, 2017a, 2017c).

A pioneer study undertaken by Nils Arnerborg group suggested that direct microbial interactions mediated by a cell-to-cell contact mechanism could be involved in the early death of non-*Saccharomyces* yeasts during wine fermentations (Nissen et al. 2003; Nissen & Arneborg 2003). Since GAPDH is not only a glycolytic enzyme located in the cytosol, but also a cell wall-associated protein in *S. cerevisiae* (Delgado et al. 2001, 2003), we wonder if the GAPDH-derived AMPs secreted by *S. cerevisiae* strains could be involved in death mediated by cell-cell contact. Therefore, we investigated the reasons underlying cell-to-cell contact mechanism. Our results showed that 48 h-grown cells, conversely to 12 h-grown cells, of *S. cerevisiae* induce death of two non-*Saccharomyces* species (*L. thermotolerans* and *H. guilliermondii*) by direct cell-to-cell contact (Branco et al. 2017b). These results confirmed the previous results of Nissen et al. (2003) and Nissen & Arneborg (2003), showing that, indeed, death of *L. thermotolerans* during mixed-culture fermentations with *S. cerevisiae* is mediated by a cell-to-cell contact

mechanism. To confirm that the GAPDH-derived AMPs were were present in the cell wall of *S. cerevisiae* cells, we extracted membranes from 12 h-grown and 48 h-grown cells of different *S. cerevisiae* strains. Indeed, immunological assays and proteomic analysis revealed the presence of the GAPDH-derived AMPs on the surface of 48 h-grown cells of *S. cerevisiae* in higher amounts than on the surface of 12 h-grown cells of *S. cerevisiae*. Additionally, two spots from the membrane proteome (2D-gels) of 48 h-grown cells of *S. cerevisiae* were analysed by mass spectrometry (MALDI-TOF/MS) and results confirmed the presence of peptides derived from the GAPDH isoenzyme 1 in both spots, namely one peptide matching the exact sequence of the AMP1 (i.e. ISWYDNEYGYSAR) (Branco et al. 2017b). These results seem to clarify why non-*Saccharomyces* yeasts begin to die-off during wine fermentation only after cells attain the stationary growth phase (24-48 h), as previously found by Nissen and Arneborg (2003) and Pérez-Nevado et al. (2006). Besides, our study revealed that GAPDH accumulation in the cell-wall of *S. cerevisiae* seems to have a defensive function, although we still do not know how GAPDH is transported to the cell wall of *S. cerevisiae*, neither what triggers the formation of these GAPDH-derived peptides.

Experimental evidence indicates that mechanisms of secretion other than the endoplasmic reticulum-Golgi pathway can drive proteins outside the plasma membrane, which is the case of different glycolytic enzymes such as GAPDH and enolase that have been found on the surface of yeasts cell (Delgado et al. 2001; Nombela et al. 2006). The relevance of these secretion signals-less proteins in virulence and in the cell-wall dynamics of yeasts has remained unknown (Nombela et al. 2006). Work presented in chapter V (Branco et al. 2017b) raised, for the first time, experimental evidence that the presence of GAPDH on the surface of *S. cerevisiae* cells seems to be related with its antagonist effect against non-*Saccharomyces* wine yeasts. Besides, a study by Silva et al. (2011) reported that GAPDH is a specific substrate of yeast metacaspases, which play an important role in regulating apoptosis, and showed that the *in vivo* cleavage of GAPDH by metacaspases originates several GAPDH-derived fragments, namely some equal to the ones identified in the present work. Thus, we tested the antagonistic effect of a *S. cerevisiae* mutant strain deleted in the metacaspases gene *YCA*1 (*S. cerevisiae* Δ*yca*1) against a sensitive non-*Saccharomyces* yeast (Branco et al. 2014). Our results showed that the antagonistic effect of that mutant strain (Δ*yca*1) against *H. guilliermondii* during mixed-culture fermentations was less pronounced than the effect exerted by the respective wild type strain. Moreover, the cell surface

peptidic fraction of the Δ*yca*1 strain that corresponds to the fraction where the GAPDH-derived AMPs were identified did not show antimicrobial effect against *H. guilliermondii* (Branco et al. 2017b) Nevertheless, further work is required in order to discover how and why *S. cerevisiae* produces these peptides. Since the GAPDH-derived AMPs are produced in higher amounts only after *S. cerevisiae* cells attains the stationary growth phase (i.e. after 24 h) (data not shown) maybe this stress response results from scarcity of substrates and/or from apoptotic cells in which the GAPDH protein accumulated in the cell-wall is cleaved by metacaspases originating the GAPDH-derived AMPs.

In the last years, a growing number of studies have been carried out in order to understand the spoilage ability of *D. bruxellensis* and to establish the required control measures (Chandra et al. 2016; Loureiro & Malfeito-Ferreira 2003; Suárez et al. 2007). The microorganisms present in wine fermentations can be controlled using sulphur dioxide, which is the main preservative used in the wine industry. However, the addition of sulphur dioxide in excessive doses must be avoided due to its impact on human health (Ribéreau-Gayon et al. 2006). Thus, the last task of the present work, consisted in constructing a genetically-modified *S. cerevisiae* strains able to over-express the GAPDH-derived AMPs that could be used to prevent the growth of wine contaminants, particularly of *D. bruxellensis* (Branco et al. 2017d). With that purpose, a laboratory *S. cerevisiae* wine strain (strain k1) was genetically-manipulated and the partial genes codifying AMP1 and AMP2/3 were inserted in a plasmid. In addition, we also evaluated the antimicrobial activity of sulphur dioxide in combination with the GAPDH-derived peptides to verify the possibility of reducing the concentrations of this wine preservative. Results were promising since genetically-manipulated *S. cerevisiae* strains exhibited a higher antagonistic effect against one *D. bruxellensis* strain during mixed-culture wine fermentations and we were able to obtain recombinant *S. cerevisiae* strains that over-express and over-produce saccharomycin by comparison to the respective wild type strain (Branco et al. 2017d). Thus, these results give us the expectation that the implementation of a large-scale production of these AMPs is possible, since the recombinant approach is relatively low cost and easy to scale up. Furthermore, our results demonstrate that the concentration of sulphur dioxide that effectively induce death of the major contaminate of wine industry, *D. bruxellensis*, can be reduced if the GAPDH-derived AMPs are added. Nevertheless, more studies, using other *D. bruxellensis* wine strains, should be performed to accurately estimate the adequate sulphur dioxide concentration to

be used. Hence, the possibility of using these AMPs as natural alternative biopreservative in alcoholic fermentations, wine and/or other food products looks promising to help the reduction of economic losses in wine industry due to microbial contaminations, while reducing the concentration on non-healthy preservatives.

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