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Yeast adaptive response and tolerance to acetic acid stress: focus on the cell envelope

Ricardo Aguilar Andrade Ribeiro

Supervisor: Doctor Isabel Maria de Sá Correia Leite de Almeida

Thesis approved in public session to obtain the PhD Degree in Biotechnology and Biosciences

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ABSTRACT

The experimental eukaryotic model and cell factory *Saccharomyces cerevisiae* is an invaluable platform to study the mechanisms underlying yeast adaptive response, and tolerance to stresses that occur in industrial-relevant bioprocesses. The implementation of a circular bioeconomy demands the development of Advanced Yeast Biorefineries to produce biofuels or other bioproducts from organic residues. In this transition to a sustainable biobased economy, some non-*Saccharomyces* yeast species are gaining attention due to their high catabolic and biosynthetic versatility and tolerance to relevant bioprocess-related stresses.

Acetic acid is a major inhibitory compound in industrial bioprocesses, it is widely used as a preservative in food and beverages and is also a byproduct of alcoholic fermentation, that, together with high concentrations of ethanol and other toxic metabolites, leads to fermentation inhibition or arrest. Among the proposed mechanisms underlying adaptation and tolerance to this stress, is the alteration of the properties of the cell wall. The understanding of the molecular mechanisms underlying yeast adaptive response and tolerance to stress is essential to guide the genetic engineering of yeasts to develop more robust strains capable to cope with the challenging conditions to which they are exposed during industrial bioprocesses to guarantee their efficient growth and productivity and assure their economic viability. Stressing conditions trigger intricate cellular responses and, among them, are those involving cell wall remodeling, whose biochemical and biophysical properties can be tuned in order to better sense, respond and adapt to stress. The objective of this thesis work was to examine the role of the cell wall, its physico-chemical properties and cell-wall-biosynthesis and regulation-related signalling pathways during the different phases of the adaptive response of a cell population of *S. cerevisiae* to sudden exposure to acetic acid stress. Given the existing indications that alterations at the level of plasma membrane composition and biophysical properties can result in responses at the level of cell wall, the hypothesized crosstalk between plasma membrane and the cell wall was examined. For this, the ergosterol content was altered by altering the expression of the plasma membrane transporter ABC transporter Pdr18 involved in ergosterol transport and a determinant of tolerance to acetic acid and other stresses. The cell wall molecular and biophysical properties were characterized in the parental strain and in the deletion mutant *pdr18*Δ during the different phases of adaptation and growth under acetic acid stress. An increase of the cell wall resistance to lyticase activity of the parental strain was observed during acetic acid-induced growth latency. This alteration was correlated with the increase of cell wall

stiffness (assessed by AFM) , the increase of the content of cell wall β-glucans (assessed by fluorescence microscopy) and the slight increase of the transcript levels from the *GAS1* gene, encoding a β-1,3-glucanosyltransferase involved in elongation of β1,3-glucans chains. This result reinforces the notion that the adaptive yeast response to acetic acid stress involves a coordinate alteration of the cell wall at both biophysical and molecular levels, essential to limit the futile cycle associated to the re-entry of the toxic acid form after the active expulsion of acetate from the cell interior. A crosstalk between plasma membrane ergosterol content and cell wall biophysical properties was also demonstrated. However, despite the robust and more intense adaptive response registered in cells devoid of *PDR18*, involving the acetic acid-induced transcriptional activation of cell wall biosynthetic genes and an increased cell wall stiffness, the cell wall resistance to lyticase of the *pdr18*Δ mutant was below the one registered for the parental strain. Moreover, the duration of the period required for intracellular pH recovery to more physiological values and growth resumption was higher for the less tolerant *pdr18*Δ population.

Collectively, the results from this thesis work provide an integrative view on the role of the cell wall in adaptation and tolerance to acetic acid stress, and on the importance of membrane lipid composition in cell wall homeostasis during stress.

Keywords: *Saccharomyces cerevisiae*, adaptative response to acetic acid, tolerance to acetic acid; cell envelope, cell wall

RESUMO

A levedura *Saccharomyces cerevisiae* é um modelo experimental eucariótico e uma fábrica celular, sendo uma plataforma experimental de inestimável interesse para o estudo dos mecanismos subjacentes à resposta adaptativa e à tolerância a stresses de relevo em bioprocessos industriais. Adicionalmente, a implementação de uma bioeconomia circular sustentável exige o desenvolvimento de biorefinarias avançadas com base em leveduras para produzir biocombustíveis e outros bioprodutos a partir de resíduos orgânicos. Nesta transição para uma bioeconomia sustentável, algumas leveduras não-*Saccharomyces* estão a ganhar relevância devido à sua elevada versatilidade catabólica e biossintética e à elevada tolerância a stresses de relevo em bioprocessos industriais.

O ácido acético é um inibidor de elevado impacto em bioprocessos industriais, é amplamente utilizado como conservante de alimentos e bebidas e é também um subproduto da fermentação alcoólica que, em conjunto com elevadas concentrações de etanol e outros metabolitos tóxicos, contribui para a inibição ou paragem das fermentações. Entre os mecanismos propostos como subjacentes à adaptação e tolerância a este stress, destaca-se a alteração das propriedades da parede celular. A compreensão destes mecanismos moleculares é essencial para guiar a construção racional de leveduras no sentido de desenvolver estirpes com maior robustez face às múltiplas agressões a que estão expostas durante os bioprocessos industriais, de modo a garantir a sua máxima produtividade e viabilidade económica dos bioprocessos. As condições de stress desencadeiam respostas celulares complexas e, entre elas, as que envolvem a remodelação da parede celular, cujas propriedades bioquímicas e biofísicas podem ser moduladas para melhor perceção, resposta e adaptação ao stress. O objetivo do trabalho desenvolvido durante esta tese de doutoramento foi o de examinar o papel da parede celular, as suas propriedades físico-químicas e as vias de sinalização relacionadas com a sua biossíntese e regulação, durante as diferentes fases da resposta adaptativa de uma população celular de *S. cerevisiae*, subitamente sujeita a stress induzido por ácido acético. Uma vez que existem indicativos de que alterações que ocorrem ao nível da composição da membrana plasmática e das propriedades biofísicas podem suscitar respostas ao nível da parede celular, a hipótese de uma resposta coordenada entre a membrana plasmática e a parede celular foi examinada. Para o efeito, o conteúdo de ergosterol foi alterado através da expressão do transportador de ergosterol Pdr18, presente na membrana plasmática e pertencente à superfamília ABC, e um determinante de tolerância a ácido acético e outros stresses.

Foram caracterizadas as propriedades moleculares e biofísicas da parede celular durante as diferentes fases de adaptação e crescimento sujeito a stress induzido por ácido acético na estirpe parental e no mutante de eliminação *pdr18*Δ. Durante o estudo, observou-se um aumento da resistência da parede celular à atividade de liticase durante o período de latência de crescimento induzida por ácido acético. Esta alteração foi correlacionada com o aumento da rigidez da parede celular (avaliado por AFM), o aumento do conteúdo da parede celular em β-glucanos (avaliado por microscopia de fluorescência) e o ligeiro aumento dos níveis de transcritos do gene *GAS1*, que codifica uma β-1,3-glucanosiltransferase envolvida no alongamento das cadeias de β1,3-glucanos. Este resultado reforça a ideia de que a resposta adaptativa de levedura ao stress induzido por ácido acético envolve uma alteração coordenada da parede celular a nível biofísico e molecular, essencial para limitar o ciclo fútil associado à reentrada da forma tóxica do ácido após a expulsão ativa do contra-ião acetato do interior da célula. Foi ainda demonstrada a existência de uma "conversa cruzada" entre o conteúdo de ergosterol da membrana plasmática e as propriedades biofísicas da parede celular. Apesar da resposta adaptativa robusta e mais intensa observada em células do mutante com o gene *PDR18* eliminado, envolvendo a ativação transcricional dos genes biossintéticos de parede celular induzida por ácido acético e aumento da rigidez da parede celular, a resistência da parede celular à liticase do mutante de eliminação *pdr18*Δ revelou-se inferior à da estirpe parental. Acresce que a duração do período necessário para a recuperação do pH intracelular para valores mais fisiológicos e para a retoma do crescimento foi superior no caso da população menos tolerante de células *pdr18*Δ.

Coletivamente, os resultados do trabalho desenvolvido durante esta tese fornecem uma visão integrada do papel da parede celular na adaptação e tolerância ao stress induzido por ácido acético, e da importância da composição lipídica da membrana celular na homeostase da parede celular durante esse stress.

Palavras-chave: *Saccharomyces cerevisiae*, resposta adaptativa ao ácido acético, tolerância ao ácido acético; envelope celular, parede celular

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

ABC	ATP-Binding-Cassette
AFM	Atomic Force Microscopy
ATP	Adenosine TriPhosphate
cAMP	Cyclic Adenosine MonoPhosphate
cDNA	Complementary DeoxyriboNucleic Acid
CFW	CalcoFluor White
CHP	Cumene HydroPeroxide
CWI	Cell Wall Integrity
CWP	Cell Wall Protein
DMT	Derjaguin-Muller-Toporov
ESR	Environmental Stress Response
EUROSCARF	European *Saccharomyces cerevisiae* archive for functional analysis
FACS	Fluorescence-Activated Cell Sorting
FITC	Concanavalin A conjugated with Fluorescein
FTIR	Fourier Transform InfraRed
GDP	Guanosine DiPhosphate
GEFs	Guanine nucleotide Exchange Factors
GlcNAc	N-Acetylglucosamine
GPI	GlycosylPhosphatidylInositol
HOG	High Osmolarity Glycerol
HSR	Heat Shock Response
IgG	Immunoglobulin G
LoaOOH	Linoleic Acid Hydroperoxide
MAPK	Mitogen-Activated Protein Kinase
MAPKKK	Mitogen Activated Protein (MAP) Kinase Kinase Kinase
MDR/MXR	MultiDrug/MultiXenobiotic Resistance
MFS	Major Facilitator Superfamily
MIPC	MannosylInositol PhosphorylCeramide
MM4	Minimal growth Medium supplemented with amino acids and uracil

OD_{600nm}	Optical Density at 600nm
PBS	Phosphate Buffered Saline
PDC	Programmed cell death
PDR	Pleiotropic Drug Resistance
pHi	Intracellular pH
PKA	Protein Kinase A
pK_a	log(K_a), where K_a is the acid dissociation constant
qRT-PCR	Quantitative Real-Time reverse Transcription Polymerase Chain Reaction
RNA	RiboNucleic Acid
ROI	Regions Of Interest
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
TOR	Target Of Rapamycin
UDP	Uridine DiPhosphate
VFA	Volatile Fatty Acids
YPD	Yeast extract Peptone Dextrose

THESIS MOTIVATION AND OUTLINE

The budding yeast *Saccharomyces cerevisiae* is one of the most widely used microorganisms in industrial processes [1]. It has been considered a major cell factory due to its long-term traditional use in the production of fermented food and beverages, ease of handling and genetic manipulation [1–4]. Currently, some non-*Saccharomyces* (non-conventional) yeasts are gaining increasing attention due to promising features towards the implementation of a circular bioeconomy such as the ability to efficiently catabolize a wide range of carbon sources, remarkable metabolic capacities and high robustness against multiple stresses occurring during industrial bioprocesses [5].

During industrial bioprocesses, yeast cells must cope with different challenging conditions and assure adequate response mechanisms to assure growth and viability, as it is the case in their natural environments [6]. For bioprocesses to be sustainable and economically effective, it is fundamental to understand the mechanisms underlying yeast response and tolerance to relevant stresses for guiding the rational construction of superior industrial strains and the cultivation conditions.

Acetic acid is a short-chain weak organic acid used as a food preservative in the Food Industry. It is also considered a major inhibitory compound impacting industrial bioprocesses, in particular those using as feedstock lignocellulosic biomass hydrolysates[7,8]. This weak acid is also a byproduct of *S. cerevisiae* alcoholic fermentation and, together with high concentrations of ethanol and other toxic metabolites, contributes to growth and fermentation inhibition or even arrest, depending on the stress level and tolerance of the industrial strain [7–9]. To sense, survive, and adapt to different stresses, yeasts rely on a network of signalling pathways to modulate the global transcriptional response and elicit coordinated changes in the cell [10]. In particular, these pathways cooperate and tightly regulate the composition, organisation and biophysical properties of the cell wall [11]. The intricacy of the underlying regulatory networks reflects the major role of the cell wall as the first line of defence against a wide range of environmental stresses [11–13]. However, the involvement of cell wall in the adaptation and tolerance of yeasts to multiple stresses of biotechnological relevance has not received the deserved attention. The objective of this PhD thesis work was to examine the role of the cell wall, related signalling pathways and physico-chemical properties following *S. cerevisiae* sudden exposure to acetic acid stress and during the adaptation period and eventual growth of adapted cells under stress. Given that there were indications that changes occurring at the level of plasma membrane can also elicit responses at the cell wall level[14–20], the hypothesized crosstalk between plasma membrane ergosterol content, related with the level of expression of the plasma membrane ergosterol transporter

Pdr18, and cell wall biophysical properties was examined. Results confirmed the working hypothesis and suggested a coordinated response, at the level of the cell envelope, to counteract acetic acid deleterious effects.

The first chapter of this PhD thesis is a general introduction to the thesis work concerning the involvement of the yeast cell wall in the response and tolerance to stresses of Biotechnological relevance, providing a comprehensive overview of this field. Based on a review paper accepted for publication in the journal *Frontiers in Microbiology*, this chapter is dedicated to the complex molecular regulatory mechanisms involved in fine-tuning cell wall physicochemical properties during the stress response of *S. cerevisiae* and their implication in stress tolerance. A few successful attempts to improve stress tolerance through the manipulation of cell wall biosynthetic pathway are also described. The available information for non-conventional yeast species is also included since the understanding of all those issues is instrumental to explore or control their biological activity in Biotechnology, in particular, envisaging the transition towards a circular bioeconomy. The review paper was prepared by the PhD candidate, under the guidance of the PhD supervisor, Prof. Isabel Sá-Correia, who contributed to the text writing and revision. The co-author Nuno B. Melo has contributed to the literature search, preparation of figure 2 (figure 1.2 of this thesis), and to the first draft.

The second chapter of this thesis work provides a coordinate and comprehensive view of the time course of the alterations occurring at the level of the cell wall during adaptation of a yeast cell population to sudden exposure to a sub-lethal stress induced by acetic acid. The alteration of the molecular composition and the physical properties of the plasma membrane and the cell wall leading to decreased cell envelope permeability are among the proposed mechanisms for yeast adaptation to this acetic acid stress and considered essential to reduce the diffusion rate of this weak acid from the exterior to the intracellular medium [7,21–23]. Such response is useful to counteract the re-entry of the toxic non dissociated form of the acid, following the active expulsion of the counter-ion, acetate, from the cell interior, in this way avoiding the associated futile cycle [7,24]. However, biophysical and biochemical changes at the level of the cell wall in response to stress, in particular to acetic acid stress were unclear when this thesis work began. In this study, it was assessed the time-course of the alterations occurring at the level of yeast cell wall architecture during adaptation and growth of *S. cerevisiae* to acetic acid, based on cell wall susceptibility to lyticase activity and on cell wall stiffness (Young's modulus) assessed by atomic force microscopy. The transcriptional activation of cell wall biosynthesis-related genes was assessed by qRT-PCR and the content of different cell

wall polysaccharides by fluorescence microscopy. Collectively, results reinforce the notion that the adaptive yeast response to acetic acid stress involves a coordinate alteration of the cell wall at the biophysical and molecular levels [25]. This work was published as an original research article in the journal "*Scientific Reports*", (Ribeiro *et al.*, 2021)(doi: 10.1038/s41598-021-92069-3). The PhD candidate was the major contributor to this manuscript with collaborations from teams from two other laboratories where the candidate explored AFM and fluorescence microscopy techniques.

The third chapter´s objective was to examine the effect on cell wall remodeling of alterations occurring at the plasma membrane ergosterol composition during yeast adaptation and tolerance to acetic acid stress. For this, the hypothesized influence of the expression of the multidrug resistance plasma membrane ABC transporter Pdr18 in cell wall homeostasis under acetic acid stress was examined. Pdr18 is a multi-stress tolerance determinant, in particular a determinant of acetic acid tolerance due to its biological role as an ergosterol transporter at the plasma membrane level [23,26–29]. To test the hypothesis, the effect of *PDR18* expression in counteracting the decrease of plasma membrane ergosterol content and intracellular pH under acetic acid stress was assessed as well as in altering cell wall resistance to lyticase activity, cell wall stiffness (by atomic force microscopy), and cell wall polysaccharide composition (by fluorescence microscopy). Results revealed a crosstalk between plasma membrane ergosterol content and cell wall biophysical properties, suggesting a coordinated response to counteract the deleterious effects of acetic acid[30]. This study was published as an original research article in the "*Journal of Fungi*", (Ribeiro *et al.*,2022)(doi: 10.3390/jof8020103). The PhD candidate was the major contributor to this manuscript with collaborations from teams from two other laboratories where the candidate explored AFM and fluorescence microscopy techniques.

The fourth chapter is the final critical discussion of the results obtained during the thesis work and also includes future perspectives.

1. General Introduction

This chapter is based on the article accepted for publication:

Ribeiro RA., Bourbon-Melo N., Sá-Correia I. *The Cell Wall and the Response and Tolerance to Stresses of Biotechnological Relevance in Yeasts*. ***Frontiers in Microbiology***. 2022;13:953479. doi:10.3389/fmicb.2022.953479 *(in press)*

1.1 ABSTRACT

In industrial settings and processes, yeasts may face multiple adverse environmental conditions. These include exposure to non-optimal temperatures or pH, osmotic stress, and deleterious concentrations of diverse inhibitory compounds. These toxic chemicals may result from the desired accumulation of added-value bio-products, yeast metabolism, or be present or derive from the pre-treatment of feedstocks, as in lignocellulosic biomass hydrolysates. Adaptation and tolerance to industrially relevant stress factors involve highly complex and coordinated molecular mechanisms occurring in the yeast cell with repercussions on the performance and economy of bioprocesses, or on the microbiological stability and conservation of foods, beverages, and other goods. To sense, survive, and adapt to different stresses, yeasts rely on a network of signalling pathways to modulate the global transcriptional response and elicit coordinated changes in the cell. These pathways cooperate and tightly regulate the composition, organization and biophysical properties of the cell wall. The intricacy of the underlying regulatory networks reflects the major role of the cell wall as the first line of defence against a wide range of environmental stresses. However, the involvement of cell wall in the adaptation and tolerance of yeasts to multiple stresses of biotechnological relevance has not received the deserved attention. This chapter of this thesis work provides an overview of the molecular mechanisms involved in fine-tuning cell wall physicochemical properties during the stress response of *Saccharomyces cerevisiae* and their implication in stress tolerance. The available information for non-conventional yeast species is also included. These non-*Saccharomyces* species have recently been on the focus of very active research to better explore or control their biotechnological potential envisaging the transition to a circular bioeconomy.

1.2 INTRODUCTION

Saccharomyces cerevisiae is an essential eukaryotic cell model that also has a plethora of industrial applications [1]. Since early in human civilisation, it has been extensively used in the production of fermented food and beverages, which nowadays include bread, chocolate, wine, beer, cider, sake, spirits (rum, vodka, whisky, brandy), and other alcoholic beverages arising from the fermentation of fruits, honey, and tea [1,2]. Nowadays, many enzymes, pharmaceuticals, nutraceuticals, and other added-value bioproducts can be produced in engineered yeast cell factories [4,31–37]. The implementation of a circular bioeconomy requires the development of Advanced Yeast Biorefineries to produce biofuels (e.g., bioethanol and biodiesel), chemicals, materials, and other bioproducts from organic residues from agriculture, forestry, and industry residues [38–44]. For the transition to a biobased economy, the use of non-conventional yeasts is gaining momentum since strains of this heterogenous group of non-*Saccharomyces* species are advantageous alternatives to *S. cerevisiae* whenever they can natively express highly interesting metabolic pathways, assimilate a wider range of carbon sources, and/or exhibit higher tolerance to relevant bioprocess-related stresses [5]. Non-conventional yeasts are also being explored to enhance the flavor profiles and reduce the ethanol content of alcoholic beverages [45–47]. Spoilage yeasts, as is the case of the osmophilic and highly weak acid tolerant yeast species of the *Zygosaccharomyces* genera, are also being studied due to their high tolerance to stresses associated to food preservation [9]. However, the advanced genome-editing techniques and other genomic and bioinformatics information and tools available for *S. cerevisiae*, are arguably the major reason why this species is still considered the major yeast cell factory [3,4].

Regardless of the yeast species or the specific application, industrial yeasts typically encounter stresses that trigger intricate cellular responses involving multiple players [48–50]. Among these players is the cell wall. The cell wall maintains cell shape and integrity and, together with the plasma membrane, is part of the cell envelope and the first line of defence against multiple adverse environmental conditions [13]. The cell wall is a highly dynamic organelle whose biochemical and biophysical properties can be finely tuned as yeast cells encounter different stresses throughout the course of industrial bioprocesses [12,13]. Considerable cell energy must be invested for maintaining such complex and energetically expensive response, considering that over 1200 genes are estimated to be involved, directly or indirectly, in cell wall synthesis and regulation[51]. A deeper mechanistic insight into how the cell enacts such changes at the level of the cell wall is

expected to lead to a better understanding of yeast's adaptation to stresses of industrial relevance that ultimately may lead to improved bioprocess productivity and product yield. This understanding is also instrumental to guide the improvement of food preservation practices, in particular those involving the use of weak acid food preservatives, temperature- and osmotic- induced-stresses for the microbiological stabilization and conservation of foods, beverages and other goods.

The suggested role for the cell wall in yeast stress response and tolerance, mostly emerged from the datasets obtained from the numerous genome-wide expression analyses or chemogenomic analyses under stress reported in recent years. However, the cell wall has not received the attention it deserves as a tolerance determinant towards multiple stresses. The objective of this chapter of this thesis work is to gather the literature available on the topic and provide a critical opinion and a comprehensive view on the current knowledge on the role of the cell wall and related signalling pathways in yeast adaptation and survival under industrially relevant stresses. Attempts of stress tolerance improvement through the manipulation of cell wall biosynthetic pathway are also included. Most of the published knowledge pertains to the yeast model *S. cerevisiae* but the information available in the scientific literature for other yeast species of biotechnological relevance is also offered.

1.3 THE YEAST CELL WALL: COMPOSITION, STRUCTURE, FUNCTION AND BIOSYNTHESIS

The cell wall of *S. cerevisiae* represents up to 30% of the cell's dry weight (w/w) being almost entirely composed of polysaccharides (≈85%) and proteins (≈15%) [52] with a thickness of 115nm - 120nm, as determined by atomic force microscopy and ultrathin-sectioning electron microscopy [53,54]. It is a layered structure with two different layers (**Figure 1.1**), distinguishable by ultrathin-sectioning electron microscopy [54–56].

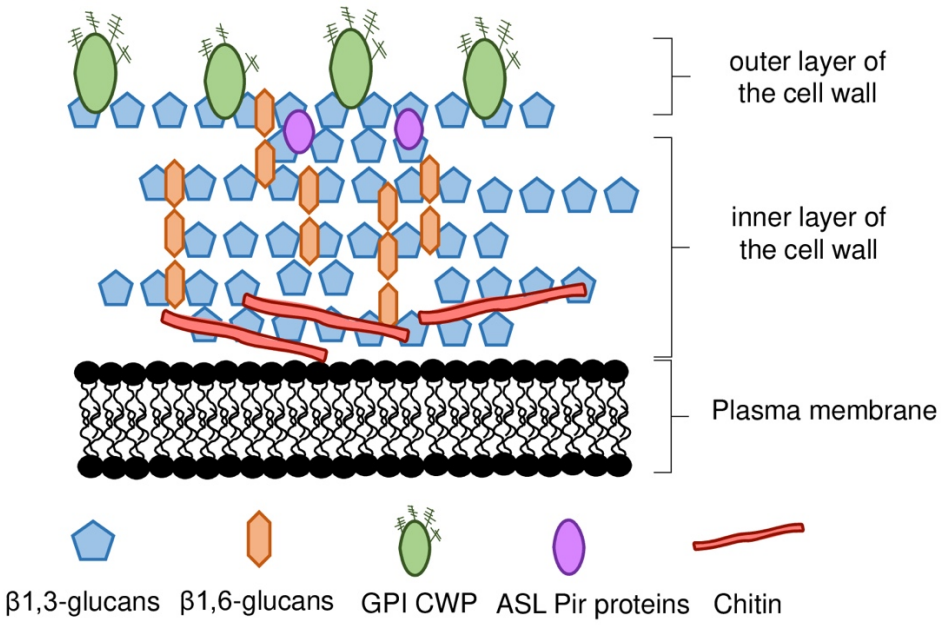

Figure 1.1 - Schematic representation of S. cerevisiae cell wall. The inner layer of the cell wall is mostly composed of β1,3-glucan chains branched with β1,6-glucans, and chitin. The outer layer is composed of mannoproteins, most of which are linked to the inner wall by a GPI anchor, whereas ASL-Pir proteins seem to be uniformly distributed throughout the inner layer. ASL-CWP: alkali-sensitive linkage cell wall protein. GPI-CWP: glycosylphosphatidylinositol cell wall protein. Details in the main text.

The electron-dense outer layer mostly consists of mannoproteins whereas, in the more electron-transparent inner layer, glucans are the major component and chitin is in a lesser extent [54–56]. Most of the cell wall mechanical strength derives from its inner layer, in which the β-linked glucans are the major components of the polysaccharide fraction, and alone represent 30 to 60% of the cell wall dry weight [57]. Approximately 30-60% of the cell wall dry biomass is composed by β-glucans, around 85% of these are 1,3-β-glucans and the remaining are 1,6-β-glucans [56,58] (**Figure 1.1**). The β1,3-glucan chains are composed of ≈1500 glucose units and are assembled in coiled spring-like structure that confer elasticity and tensile strength to the cell wall [12]. The β1,6-glucans chains are shorter than the β1,3-glucan chains and, although quantitatively being a minor component of the wall, β1,6-glucans have a central role in cross-linking β1,3-glucans

together [56,59]. Additionally, β1,6-glucans can be connected to mannoproteins with a glycosylphosphatidylinositol (GPI) anchor and to chitin [56,60]. The reducing ends of β1,3-glucans chains can be linked to a side-branching β1,6-glucan on β1,3-glucans chains, forming a fibrillar structure that serves as backbone and anchorage point for other constituents of the cell wall [61] (**Figure 1.1**). The nonreducing ends of 1,3-β-glucans are linked to the reducing ends of chitin through a β-1,4 link [56,59,60].

Chitin is a linear polymer of β1,4-linked *N*-acetylglucosamine (GlcNAc), representing less than 2% of *S. cerevisiae* cell wall dry weight (**Figure 1.1**). Chitin can occur both in the free form, or bound to β-glucans [56,59,60]. Chitin is normally concentrated as a ring between the mother cell and the emerging bud and in the lateral walls of the mother cell after septation [62]. In response to stress, chitin levels can increase to as much as 20% of the cell wall dry weight [56,63–68]. Changes occurring in cell wall nanomechanical properties, such as cell surface stiffness, are mostly dependent on the crosslinking between β-glucans and chitin [69] and occur in response to stresses of industrial relevance [25,30,69–71].

The outer layer is composed by cell wall mannoproteins (**Figure 1.1**). These mannoproteins are heavily glycosylated, modified with both N-and O-linked carbohydrates, commonly formed by mannose [72,73]. The outer layer serves an important protective role in the cell by limiting the access of external aggressors, such as foreign enzymes, to the inner layer [72]. The proteins that constitute this layer are involved in a wide range of functions often related to cell-to-cell interactions (e.g., mating, flocculation, biofilm formation, etc.) [12]. Increased cell wall hydrophobicity influences flocculation leading to increased robustness to inhibitory chemical compounds [74]. This was associated with changes in the expression of *MOT3* gene*,* encoding a transcription regulator that controls the expression of a cell wall protein encoding gene, *YGP1,* that influences cell wall hydrophobicity [74].

There are two major classes of cell wall proteins (CWPs), namely the glycosylphosphatidylinositol CWPs (GPI-CWPs), and the alkali-sensitive linkage CWPs (ASL-CWPs), which include proteins of the internal repeats (Pir) family [12,72] (Figure 1). While GPI-CWPs are typically linked to β1,6-chains by a GPI anchor, ASL-CWPs are directly linked to β1,3-glucans through an alkali-labile bond [61]. They also differ in their distribution throughout the cell wall. While GPI-CWPs are found in the outer layer, Pir-CWPs seem to be uniformly distributed throughout the inner layer, which is consistent with their direct association with β1,3-glucans [72,75] (**Figure 1.1**).

Cell wall biogenesis involves cell wall polysaccharide synthases and enzymes involved in cell wall remodeling, assembly and degradation [72,76] (**Figure 1.2**). The β1,3-glucans

are synthetized as a linear polymer by the *FKS* family of genes (*FKS1-3)*. The *FKS1* and *FKS2* genes code for β1,3-glucan synthases and differ mostly on the expression pattern, whereas *FKS3* remains poorly characterized [61,77–80]. While *FKS1* expression is prevalent under optimal growth, *FKS2* expression is induced in response to different stresses such as glucose depletion, alternative carbon sources (e.g., acetate, glycerol, galactose), high extracellular Ca^{2+} or heat stress [77–80]. Several genes influence β-1,6-glucan levels, in particular the *KRE6* and *SKN1* genes, encoding proteins involved in β1,6-glucan synthesis, and the *KRE9* and *KHN1* encoding cell wall proteins presumably involved in β1,6-glucan cross-link to other components of the cell wall [61,81]. Chitin is synthetized as a linear polymer by the chitin synthases encoded by the *CHS1-3* gene family. The gene *CHS3,* encoding chitin synthase III, is by far responsible for the majority of chitin synthesis, whether it is during optimal growth conditions or during stress response when increased chitin deposition in the cell wall occurs [56,61,72,82]. Also, cell wall protein mannosylation requires several genes encoding proteins with mannosyltransferase activity [56,83]. The *GAS* family (*GAS1*-5), encodes β1,3-glucanosyltransferases involved in β1,3-glucan branching and elongation, in which *GAS1* has a major role and is also required for β1,6-glucan linkage with β1,3-glucan chains [56,63,84,85]. Shortening of glucan chains is also required for cell wall remodeling and involves *BGL2* and *EXG1-2* encoding a major endoglucanase and major exoglucanases, respectively [56,61,85,86]. The transglycosylases GPI proteins encoded by the *CRH1-2* genes have a major role in the cross-linkage between chitin and glucans [56,62,87–89]. Endochitinases, encoded by *CTS1-2,* are required for septation and cell separation [56,90,91].

S. cerevisiae cell wall appears to have many organizational similarities with the walls of other ascomycetous yeasts and even of basidiomycetous yeasts [92–96]. However, there are differences between the cell walls of *S. cerevisiae* and other fungi. For instance, the cell wall of *S. cerevisiae* does not contain α-glucans, chitosan, or melanin, and its chitin content is relatively low, especially when compared to other yeasts or filamentous fungi (e.g., *Candida albicans, Aspergillus niger, Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*) [96–98].

1.4 CELL WALL-RELATED SIGNALING PATHWAYS INDUCED BY STRESS

Yeast cells sense and respond to environmental stresses through the induction and activity of different signalling pathways [10]. Depending on the type of stress, specific pathways can be triggered and directly, or indirectly, elicit changes in the composition and architecture of the cell wall [11] (**Figure 1.2**). The CWI signalling pathway is one of the most well-described pathways, characterized for its role in cell wall maintenance and

homeostasis, particularly in response to environmental external stimuli that may damage the cell wall [11,99,100] (**Figure 1.2**). CWI pathway relies on a family of plasma membrane sensors, namely Wsc1-3, Mid2, and Mtl1. Stimuli that may impose a stress to the cell are detected by these sensors and triggers a phosphorylation cascade that elicit transcriptional changes to enable the cell to adapt to stress conditions [100,101] (**Figure 1.2**). For the Wsc1 sensor, the signalling transduction to the downstream components of the pathway is influenced by the spatial distribution of these sensors within the plasma membrane, requiring a functional extracellular cysteine-rich domain to form clusters in specific microdomains or rafts [102,103]. Industrially relevant stresses reported to trigger a response by the referred CWI pathway membrane sensors are highlighted in **Table. 1.1**. Together with phosphatidylinositol 4,5-biphosphate, PIP_2, which recruits the guanine nucleotide exchange factors (GEFs) Rom1 and Rom2, the surface sensors activate the Rho1 GTPase [11,100]. Rho1 regulates both β1,3-glucan synthases encoded by *FKS1* and *FKS2*, and β1,6-glucan synthase activities, and is considered a key regulator of CWI signalling [11]. Rho1 activates Pkc1 and sets in motion a series of phosphorylation events which sequentially activate Bck1, Mkk1/2, and the pathway's MAPK, Slt2 [11] (**Figure 1.2**). Finally, Slt2 activates two transcription factors, Rlm1 and SBF (Swi4/Swi6 complex), that coordinate the CWI transcriptional response [11,56] (**Figure 1.2).** This response mostly involves the activation of cell wall biogenesis genes. The Rlm1 transcription factor is responsible for the expression of most of the genes induced in response to cell wall stress, such as the *CHS3* gene, and also regulates the expression of *FKS1* [72,104]. Importantly, Rlm1 activates *MLP1*, coding for a Slt2 pseudo-kinase paralog, that, together with Slt2, activates the SBF complex for transcription of a subset of cell wall stress-activated genes. Among them are genes related with glucan synthesis (*FKS1*, *FKS2*) or glucan elongation and branching (*GAS1*) [11,80,105–107].

Table 1. 1 - Cell wall integrity membrane sensors implicated in the sensing of industrially relevant stresses

CWI membrane sensor	Industrially relevant stress	Bibliographic References
Wsc1	Hyper-osmolorarity	García-Rodrigues *et al.*, 2005 [108]
	Heat	Rajavel *et al.,* 1999 [109]
	Acetic acid	Mollapour *et al.*, 2009 [110]
	Alkaline pH	Serrano *et al.*, 2006 [111]; Kwon *et al.*, 2016 [112]
	Diamide and H_2O_2	Vilella *et al.*, 2005 [113]
Wsc2	H_2O_2	Vélez-Segarra et al., 2020 [114]

Wsc1/Wsc2	Impaired mannosylinositol phosphorylceramide metabolism	Tanaka and Tani., 2018 [17]
Mid2	Hyper-osmolorarity Heat Low pH - media acidified with a strong acid H_2O_2	García-Rodrigues *et al.*, 2005 [108] Rajavel *et al.,* 1999 [109] Claret *et al.*, 2005 [115] Jin et al., 2013[116]
Mtl1	Diamide and H_2O_2	Vilella *et al.*, 2005 [113]; Jin *et al.*, 2013 [116]

This brief description of the CWI pathway as a simple linear cascade of events can be deceiving. In fact, the CWI pathway receives lateral influences from cAMP-Protein Kinase A (PKA) signalling, TOR signalling, calcineurin signalling, the HOG pathway, and likely from others not yet clarified [117–122] (**Figure 1.2**). This complex network is what enables the CWI pathway to be activated by numerous types of stressors, ensuring an adequate response to each stress or group of stresses (**Figure 1.2**). The existence of a complex interplay between the CWI pathway and other signalling pathways is also being revealed for other yeast species [123–126].

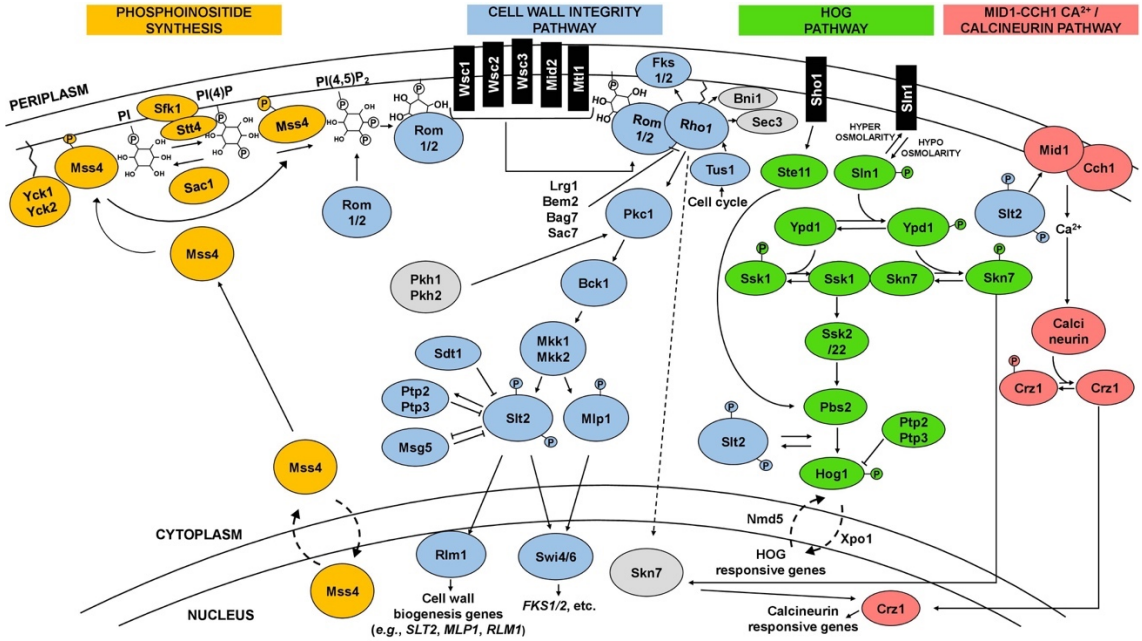

Figure 1.2 - Schematic representation of the CWI, HOG, and calcineurin signalling pathways and interactions. The cell wall integrity pathway (CWI), in blue, is the main pathway involved in the maintenance of yeast cell wall integrity. It is frequently activated by a signal starting at plasma membrane sensors (Wsc1-3, Mid2, Mtl1), which then trigger a cascade culminating in the phosphorylation of Slt2. Phosphorylated Slt2 activates the transcription factor Rlm1 and the SBF complex (Swi4/6), triggering a transcriptional response that elicit changes at cell wall level. Phosphoinositide synthesis (in orange) plays an important role in the activation of the CWI pathway by recruiting Rom1/2 to the plasma membrane, which activates the main regulator of the pathway, the GTPase Rho1. The CWI pathway receives input and interplays with other pathways, including the high osmolarity glycerol (HOG) pathway (in green) and the calcineurin pathway (in pink), represented in the figure, but also with the protein kinase A (PKA) and TOR signalling pathways. Details in the text.

Changes occurring at the level of plasma membrane can also elicit responses at the level of the cell wall. Plasma membrane stretching appears to be the main factor in activating the CWI pathway in response to a number of stresses, including high osmotic pressure and supra-optimal temperature [14,15]. This is consistent with the CWI pathway-activating sensors being located at the membrane and the likely role of Wsc1 as a mechanosensor [127]. Alterations of plasma membrane lipid composition have also been shown to impact the CWI pathway, namely, a defective biosynthesis of the complex sphingolipid mannosylinositol phosphorylceramide (MIPC) leads to increased abundance of the phosphorylated form of Slt2 and sensitivity to cell wall-perturbing agents [16]. This phenotype is partly suppressed by the upregulation of ergosterol biosynthesis, suggesting that MIPC and ergosterol are coordinately involved in maintaining the integrity of the cell wall [17]. Also, a reported crosstalk between plasma membrane ergosterol content (related with the level of expression of the plasma membrane ergosterol transporter Pdr18), and cell wall biophysical properties suggested

a coordinated response to counteract acetic acid deleterious effects, reinforcing the notion that plasma membrane lipid composition influences cell wall integrity during stress[30].

For the main and better studied stresses encountered during industrial bioprocesses, this chapter of this thesis work provides, whenever possible, an integrative view of the pathways and responses that cooperatively maintain cell wall integrity and, in so doing, helps yeasts to resist to multiple stresses.

1.5 YEAST RESPONSE AND TOLERANCE TO INDUSTRIALLY RELEVANT STRESSES INVOLVING THE CELL WALL

1.5.1 THE CELL WALL IN THE RESPONSE TO OXIDATIVE STRESS

All aerobically growing organisms suffer exposure to oxidative stress, caused by reactive oxygen species (ROS) capable of damaging cellular DNA, lipids, carbohydrates, and proteins, threatening cell integrity [118]. Consequently, mechanisms to protect cell components against ROS were evolved and the antioxidant defences can be induced either by respiratory growth or in the presence of pro-oxidants [128]. Yeasts have several inducible adaptive stress responses to oxidants regulated at the transcriptional and posttranscriptional levels [128,129]. Stresses commonly arising during industrial fermentations, such as supra-optimal temperatures or presence of inhibitory concentrations of ethanol, acetic acid or lactic acid lead to oxidative stress that occurs when cellular defense mechanisms are unable to cope with existing ROS [130–134].

The cell wall or, more broadly, the cell envelope, has been associated with oxidative stress toxicity and tolerance. This involvement has been unveiled by studies focused on *S. cerevisiae* exposure to pro-oxidant agents such as hydrogen peroxide, lipid hyperoxides, diamine, catecholamines, and organic hydroperoxides, such as cumene hydroperoxide (CHP) and linoleic acid hydroperoxide (LoaOOH) [113,135–137]. Oxidative stress resulting from exposure to these agents induces distinct responses in *S. cerevisiae*. For instance, a quantitative proteomics study reported differences in the activation of the CWI pathway between hydrogen peroxide, CHP and diamide [138]. Decreased cell permeability, a property influenced by the thickness and composition of the cell wall and plasma membrane, results in increased resistance to pro-oxidant compounds by limiting their diffusion into the cell [139]. Furthermore, membrane lipid composition is a determinant of oxidative stress resistance, with cells containing a higher

level of saturated fatty acids being more resistant than cells with a higher level of polyunsaturated fatty acids [129].

Diamide induces the formation of disulfide bonds in the three-dimensional structure of CWPs, causing changes in the morphology of the cell outer layer and the increase of cell wall thickness [113,140]. CHP causes oxidative damage to the cell wall periphery leading to the upregulation of genes related to cell wall biogenesis (*HSP150*), CWI pathway regulation (*RHO1,ROM2*) and β1,6-glucan synthesis (*KRE5,KRE6,KHN1*) [141] (**Figure 1.3**). The transcriptional reprogramming of yeast in response to oxidative stress is mainly regulated by two oxidative stress-responsive transcription factors, Yap1 and Skn7, and the general stress transcription factors, Msn2/Msn4 [142]. Notably, Skn7 has an important role in the regulation of genes involved in the maintenance of cell wall integrity, including genes coding for cell wall proteins [11,143,144]. The CWI pathway sensors Wsc1, Wsc2, Mtl1, and Mid2 play an important role in the sensing of oxidative stress induced by H_2O_2 [113,114,116]. Surviving and overcoming oxidative stress induced by H_2O_2, as for diamine, requires Pkc1 and the upstream element of the CWI pathway Rom2 [113] (**Figure 1.3**).

In *Yarrowia lipolytica*, a promising oleaginous yeast [145], oxidative stress induced by H_2O_2 leads to changes in the morphology of the cell wall, with the formation of globular surface structures in the cell wall surface [146]. When exposed to soluble complexes of UO_2, the mRNA levels from genes involved in elongation of β1,3-glucan and chitin synthesis were reduced in a tropical marine strain of *Y. lipolytica,* able to immobilize in the cell surface uranium often associated with oxidative stress [147]. These complexes, formed above pH 7.0, are prevalent in aquatic environments such as rivers or sea water, being this strain considered promising for the bioremediation of uranium-contaminated aquatic environments [147,148].

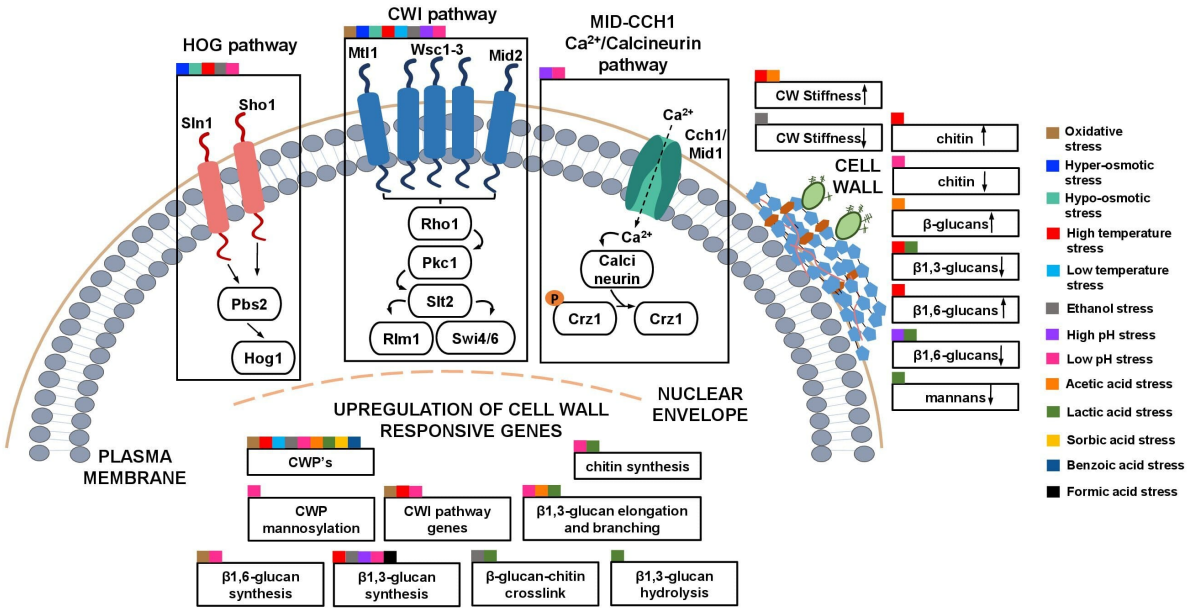

Figure 1.3 - Schematic model of the described S. cerevisiae adaptive responses common to multiple industrial relevant stresses involving the cell wall. The cell wall integrity pathway (CWI) and other signaling pathways (HOG and Calcineurin pathway) that, in collaboration with the CWI pathway and the upregulation of cell wall genes, lead to changes in the composition and nanomechanical properties of the cell wall in response to different stresses are highlighted. Squares of different colors indicate the different industrial-relevant stresses for which data is available in the literature.

1.5.2 OSMOTIC STRESS

Hyper-osmotic stress is common in industrial bioprocesses [149]. Very high gravity fermentations are frequently used to enhance product titres in specific sectors, such as in first-generation bioethanol production by increasing initial sugar concentration and, consequently, osmotic pressure [150]. In winemaking and baking, yeasts also have to grow in hyper-osmotic environments and their performance depends on the ability to adapt and respond to this stress [151,152]. Also, the increase of osmotic pressure induced by the addition of stressful concentrations of sugars or salts is beneficial for food preservation [153]. In yeasts, the High Osmolarity Glycerol (HOG) and the CWI signalling pathways are central to osmotic stress-induced response and control of cellular turgor [154,155]. Although there are many similarities in how the yeast cell responds to osmotic stress caused by different agents, thus allowing the description of a generalised stress response, there are also specific features for each osmolyte or salt stress [153].

Sudden exposure to increased osmolarity leads to cell shrinkage due to water efflux and likely causes the release of plasma membrane material, as well as changes in cell wall structure [156–158]. The decrease in cell volume is accompanied by changes in cell morphology and surface roughness [158,159]. Intracellularly, a compensatory response is

triggered, recruiting water from the vacuole into the cytoplasm [160]. These changes occur immediately after an osmotic upshift, likely caused by rapid biophysical forces [161]. To survive such a shock, the properties of the cell wall have to be modified to have enough elasticity to respond to shifts in osmolarity, while maintaining enough rigidity to preserve cell morphology and integrity [161]. The rapid changes of cellular volume and shape occurring in response to hyperosmotic shock that are fully reversible when cells are introduced in an isosmotic solution, are evidences of the remarkable plasticity of the cell wall [158].

The HOG pathway (**Figure 1.2-1.3**) is a MAPK signal transduction system and the major pathway in the adaptation of yeast cells to increased osmolarity [154]. The pathway relies on two osmolarity sensors, Sln1 and Sho1. During an osmotic upshift, Sln1 is transiently inhibited, diminishing the levels of phosphorylated Ssk1. Unphosphorylated Ssk1 can then interact with Ssk2/Ssk22, which in turn phosphorylates Pbs2. The Sho1 osmosensor also transmits a signal to Pbs2 via the MAPKKK Ste11 [162]. The phosphorylated Pbs2 activates Hog1 and modulates the expression of several genes [162], including the transcriptional activation of *GPD1* and *GPP2* genes, encoding a glycerol 3-phosphate dehydrogenase and a glycerol-1-phosphatase respectively, involved in glycerol production [162]. In response to hyper-osmotic stress, Hog1 phosphorylates Rgc2, a regulator of the Fps1 glycerol channel, causing the dissociation of Rgc2 from Fps1 and the consequent glycerol channel closure [163]. Together, the blockade of glycerol efflux by the closing of the Fps1 channel and the increase in glycerol biosynthetic capacity lead to glycerol accumulation in the cytoplasm and effectively counteract the loss of turgor pressure caused by an osmotic upshift [118].

A coordinated interplay between the HOG pathway and the CWI pathway allows yeast cells to better adapt to hyper-osmotic stress conditions [108,164] (**Figure 1.3**). The CWI pathway appears to be indirectly activated by an increase in turgor pressure caused by the accumulation of glycerol, leading to transient phosphorylation of Slt2 that depends on a functional HOG pathway [108]. Nevertheless, the CWI sensors Mid2 and Wsc1 seem to be involved in the sensing of Hog1-driven accumulation of glycerol [108]. Further evidences of the interplay between the HOG pathway and the CWI pathway were reported [164]. Chemogenomic studies have implicated genes related to β1,3-glucan synthesis (*FKS1*), β1,6-glucan synthesis (*KRE6*) and cell wall mannosylation (*MNN10*, *ANP1*) as determinants of tolerance to high-glucose and sucrose concentrations [165,166], known to induce hyper-osmotic stress [150].

In *Y. lipolytica,* hyper-osmotic stress was also found to lead to cell wall remodeling [167]. Yl*HOG1* deletion impacts filamentous growth, cytokinesis, and resistance to cell wall perturbing agents [168]. A quantitative proteomic analysis has shown that, among the

upregulated proteins under hyper-osmotic stress is Pil1, involved in formation of membrane-associated protein complexes commonly referred as eisosomes and distributed across the cell surface periphery, and *UTR2* coding for a cell wall protein, involved in glucan-chitin crosslinking in *S. cerevisiae* [167]*.* Other non-*Saccharomyces* species, such as *Zygosaccharomyces rouxii*, *Debaryomyces hansenii* and *Pichia sorbitophila* are notable for their osmotolerance [169]. The spoilage yeast of high-sugar or high-salt foods *Z. rouxii* appears to react to hyperosmolarity similarly to *S. cerevisiae*, accumulating intracellular glycerol through the increased expression of Zr*GPS1* and decreased expression of Zr*FPS1* [170–172]. Among *Z. rouxii* upregulated genes under high osmolarity stress are *FKS1*(encoding a β1,3-glucan synthase), *UTR2* (encoding a cell wall transglycosylase), *KRE9* (encoding a glycoprotein involved in β-glucan assembly), *CHS1* (encoding a chitin synthase) and *KAR2* (encoding an ATPase involved β1,6-glucan synthesis and also involved in the translocation of proteins into the endoplasmic reticulum) [171]. The upregulation of the encoding genes helps to explain why the cell wall became thicker as the cell volume decreases, resulting in a smaller amplitude of cell size variation [171].

In a *Zygosaccharomyces mellis* strain, isolated from honey and tolerant to high-glucose concentrations [173,174], the genes *KRE5* (involved in β1,6-glucan synthesis) and *SLT2* (encoding a kinase of the CWI pathway), are upregulated under hyper-osmotic stress [174]. This indicates that, in *Z. mellis*, the maintenance of cell wall integrity under this stress is also important. In *D. hansenii*, the cell wall was also shown to play a critical role in osmosensing and genes involved in cell wall protein mannosylation (*MNN1, PMT2, PSA1* and *MNT1*) are upregulated in response to hyper-osmotic stress [175,176].

1.5.3 HEAT STRESS

Another obstacle that can be faced by yeast cell factories is temperature stress, in particular heat stress. Heat stress is common during alcoholic fermentations to produce alcoholic beverages or bioethanol due to their exothermic nature. If the temperature is not controlled, a significant rise in temperature occurs [1,177]. Given that in the presence of other stresses, in particular of ethanol or acetic acid stress, the optimum and the maximum temperatures for growth decrease as the stress level increase, even temperatures close to the optimal range of growth can become lethal temperatures, depending on the level of stress [28,178]. Despite the importance of cold stress, the related literature is scarce in the context of this chapter of this thesis work, so this part of the chapter is essentially dedicated to heat stress.

At supra-optimal temperatures in the absence of any other stress (higher than 35º-37 ºC), *S. cerevisiae* activates the heat shock response (HSR) and undergoes physiological changes which include membrane and cell wall restructuring [73,179]. During heat stress, β1,6-glucan and chitin content increases by 20% and 100% respectively, and β1,3-glucans levels decrease by 45% [73] (**Figure 1.3**). The increased β1,6-glucan synthesis under heat stress is in accordance with a higher number of cross-linkages between this polymer and chitin, presumably compensating cell wall weakening during this stress [73,180]. Heat stress also induces changes in the morphology of the yeast cell surface with the formation of circular structures in the surface of heat-stressed cells [71]. The emergence of these structures is, apparently, related to a dysfunction in the budding machinery, accompanied by a concomitant increase in chitin and cell wall stiffness, regulated by the CWI pathway [71] (**Figure 1.3**) activated in response to heat stress [181–183]. Together with Msn2/4, Hsf1 is a transcription factor responsible for the bulk of the HSR [184,185]. Heat stress also promotes glycerol efflux by the opening of Fps1 channels regulated by the CWI pathway [186]. The resulting turgor loss stimulates the activation of the HOG pathway, promoting glycerol production and the re-establishment of turgor pressure [186]. Heat shock leads to the intracellular accumulation of trehalose in *S. cerevisiae* [187,188]. Since trehalose and thermotolerance are closely related, trehalose was suggested to act as a thermoprotectant [189]. The intracellular accumulation of trehalose causes a decrease of the specific growth rate that may trigger the environmental stress response (ESR) and higher thermotolerance [190]. The accumulation of trehalose increases cytosolic osmolarity and turgor pressure, mimicking hypotonic stress, and causing plasma membrane stretching known to lead to activation of the CWI pathway [15,191]. Consistently, preventing trehalose synthesis by deletion of the *TPS1* gene led to decreased activation of the CWI pathway upon exposure of yeast cells to heat stress [191]. Interestingly, a recent study suggests that the UDP-Glucose Pyrophosphorylase Ugp1 is required for heat stress response by influencing trehalose and glucan content [192].

Although the mechanism behind heat-induced activation of the CWI pathway is not clear, the Wsc1 and Mid2 CWI sensors appear to be involved in the activation of CWI pathway, reinforcing the notion that this stress is ultimately transmitted to the cell surface [11,109,179,193,194]. In fact, Wsc1 sensors form clusters in the plasma membrane upon heat stress (the so-called Wsc1 sensosomes) enhancing the CWI pathway signalling capability [102]. The expression of the CWI pathway *SLT2* gene, the *HSP150* gene encoding a protein required for cell stability and the cell wall protein-encoding gene *YGP1* are induced in response to heat stress [195,196] (**Figure 1.3**). Also, *FKS2,* encoding a β1,3-glucan synthase, is upregulated during heat stress, being its expression regulated by both CWI and Calcineurin pathways [79] (**Figure 1.3**).

The ability to withstand elevated temperatures while maintaining high growth rates and ethanol productivity can be beneficial for bioethanol production and could alleviate some of the costs associated with the cooling of the bioreactors often required to allow an adequate fermentative performance [5,197]. Two prominent examples of thermotolerant yeast species, shown to be able to grow relatively well above 40 ºC, are *Kluyveromyces marxianus* and *Ogataea polymorpha* (formerly *Hansenula polymorpha*) [5,197]. In *K. marxianus*, high temperatures were shown to lead to upregulation of genes associated with changes in plasma membrane composition [198]. Very few is reported concerning changes occurring in *K. marxianus* cell wall under heat stress but a strain isolated from agave when grown at 42°C exhibit increased sensitivity to lyticase than when grown at 30°C, indicating that heat stress may affect cell wall integrity [199]. In *Ogataea* species, thermotolerance has been attributed to a structural predisposition of the cell envelope, related with membrane and cell wall composition, allowing a higher cell envelope stability [197]. Among the candidate genes that contribute to heat tolerance in *O. polymorpha* is an ortholog of *S.cerevisiae PSA1,* coding for GPD-mannose pyrophosphorylase, involved in cell wall biosynthesis [200]. In the genus *Ogataea*, Mpk1 appears to be involved in CWI signalling in response to heat stress, but, different *Ogataea* strains exhibit different growth phenotypes when *MPK1* is absent [201]. Compared to *O. polymorpha*, *O. parapolymorpha* has a thinner β-glucan and chitin layer with short mannan chains and the derived deletion mutant *mpk1*Δ exhibit more severe growth defects during heat stress and higher susceptibility to cell wall-perturbing agents, leading the authors to consider these differences related to cell wall structural differences [201].

The characterization of the yeast response to low temperatures is also important in industry-related bioprocesses [202], in particular in many wine and beer fermentations, resulting in the retention of more volatile compounds that influence the sensory properties of the product [202]. Furthermore, cold-adapted spoilage yeasts can potentially impose health risks to the consumers and economic burden in the Food industry, being able to grow and proliferate at temperatures at which food products are refrigerated [203]. Among the genes that are upregulated in response to cold temperatures are genes encoding cell wall mannoproteins (*TIR1-2, TIR4*, *TIP1* and *DAN1*) [204–209] (**Figure 1.3**). Increased resistance to the cell wall-perturbing compound SDS in cold-shock-stress-induced cells has also been attributed, at least partially, to the upregulation of *DAN1* [209]. The CWI pathway *WSC1*, *BCK1* and *SLT2* genes encoding a CWI sensor, the MAPKKK Bck1 and the MAPK Slt2, respectively, have also been implicated in response and adaptation to cold temperature-induced-stress, being Slt2 phosphorylation partially dependent on the Wsc1 CWI sensor [210] (**Figure 1.3**).

Psychrophilic yeasts, yeasts adapted to low temperatures, with an optimal growth performance at 15°C, are studied due to its biotechnological potential, in particular for the production of cold-active enzymes [211]. Interestingly, the absence of the *SWI4* or *SWI6* genes, encoding transcriptional activators of the CWI pathway in *Metschnikowia australis* W7-5, leads to impaired growth at temperatures as low as 5°C, suggesting that these genes are determinants of tolerance to low temperature in this species [212].

1.5.4 ETHANOL-AND METHANOL-INDUCED STRESSES

Ethanol toxicity is arguably the major environmental stress limiting industrial titres and overall productivity in a wide range of industrial yeast fermentations [1,49]. Accumulation of high concentrations of ethanol, whether during bioethanol production, wine and brewing industries, or other alcoholic fermentations, often results in decreased fermentation yield or complete cessation of yeast metabolic activity and fermentation arrest [177].
Due to its liposolubility, ethanol disrupts plasma membrane lipid organization, increasing its fluidity, nonspecific permeability, and compromising transmembrane electrochemical potential (van Uden, 1985). Among the adaptive responses is the alteration of plasma membrane composition (e.g., increase in ergosterol content and in unsaturated/saturated fatty acid ratio) leading to increased lipid order and counteracting plasma membrane fluidisation [213], together with and adaptive response at the level of the cell wall [214,215]. Remarkably, the impairment of plasma membrane integrity was shown to influence the nanomechanical properties of the cell wall during ethanol stress [70] (**Figure 1.3**). Specifically, ethanol-induced changes to the plasma membrane, compromising the proper delivery of plasma membrane-anchored GPI-CWPs to the membrane via the secretory pathway, affect their crosslinking to cell wall polysaccharides resulting in reduced cell wall stiffness [70] (**Figure 1.3**).

Several chemogenomic studies have implicated genes related to cell wall biosynthesis, cell wall remodeling, and CWI pathway as determinants of ethanol tolerance [134,216–219]. Additionally, suitable supplementation of the fermentation medium with potassium, zinc or inositol was shown to improve tolerance to ethanol stress [220–222]. Genes reported as determinants of ethanol tolerance include *MNN10*, *MNN11*, *ANP1*, and *HOC1*, encoding four subunits of the polymerase complex responsible for the elongation of the mannose backbone present in CWPs, and *LDB7*, encoding a component of the chromatin structure remodeling complex, involved in the regulation of the mannosylphosphate content of mannoproteins [134,217,219]. Moreover, the cell wall protein-encoding genes *PIR3*, *SED1*, *SPI1*, and *YGP1*, as well as the GPI-CWPs-encoding genes of the *TIR* family (*TIR1-3*

and *TIP1*), were found to be transcriptionally responsive to ethanol stress [121,223–225] (**Figure 1.3**). The overrepresentation of genes related to the mannoprotein-rich outer layer of the cell wall in response to ethanol-induced stress suggests that mannoproteins have a role in yeast adaptation to this stress, limiting ethanol's access to the plasma membrane and thus counteracting ethanol-induced membrane permeabilization and subsequent deleterious effects. Due to its amphiphilic nature, ethanol can bind to the exposed proteins at the outer layer of the cell wall, likely altering its organization and increasing cell wall porosity [121,226,227].

Genes related to chitin and glucan synthesis, namely *CHS1* (chitin), *FKS1* (β1,3-glucans), *ACF2* (β1,3-glucans), and *KRE6* (β1,6-glucans), as well as genes involved in cell wall integrity (*PUN1*) and cell wall organization (*SIT4*), were also found to be involved in ethanol tolerance [134,217,219,228]. The upregulation of *FKS1* and also of *SED1* and *SMI1* genes, involved in cell wall biosynthesis depends on the Znf1 transcription factor, recently implicated in adaptation to ethanol stress [229]. Consistent with a transcriptional response involving multiple components of the cell wall under ethanol stress, elements of the CWI signalling pathway, namely the membrane sensors Mid2 and Wsc1, the MAPKKK Bck1, and the MAPK Slt2, were found to be crucial for maximum tolerance to ethanol stress [134,216,219]. Also, yeasts deficient in either one of the two Slt2-activated SBF subunits, Swi4 and Swi6, reveal higher sensitivity to ethanol [121]. The SBF complex controls the expression of *FKS2* and *CRH1*, coding for a β1,3-glucan synthase and a chitin transglycosylase involved in glucan-chitin crosslinking, respectively; these genes were shown to be upregulated during ethanol stress [121] (**Figure 1.3**). Additionally, the HOG pathway also seems to contribute to the regulation of the transcriptional response of cell wall genes during ethanol stress, since the dysfunction of the HOG pathway leads to decreased expression of *FKS2* and *CRH1* [121]. Furthermore, the wall of yeast cells exposed to ethanol exhibits higher resistance to lyticase and zymolyase treatment [121]. In summary, the mechanism underlying ethanol tolerance involves a collaborative role of the CWI and HOG signalling pathway in the transcriptional regulation of cell wall genes leading to cell wall changes [121] (**Figure 1.3**).

Not many non-conventional yeasts can compete with *S. cerevisiae* when it comes to ethanol production and tolerance, but some have other interesting features that could make their use in the bioethanol industry appealing. *K. marxianus*, known for its thermotolerance and ability to metabolise several carbon sources [5], has a fair number of studies dedicated to understanding the response to ethanol stress [230–233], although not much is known about ethanol-induced changes to its cell wall. However, the adaptive evolution under ethanol stress was found to lead to the improvement of multiple pathways, including cell wall biogenesis, suggesting that cell wall remodeling is part of a

strategy to mitigate the toxic effects of ethanol, also in this species [232]. The increase in cell wall thickness also occurs in *Saccharomyces boulardii* under ethanol stress [234]. *S. boulardii* is known for its probiotic capacities as a biotherapeutic agent in infections and medical disorders [235–237] and is a promising species to be used in certain crafts of beer fermentation [234]. In *Issatchenkia orientalis,* with potential to be used in winemaking, cell wall-related genes *GAS4* (β-1,3-glucanosyltransferase involved in glucan elongation), *FLO1* (CWP involved in adhesion events important for flocculation) and *IFF6* (GPI-CWP involved in cell wall organization) were found to be up-regulated under ethanol stress [238].

A recent chemogenomic analysis reported that, in *S. cerevisiae*, methanol and ethanol, share genetic determinants of tolerance involved in cell wall maintenance [228]. Methanol is a feedstock alternative to sugar-based raw substrates for biorefinery processes and a toxic compound commonly found in crude glycerol, a by-product of the biodiesel industry, and in hydrolysates from pectin-rich biomass residues [228,239,240]. Methanol and ethanol tolerance genes include *FKS1* and *SMI1* (β1,3-glucan synthesis), *ROT2* (β1,6-glucan synthesis), *MNN11* (mannosylation of CWPs), and *WSC1* (CWI pathway membrane sensor). However, *KRE6*, *CWH41* (β1,6-glucan synthesis) and *GAS1* (β1,3-glucan chain elongation and branching) were found to be required for maximum tolerance to methanol but not for equivalent inhibitory concentrations of ethanol [228]. This suggests that, despite the similarities of these alcohols, equivalent inhibitory concentrations might impact the cell wall differently and, as such, elicit not fully overlapping remodeling responses [228]. The CWI pathway is implicated in methanol adaptation in *Komagataella phaffii* (formerly *Pichia pastoris*), involving the upregulation of the *SLT2* homolog encoding gene in *K. phaffii* [241]. The homolog of Wsc1 and Wsc3 CWI sensors in the methylotrophic yeast *K. phaffii* were implicated in sensing methanol, interacting upstream with the *K. phaffii* homolog of Rom2, a guanine nucleotide exchange factor of *S. cerevisiae* CWI pathway for activation of methanol-inducible genes [242]. Additionally, differences in methanol metabolism, vector transformation efficiency, growth and heterologous protein production between different *K. phaffii* strains were related with cell wall integrity [241], providing another example of the important role of cell wall.

1.5.5 LOW-OR HIGH-PH-INDUCED STRESS

Cell wall composition and architecture and the CWI pathway were also implicated in yeast adaptive response to acid and alkaline stress conditions [111,115,243]. However, the mechanisms underlying yeast response and tolerance to acidic conditions are complex and dependent not only on the pH value but also on the nature of the acid used to adjust low pH. Strong inorganic acids, such as sulphuric acid and hydrochloric acid, are fully dissociated at any external pH, while weak organic acids dissociation depends on medium pH and their pK_a, the toxic form being the liposoluble non dissociated form [244–246]. Since the plasma membrane of unstressed cells is very poorly permeable to H^+, the effect of strong acids relies, essentially, on the concentration of H^+/medium pH [244,246,247]. For this reason, pH stress and stress induced by organic acid stress at low pH are discussed separately.

1.5.5.1 ACID PH-INDUCED STRESS

In bioethanol production, yeast biomass is reused after being washed between successive batches using inorganic acids. It is a common procedure, frequently carried out at a pH below 3.0 as a means of eliminating contaminant bacteria from pitching yeast. This disinfection treatment, together with the presence of toxic metabolites and other stressful conditions occurring during fermentation, may lead to the loss of cell viability and limit fermentation yield [247–249]. A scalable and economic solution to control bacterial contamination during alcoholic fermentation is to run the fermentations at low pH (< 4.0), at which growth and viability of most bacteria are drastically reduced [250]. Thus, understanding how yeast strains tolerate low pH set up with strong acids may enable the improvement of ethanol yield and reduce production costs [251]. It is likely that during fermentation, organic acids (weak acids) may also play a role since they are produced during yeast metabolism and may be already present in the fermentation medium (e.g., when lignocellulosic biomass hydrolysates are used).

In response to acid pH, the cell wall structure and composition suffers alteration [252,253] leading to deformation of surface morphology[254]. Cell wall chitin levels decrease at growth pH values below 5.0, likely as a result of increased chitinase activity [252] (**Figure 1.3**). Many of the genes found to be upregulated at low pH are related to cell wall biogenesis, including *FKS1* (β1,3-glucan synthase), *GAS1* (β-1,3-glucanosyltransferase involved in cell wall remodeling-elongation of (1→3)-β-D-glucan chains and branching), *CHS1* (chitin synthase), *NCW2* (GPI-protein involved in chitin-glucan assembly), *KRE6*

(glucosyl hydrolase required for β1,6-glucan synthesis) and *MNN9* (mannosyltransferase subunit involved in wall protein mannosylation) [243,249] (**Figure 1.3**). Interestingly, using QTL mapping to uncover the genetic basis of a bioethanol industrial strain Pedra-2 (PE-2) tolerance, a prevalent non-synonymous mutation (A631G) in *GAS1* was identified during growth at low pH induced by sulfuric acid exposure, reinforcing the idea of the relevant role of this GPI-protein in yeast tolerance in acidic environments [255]. Together with the up-regulation of genes related with 1,3-β-glucan synthesis, elongation, and anchoring, low pH stress leads to increased cell wall resistance to compounds with β1,3-glucanase activity and to the establishment of more alkali-sensitive linkages between CWPs and the β1,3-glucan network [243,247,249,253,254]. This suggests that low pH established by a strong inorganic acid affects the β-glucan fraction of the cell wall.

As for other stresses, response to acid pH imposed by inorganic acids involves a crosstalk between different signalling pathways. The CWI signalling pathway is involved in response to low pH in *S. cerevisiae* and has been proposed as the main mechanism for tolerance to acid pH [115,243] (**Figure 1.3**). Its activation is mainly mediated by Mid2, but also by Wsc1, with the latter appearing to have a more prominent role in activating the general response of the cell to this stress [115,243]. The proposed model is that cell wall injury due to acid stress results in lower turgor and consequently mimics the effect of hyper-osmotic shock, justifying the activation of the HOG pathway[243] (**Figure 1.3**). The HOG pathway then appears to have a dual role. First, Hog1 activates the protein complex Msn2/4, which induces the expression of Environmental Stress Response (ESR) genes, including *RGD1*, a major regulator of yeast survival at low pH stress, that encodes a protein implicated in the activation of CWI pathway under acid stress [115]. Second, the Hog1 kinase may help to establish a positive feedback loop at the downstream module of the CWI pathway by cooperating with the Slt2-activated Rlm1 transcription factor to increase the expression of *SLT2* [115,243]. Therefore, the HOG pathway can activate the CWI pathway while bypassing its membrane sensors. The Ca^{2+}/calmodulin-dependent calcineurin pathway is also involved in the response to acid pH, and interacts with the CWI pathway by activating Cch1/Mid1 calcium channels by Slt2, the Crz1 transcription factor by Rho1-Skn7, and through the cooperation between Slt2 and Crz1 for the expression of *FKS2* in response to cell wall injury[243] (**Figure 1.3**). This Ca^{2+}-dependent response is likely responsible for the increment of CWI protein trafficking and their localisation at cell surface to repair the structural changes caused by medium acidification[247]. Together, CWI, HOG, and calcineurin signalling pathways ensure the

post-translational activation of the transcription factors needed to promote cell wall maintenance and regeneration to survive acidic pH stress.

1.5.5.2 ALKALINE PH STRESS

The yeast *S. cerevisiae* proliferates better at acidic than at neutral or alkaline pH and medium alkalinisation has widespread effects in yeast physiology [256]. An increase of pH from 4.0 to 6.0 leads to the decrease of the relative proportion of β1,6-glucans in the β-glucan fraction of the cell wall [58] and cells grown at pH 6.0 are more susceptible to zymolyase treatment [58] (**Figure 1.3**). The CWI pathway is necessary for tolerance to alkaline pH, as shown by the strong alkali-sensitive phenotype of the *bck1*Δ, *slt2*Δ, *swi4*Δ, and *swi6*Δ mutants [111]. The alkaline stress-mediated activation of Slt2 was also shown to depend on the CWI Wsc1 membrane sensor [111,112] (**Figure 1.3**). Both *FKS2* and *GAS1* encoded proteins, involved in the synthesis and elongation of β1,3-glucans respectively, are required to resist alkaline stress, and likely play a major role in altering the ratio between different types of glucans in the cell wall [111]. The Ca^{2+}-dependent calcineurin response has also been implicated in the regulation of cell wall synthesis during alkaline stress by the upregulation of *FKS2* expression via the calcineurin-activated transcription factor Crz1 [257] (**Figure 1.3**).
Changes in cell wall composition were also implicated in *Y. lipolytica* adaptation to high pH stress [258]. In particular, the structural mannoprotein YlPir1 is abundant in the cell wall in unstressed conditions but absent when *Y. lipolytica* cells are exposed to high pH stress [258]. This readjustment is consistent with the fact that mannans, unlike other main polysaccharides of the cell wall, are prone to alkaline hydrolysis, and therefore unstable at high pH [258].

To summarise, there are major differences between the signalling responses elicited by acidic and alkaline pH stresses involving the cell wall. First, Mid2 appears to be the main sensor activating the CWI pathway in response to acidic pH, while Wsc1 is the main sensor in alkaline pH [115,117,256]. Second, acidic pH stress mostly leads to the transcription of Rlm1-dependent genes, while alkaline pH stress favours transcription of SBF-dependent genes. Third, while the CWI pathway manages acidic stress in a Hog1-dependent manner, response to alkaline stress is Hog1-independent [117].

1.5.6 ORGANIC ACID-INDUCED STRESS

The response and tolerance of the yeast cell to the various industrially relevant weak acids and the underlying toxicity mechanisms are not fully shared by all the acids, with specific mechanisms for a weak acid/group of weak acids [259]. In general, and broadly speaking, the higher the lipophilicity of each of a weak acid is, the higher its toxicity. The straight medium chain weak acids (e.g., butyric, hexanoic, octanoic, and decanoic acids) and sorbic and benzoic acids are more lipophilic and toxic than the short-chain volatile fatty acids (VFA) formic, acetic, and propionic acids [259,260].

1.5.6.1 ACETIC ACID STRESS

Acetic acid is widely used as a food preservative in the food industry and is also a major inhibitory compound present in lignocellulosic biomass hydrolysates limiting the use of this low cost and abundant biomass [7,8]. Acetic acid is also produced by yeast metabolic activity and can lead, together with ethanol and other yeast toxic metabolites, to decreased ethanol yield and even fermentation arrest depending on the level of stress [7–9]. Elucidation of the mechanisms underlying yeast adaptation and tolerance to acetic acid is instrumental to pave way for strain and process optimisation in several important biotechnological and food industries.

When the external pH is below acetic acid pK_a (below 4.75 at 25°C) [261], the undissociated form of the acid (CH_3COOH) is able to passively diffuse through the plasma membrane lipid bilayer [7,9,259,262]. Once in the near-neutral cytosol, acetic acid dissociates into the negatively charged acetate counterion, CH_3COO^-, releasing protons, H^+. Being unable to cross the hydrophobic lipid layer due to the electric charge, these ions accumulate in the cytosol, resulting in decreased intracellular pH, increased turgor pressure and oxidative stress, disrupting normal metabolism [7,9,30,259].

Several genome-wide studies have sought to shed light into the global mechanisms involved in the response and tolerance of *S. cerevisiae* to acetic acid [263–269]. Increased cell wall impermeabilization in adapted yeast cells can reduce the passive diffusion of the weak acids into the cytosol, in this way restraining the futile cycle associated with the re-entry of the liposoluble acid form after the active expulsion of its counter-ion from the cell interior [24]. Recently, it was reported a coordinate and comprehensive view on the time course of the alterations occurring at the level of the cell wall during adaptation of a yeast cell population to sudden exposure to a sub-lethal stress induced by acetic acid [25]. Yeast cell wall resistance to lyticase activity was found to increase during acetic acid-induced growth latency, corresponding to the period of yeast population adaptation to sudden exposure to acetic acid. This response was correlated with the increase of cell

stiffness, assessed by atomic force microscopy [25] (**Figure 1.3**). The increased content of cell wall β-glucans, also assessed by fluorescence microscopy, and the slight increase of the transcription level of the *GAS1* gene encoding a β-1,3-glucanosyltransferase that leads to elongation of β1,3-glucan chains, were also implicated [25] (**Figure 1.3**). These observations reinforce the notion that the adaptive yeast response to acetic acid stress involves a coordinate alteration of the cell wall at the biophysical and molecular levels, essential to limit the futile cycle associated to the re-entry of the toxic acid form after the active expulsion of acetate from the cell interior [25].

The adaptive genomic response to acetic acid in *S. cerevisiae* is mainly regulated by the Haa1 transcription factor involved in the direct, or indirect, transcriptional activation of approximately 80% of acetic acid-responsive genes and likely involved in the response at the cell wall level [266,270]. Haa1 increased expression or Haa1 specific mutations lead to increased tolerance to acetic acid stress and to a lower intracellular accumulation of acetate [7,271]. Overexpression of *HAA1* improves cell wall robustness in response to this weak acid, as suggested by the decreased susceptibility of the cell wall to lyticase activity mediated disruption [272]. Also, *YGP1* and *SPI1,* encoding CWPs, belong to the Haa1 regulon; they are upregulated under acetic acid stress and contribute to yeast tolerance [273,274] (**Figure 1.3**). Increased mRNA levels from *YGP1* were reported in cells overexpressing *HAA1* [271]. These results suggest that not only the activation of acetate expulsion through efflux pumps is involved in acetic acid tolerance, as proposed [263,264,275–278], but a more efficient restriction of the diffusional entry of acetic acid, partially dependent of the CWP Ygp1, can also be involved [271]. The Znf1 transcription factor was also implicated in acetic acid tolerance and in the upregulation of the *YGP1* gene [279].

Genes involved in CWP mannosylation (*MNN2, MNN9, MNN11, KTR4*), chitin synthesis (*CHS1, CHS5*), β1,3-glucan synthesis (*FKS1, ROM2*), and β1,6-glucan synthesis (*KRE6*) were also reported as being required for maximum tolerance of *S. cerevisiae* to acetic acid stress [110,264]. However, the mRNA levels from *RLM1*, encoding a major transcriptional regulator of the CWI pathway, and from Rlm1-target genes, were found to decrease in cells exposed to acetic acid stress, suggesting that the CWI pathway is not the major key player in acetic acid stress response [25].

Zygosaccharomyces bailii is a common food spoilage yeast capable of adapting and proliferating in the presence of remarkably high concentrations of acetic acid [7]. During exposure to acetic acid, several genes involved in the modulation of plasma membrane composition and cell wall architecture were found to be differentially expressed [280]. Among those genes is the homologue of *S. cerevisiae YGP1*, encoding a cell wall-related glycoprotein, whose upregulation in the presence of acetic acid was shown to depend

on ZbHaa1 [280,281], showing a high degree of similarity between the responses involving the cell wall in both yeast species. In a *K. marxianus* strain isolated from agave, lyticase assays showed that the addition of KCl/KOH leads to the increase of cell wall robustness in cells grown in the presence of acetic acid [199]. However, further studies are necessary to elucidate the link between changes in cell wall during acetic acid adaptation and potassium homeostasis [199]. In *I. orientalis*, the expression of *MPG1* gene, coding for a GDP-mannose pyrophosphorylase involved in cell wall synthesis, was also found to be upregulated under acetic acid stress [282].

1.5.6.2 LACTIC ACID STRESS

Lactic acid is another important weak acid in the food industry and also in the pharmaceutical and cosmetic industries. Its industrial production is currently carried out by lactic acid bacteria [283,284] but bacteria are sensitive to low pH, requiring large amounts of neutralising agents to counteract the acidification of the fermentation media, thus compromising the recovery yield of precipitated lactic acid [285,286]. Yeasts typically fare better than bacteria in acidic environments, which has motivated attempts to produce lactic acid through the heterologous expression of lactate dehydrogenases in yeast [287–289]. The success of such strategy and its industrial application require knowledge of the mechanisms behind yeast tolerance to lactic acid stress. Exposure to lactic acid leads to a decrease in cell wall glucan content and, to a lesser extent, of the mannan content of *S. cerevisiae* cell wall [290] (**Figure 1.3**). Several genes involved in the synthesis of cell wall polysaccharides, cell wall remodeling, and synthesis of mannoproteins are transcriptionally responsive and/or are determinants of tolerance to lactic acid stress [263,265,291]. Specifically, genes required for glucan remodeling (*EXG1*, *GAS2*, *SCW10*), cross-linking between β-glucans and chitin (*CRH1*), chitin synthesis (*CHS1*), and genes encoding mannoproteins required for cell wall stability (*HSP150*, *PIR3*, *SED1*), are up-regulated in response to lactic acid stress [263] (**Figure 1.3**). Genes encoding elements of the MAPK module of the CWI pathway (*BCK1*, *SLT2*), as well as genes involved in glucan synthesis (*KRE1*, *KRE11*) and remodeling (*GAS1*), have also been implicated in lactic acid tolerance [263,291].

The Haa1 transcription factor also plays an important role in tolerance to lactic acid and is involved in the control of the expression of CWPs [259,292,293]. As found for acetic acid stress [294], exposure to lactic acid leads to Haa1 translocation from the cytoplasm to the nucleus where gene transcription, in particular of *YGP1* and *SPI1* occurs [293]. The overexpression of these two genes, encoding CWP, likely confers a stronger protective effect against lactic acid-induced toxicity [293].

Due to the high tolerance of *Zygosaccharomyces parabailii* to high lactic acid concentrations at low pH, this species was proposed as a promising novel host for lactic acid production [295]. In *Z. parabailii*, several cell wall-related genes were found to be down-regulated in the presence of lactic acid [295]. Under the stress conditions tested, during exponential growth in the presence of lactic acid, a slight decrease in glucans was reported in *S. cerevisiae* and *Z.bailii*, and a slight decrease in mannans in *S. cerevisiae* [290,296].

1.5.6.3 OTHER WEAK ACIDS

Propionic acid is commonly used to preserve baked goods and dairy [297]. Transcriptomic and chemogenomic studies have hinted at a role for the cell wall in yeast adaptation to this weak acid [278,298]. Specifically, *CWP1* (encoding a GPI-CWP), *BAG7* (β1,3-glucan synthesis), and *KNH1* (β1,6-glucan synthesis) are required to resist propionic acid stress, all of which are regulated by the transcription factor Rim101 [298], suggesting that cell wall remodeling during adaptation to propionic acid stress may be dependent on *RIM101* expression.

Sorbic and benzoic acids are two other weak acids used to preserve foods and beverages. In both cases, the *S. cerevisiae* cell wall has been implicated in stress tolerance [273,299]. Common to the responses to sorbic and benzoic acids is the induction of *SPI1*, a GPI-CWP that likely leads to the decrease of cell wall porosity and, in turn, limits the access to plasma membrane, thus reducing membrane damage, intracellular acidification, and viability loss [273,299] (**Figure 1.3**). As previously mentioned, *SPI1* is also activated in response to other weak acids and ethanol stress [223,225,273].

Formic acid in an inhibitory weak acid present in lignocellulosic hydrolysates negatively impacting lignocellulosic-based biorefining [8].

Under formic acid stress, *S. cerevisiae* cells exhibited a deformed shape, with collapsed cell wall edges, indicating that the cell wall was damaged, and an FTIR analysis suggested that chitin structure was altered [300]. *S. cerevisiae* genes involved in β1,3-glucan synthesis (*FKS1*, *ELO2*), β1,6-glucan synthesis (*TRS65*), chitin synthesis (*CHS5*), cell wall protein mannosylation (*PMT2*), cell wall integrity (*PUN1*), and CWI pathway regulation (*ROM2*) are important determinants of formic acid tolerance while *FKS3*, encoding an *FKS1-2* homolog, is upregulated in response to this acid stress [300,301] (**Figure 1.3**). Moreover, the upregulation of *EXG2* encoding a major exoglucanase, and *PKC1*, encoding a CWI pathway kinase, was reported in an industrial *S. cerevisiae* strain (S6), engineered to ferment xylose, when grown in a medium with glucose and xylose supplemented with formic acid [302].

1.5.7 ADAPTIVE RESPONSES TO SEVERAL INDUSTRIALLY RELEVANT STRESSES INVOLVING THE CELL WALL

Some of the responses involving the modification cell wall metabolism and cell wall physicochemical properties in yeasts are shared by relevant industrial stresses (**Figure 1.3**), For example, the CWI pathway is implicated in oxidative-, osmotic-, heat and cold-, ethanol- and low and high pH- induced stresses [15,71,108,111–113,115,121,134,154,164,191,210,216,219,243]. Also, the coordinated regulation involving the CWI and the HOG pathways occurs during osmotic, heat, ethanol and low pH stresses [15,108,121,154,164,186,191,243] and the Calcineurin pathway interacts with the CWI pathway during high and low pH stresses [243,257]. The reported upregulation of genes encoding cell wall proteins was also observed under several stress induced conditions (e.g., under ethanol stress in *S. cerevisiae* and *I. orientalis* and under acetic acid stress in *S. cerevisiae* and *Z. bailii* [121,223–225,238,273,274,280,281] and under oxidative, high and low temperature, low pH, lactic, sorbic and benzoic acids-induced stresses in *S. cerevisiae*) [141,195,196,204–209,273,293,299]. This common response is consistent with the important role of cell wall proteins in decreasing cell surface porosity and increasing cell wall stability when coping with stress. The upregulation of genes involved in β1,3-glucan synthesis was also reported to be shared by several stresses (heat, ethanol, low and high pH and formic acid stress) and during hyper-osmotic stress in *Z. rouxii* [79,121,171,229,243,249,257,300]. Genes involved in β1,3-glucan elongation and branching were also found to be upregulated in response to low pH, acetic and lactic acids-induced stresses and under ethanol stress in *I. orientalis* [25,238,243,249,263]. Genes involved in chitin synthesis found to be upregulated were also found to be shared by the response to low pH and lactic acid stress in *S. cerevisiae*, and to hyper-osmotic stress in *Z. rouxii* [171,243,249,263]. Genes involved in β-glucan-chitin crosslinking were also found to be upregulated under ethanol and lactic acid stresses in *S. cerevisiae*, and under hyper-osmotic stress in *Z. rouxii* [121,171,263]. The upregulation of genes involved in β1,6-glucan synthesis are shared by oxidative and low pH stress in *S. cerevisiae*, and by hyper-osmotic stress in *Z. rouxii* and *Z. mellis* [141,171,174,243,249]. Genes involved in CWI pathway were found to be upregulated in oxidative-, heat- and low pH-induced stress in *S. cerevisiae*, under hyper-osmotic stress in *Z. mellis*, and under methanol stress in *K. phaffii* [115,141,174,196,241,243]. Genes involved in CWP mannosylation were found to be upregulated in *S. cerevisiae* under low pH stress and in *D. hansenii* under hyper-osmotic stress [175,176,243,249]. Genes involved in β1,3-glucan hydrolysis were found to be upregulated under lactic acid stress in *S. cerevisiae* [263].

Of all the industrially relevant stresses herein described, exposure to several stresses were found to decrease, at least, one type of cell wall polysaccharide in *S. cerevisiae*. Specifically, a decrease in the β-glucan content was reported for heat- (β1,3-glucans), high pH- (β1,6-glucans), lactic acid- (β1,3 and β1,6-glucans) induced stresses, and a decrease in mannans was reported during lactic acid stress [58,73,290]. A slight decrease in the β-glucan content (β1,3 and β1,6-glucans) was also reported for *Z. bailii* during lactic acid stress [296]. A decrease in the chitin content was reported for inorganic acid-induced low pH in *S. cerevisiae* [252]. Nevertheless, an increase of cell wall polysaccharide content was also reported, in particular an increase in chitin and β1,6-glucans under heat stress and an increase in the β-glucans content under acetic acid stress in *S. cerevisiae* [25,73]. It is noteworthy to take in consideration that the methods used for cell wall polysaccharides quantification were not the same in different articles and both the levels of stress and the adaptation phase of the cells examined (early response, cells adapted to the stress) could be different or not clearly reported.

1.6 IMPROVEMENT OF YEAST TOLERANCE TO MULTIPLE STRESSES INVOLVING CELL WALL ENGINEERING

A few successful examples of the alteration of the physicochemical properties of the cell wall either by the genetic engineering of the yeast cell or by the adaptive laboratory evolution (ALE) of yeast cells leading to the increase of tolerance to stress(es) have been reported in the literature.

As referred above, through chemogenomic analyses, it was demonstrated that the expression of genes related with chitin and glucan synthesis, namely *CHS1* (chitin), *FKS1* and *ACF2* (β1,3-glucans), *KRE6* (β1,6-glucans), and others involved in cell wall integrity (*PUN1*) and cell wall organization (*SIT4*), are required for maximum tolerance to ethanol in *S. cerevisiae* [134,217,219,228]. Superior fermentation performance of lignocellulosic hydrolysates was reported for a recombinant *S. cerevisiae* WXY70 strain overexpressing the *CCW12* gene, encoding a cell wall mannoprotein, compared to the control strain, and *CCW12* expression was found to improve cell wall stability and tolerance to the growth inhibitors present [303]. The deletion of *GAL6,* encoding a cysteine aminopeptidase with homocysteine-thiolactonase activity, was found to lead to improved growth and higher viability of *S. cerevisiae,* in the presence of ethanol stress [304]. The *gal6*Δ cells showed increased resistance to zymolyase activity indicating the occurrence of structural changes in the cell wall [304]. A marked increased tolerance to ethanol stress associated with transcription rewiring involving cell wall synthesis was also identified in

the highly–ethanol tolerant strain *K. marxianus* FIM1 obtained by ALE in an ethanol supplemented medium [232]. Among the identified changes at the transcriptional level were the alterations related with the upregulation of cell wall metabolism involving, in particular, chitin synthesis (*CHS3*), β1,6-glucan synthesis (*KNH1*), glucanosyltransferases activity involved in β1,3-glucan branching and elongation (*GAS4*) and the cell wall integrity pathway (*BCK1, MID2, WSC3*) [232].

Numerous genes encoding proteins required for cell wall polysaccharides synthesis (*FKS1*, *ROM2*, *KRE6*, *CHS1*, *CHS5*), cell wall remodeling (*GAS1*) and protein mannosylation (*MNN2*, *MNN9, MNN11*, *KTR4*) were found to be determinants of acetic acid stress tolerance by chemogenomic analyses [263,264]. The increased expression of the *HAA1* gene, encoding a transcription factor and a major determinant of acetic acid tolerance in *S. cerevisiae*, led to the improvement of cell wall robustness under acetic acid stress, as suggested by the decreased susceptibility of the cell wall to lyticase activity mediated disruption [272]. Through Haa1 amino acid sequence engineering, a single amino acid exchange at position 135 (serine to phenylalanine) was found to lead to the upregulation of genes of the Haa1-regulon, increasing acetic acid tolerance, in particular *YGP1* [305]. The deletion of *ATG22*, encoding a vacuolar membrane protein that mediates the efflux of amino acids resulting from autophagic protein degradation, was found to delay programmed cell death (PDC) induced by acetic acid. The deletion of *ATG22* contributes to the maintenance of cell wall integrity, by preventing the decrease in total cell wall polysaccharides induced by PDC caused by severe acetic acid stress, increasing the transcript levels of CWI pathway genes [306,307]. This suggested *ATG22* as a potential target for genetic engineering strategies to improve yeast cell wall robustness and tolerance to acetic acid and other industrial stresses. Enhanced tolerance to both acetic and formic acids at low pH (pH 2.4 or below) by expressing the *GAS1* gene of *Issatchenkia orientalis* in *S. cerevisiae* was reported [308]. Also, the overexpression of *GAS1* in *S. cerevisiae* led to increased lactic acid productivity at low pH [309], reinforcing the importance of *GAS1,* encoding a beta-1,3-glucanosyltransferase, in this context [310].

As previously referred, chemogenomic studies demonstrated that genes related with cell wall polysaccharide synthesis (*FKS1*, *KRE6*) and cell wall mannosylation (*MNN10*, *ANP1*) are determinants of tolerance to osmotic stress (high glucose concentrations) in *S. cerevisiae* [165,166]. In *Pichia pastoris* (now *Komagataella phaffii*), the deletion of *YPS7*, encoding a putative GPI-linked aspartyl protease, led to increased osmotic tolerance and this gene was proposed as a promising molecular target for the engineering of yeast

robustness [311]. This species is used to produce heterologous proteins in the pharmaceutical and food industry.

In a laboratory adaptively evolved *K. marxianus* strain exhibiting a significantly improved tolerance to high temperatures, a single nucleotide polymorphism was found in the coding region of the exoglucanase gene *EXG1*, required for cell wall remodeling [312].

1.7 CONCLUDING REMARKS

The important role played by the cell wall in the adaptation and tolerance of yeasts to different stresses of biotechnological interest emerges from this chapter of this PhD thesis. Although several evidences support this idea, the truth is that these evidences largely come from genome-wide analyses of the response of yeasts to various stresses. Furthermore, the experimental conditions and levels of stress used are in general different and only part of the studies involves a time-course analysis covering the various stages of adaptation and growth under stress. Therefore, it is likely that the apparent divergences reported are due to this fact.

This chapter also provides information on how the different signalling pathways coordinate to elicit changes at the level of the yeast cell wall in response to different relevant stresses. However, it is important to highlight that, in industrial bioprocesses, several stresses are often present simultaneously, which further complicates the goal of providing a comprehensive description of what happens during adaptation to adverse process conditions. It is noteworthy to take in consideration that yeast cells exposed to mild stress develop tolerance not only to higher doses of the same stress, but also to stress caused by other agents. This phenomenon, known as cross-protection, is based on the existence of integrating mechanisms that senses and responds to different forms of stress [313]. Concerning the cell wall, the physiological observations reported and the integrated molecular responses here described provide the basis for the involvement of this dynamic organelle in cross-stress protection.

Most of this chapter is dedicated to the model yeast and cell factory *Saccharomyces cerevisiae*. However, the scarce available information in the literature concerning non-conventional yeast species of biotechnological relevance is also mentioned throughout the text. A deeper understanding of the nature of the molecular response and the changes occurring at the cell envelope level would be valuable and allow the

development of more rational strategies to construct superior yeasts for biotechnology and to control the activity of food spoiling yeasts. Genome-wide analyses are contributing to identify a wealth of cell wall-related promising targets for the improvement of yeast tolerance. However, more in depth molecular and cellular studies are instrumental to better understand the somewhat overlooked role of the cell wall in tolerance to multiple stresses in yeasts.

2. Yeast adaptive response to acetic acid stress involves structural alterations and increased stiffness of the cell wall

This chapter is based on the published article:

Ribeiro RA, Vitorino MV, Godinho CP, Bourbon-Melo n, Robalo TT, Fernandes F, Rodrigues MS, Sá-Correia I. *Yeast adaptive response to acetic acid stress involves structural alterations and increased stiffness of the cell wall.* ***Scientific Reports*****.** Jun 16 2021;11(1):12652. doi:10.1038/s41598-021-92069-3

2.1 ABSTRACT

This work describes a coordinate and comprehensive view on the time course of the alterations occurring at the level of the cell wall during adaptation of a yeast cell population to sudden exposure to a sub-lethal stress induced by acetic acid. Acetic acid is a major inhibitory compound in industrial bioprocesses and a widely used preservative in foods and beverages. Results indicate that yeast cell wall resistance to lyticase activity increases during acetic acid-induced growth latency, corresponding to yeast population adaptation to sudden exposure to this stress. This response correlates with: i) increased cell stiffness, assessed by atomic force microscopy (AFM); ii) increased content of cell wall β-glucans, assessed by fluorescence microscopy, and iii) slight increase of the transcription level of the *GAS1* gene encoding a β-1,3-glucanosyltransferase that leads to elongation of (1->3)-β-D-glucan chains. Collectively, results reinforce the notion that the adaptive yeast response to acetic acid stress involves a coordinate alteration of the cell wall at the biophysical and molecular levels. These alterations guaranty a robust adaptive response essential to limit the futile cycle associated to the re-entry of the toxic acid form after the active expulsion of acetate from the cell interior.

2.2 INTRODUCTION

The mechanistic understanding of yeast adaptation and tolerance to environmental stresses is not only a highly challenging and relevant topic in biological research but is essential for guiding the construction of superior industrial strains or for the efficient control of the deleterious activity of spoilage yeasts [314]. Therefore, the elucidation of the mechanisms underlying adaptation and tolerance to acetic acid in yeasts is of high relevance in biotechnology and food industry [7,314]. In fact, acetic acid is i) a major inhibitory compound present in lignocellulosic hydrolysates affecting their use in sustainable biorefinery processes; ii) produced during normal yeast metabolism in biotechnological processes contributing to growth and fermentation inhibition or even arrest, and iii) a widely used preservative in foods and beverages [7,8].

At a pH below acetic acid pK_a (4.75 at 25ºC) [315], acetic acid is able to passively diffuse through the plasma membrane lipid bilayer. Once inside the cell, at the near-neutral cytosol, acetic acid dissociates leading to the release of protons (H^+), causing the decrease of intracellular pH (pHi), and the accumulation of the acetate counter-ion (CH_3COO^-), the inhibition of metabolism, oxidative stress and increased turgor pressure [7,314]. In recent years, several chemogenomic [263,264], transcriptomic [263,265–267,280,316,317] and proteomic[268,269] studies allowed a more comprehensive understanding of the global mechanisms involved in *Saccharomyces cerevisiae* response and tolerance to acetic acid. Among them is the alteration of the molecular composition and physical properties of plasma membrane and cell wall, leading to the decrease of cell envelope permeability[7,21–23]. Such adaptation, at the level of the cell envelope, is essential to reduce the diffusion rate of this weak acid from the cell exterior to the intracellular medium. This response counteracts the re-entry of the acid form after the active expulsion of acetate from the cell interior, presumably catalysed by efflux pumps (e.g. Tpo2, Tpo3, Aqr1, Pdr18)[318] and, in this way, limits the associated futile cycle [7,24].

Several genes encoding proteins required for the synthesis of cell wall polysaccharides and cell wall remodeling are transcriptionally responsive to acetic acid stress and/or determinants of acetic acid stress tolerance [263–266,317] The *YGP1* and *SPI1* genes, encoding a cell wall-glycoprotein and a Glycosylphosphatidylinositol (GPI)-anchored cell wall protein, respectively, are determinants of acetic acid tolerance and directly up-regulated in response to acetic acid by Haa1, the major regulator in adaptive response and tolerance to acetic acid in *S. cerevisiae* [263–266]. Genes involved in 1-3 β-glucan synthesis (*FKS1, ROM2),* 1-6 β-glucan synthesis (*KRE6),* 1-3 β-glucan elongation and branching (*GAS1*), chitin synthesis (*CHS1, CHS5*) and cell wall protein mannosylation (*MNN2, MNN9, MNN11, KTR4)* are also reported determinants of tolerance to acetic

acid [264]. Although this seems to point towards a role for the cell wall in acetic acid stress response and tolerance, the transcript levels from the acetic acid tolerance determinant genes *CHS1*, *KTR4*, *MNN9* and *FKS1* were reported to decrease in acetic acid stressed cells [265]. Another transcriptomic analysis reports the differential-expression of 28 cell wall metabolism-related genes under acetic acid stress from which 24 were down-regulated, in particular *KRE6*, *FKS1* and its paralog *FKS2*/*GSC2* [317].

The mechanisms involving changes in the chemical structure and organization of the cell wall impact its biophysical properties [319]. For instance, the stiffness of the cell wall appears to be strongly dependent on the molecular architecture of the cell wall, particularly on the crosslinking between β-glucans and chitin, rather than on the increase of a particular cell wall polysaccharide or on cell wall thickness [69,320]. Although nanomechanical and biochemical changes occurring at the yeast cell wall in response to heat and ethanol stresses have been reported [70,71,321], the response of the cell wall to stress induced by acetic acid is so far unknown and on the focus of this study. The objective of the present work was to examine the hypothesized involvement of the remodeling of the cell wall at the molecular and biophysical levels during the time course of the adaptive response of *S. cerevisiae* to a sub-lethal concentration of acetic acid. The alterations occurring at the level of yeast cell wall architecture during adaptation were assessed based on cell wall susceptibility to lyticase activity and on cell wall stiffness assessed by atomic force microscopy (AFM). The transcriptional activation of genes involved in cell wall synthesis and remodeling was assessed by qRT-PCR and the content of cell wall polysaccharides by fluorescence microscopy. Results provide a global view on mechanisms underlying the time-course of yeast cell adaptation to acetic acid stress at the cell wall level.

2.3 MATERIALS AND METHODS

2.3.1 YEAST STRAINS AND GROWTH CONDITIONS

The *S. cerevisiae* parental strain BY4741 (*MATa, his3Δ1, leu2Δ10, met15Δ0, ura3Δ0*), obtained from the EUROSCARF collection, was used in this study. The strain was maintained at −80°C in YPD media supplemented with 30% (v/v) glycerol. Cells were cultivated at 30°C, with orbital agitation (250 rpm), in minimal growth medium supplemented with amino acids and uracil (MM4). MM4 contains 1.7 g/L yeast nitrogen base without amino acids and ammonium sulphate (Difco, Michigan, USA), 20 g/L glucose (Merck, Darmstadt, Germany), 2.65 g/L $(NH_4)_2SO_4$ (Panreac AppliChem, Connecticut, USA), 20 mg/L methionine, 20 mg/L histidine, 60 mg/L leucine and 20 mg/L uracil (all from Sigma, Missouri, USA). The medium pH was adjusted to 4.0 with HCl. For the majority of the experiments performed under acetic acid stress, MM4 medium was supplemented with 60 mM acetic acid (Fluka, Waltham, USA) using a solution of 5 M acetic acid, set to pH 4.0 with NaOH. For the majority of the growth experiments, MM4 supplemented was inoculated to an initial optical density at 600_{nm} (OD_{600nm}) of 0.1 ± 0.05 mid-exponential cells harvested by filtration (Whatman, Maidstone, UK) from cultivation in fresh MM4 medium (pH 4.0) without acetic acid supplementation. Growth was followed by measuring culture OD_{600nm}.

2.3.2 YEAST CELL WALL SUSCEPTIBILITY TO LYTICASE

To monitor yeast cell wall structural alterations during cultivation in MM4 supplemented or not with 60 mM acetic acid, a lyticase susceptibility assay (β-1,3-glucanase from *Arthrobacter luteus*; Sigma, containing β-(1$\rightarrow$3)-glucan laminaripentaohydrolase with additional β-(1$\rightarrow$3)-glucanase, protease, and mannanase activities) was conducted as described before [273]. Briefly, the parental strain was cultivated in MM4 liquid medium, either supplemented or not with 60 mM acetic acid, at pH 4.0, and were harvested by filtration at adequate time-points of the cultivation. Cells were washed with distilled water and used to inoculate 100 mL erlenmeyer flasks containing 50 mL 0.1 mM phosphate buffer (pH 6.6), to a final OD_{600nm} of 0.5. After the addition of 15000 U/mL of lyticase, cell lysis was followed by measuring the decrease of OD_{600nm} for each cell suspension and converting it to a percentage of the initial OD_{600nm} value. The susceptibility to lyticase is represented as the maximum specific lysis rate defined as the absolute value of

the slope of the straight line that best fits the semi-logarithmic plot of the linear part of the lysis curve.

2.3.3 ASSESSMENT OF THE NANOMECHANICAL PROPERTIES OF YEAST CELL WALL BY ATOMIC FORCE MICROSCOPY (AFM)

For AFM assessment of cell wall nanomechanical properties, cells of the parental strain were grown and harvested at adequate time-points as described for lyticase susceptibility assays. Cell suspensions of OD_{600nm} of 0.2 were prepared in 5 mL of bi-distillate de-ionized water. These cell suspensions were filtered through a polycarbonate membrane (Whatman) with a pore size of 3 µm, similar to the yeast cell longitudinal size, and washed once with 15 mL bi-distillate de-ionized water. The filter was allowed to dry on air and then deposited on a piece of double-faced tape and placed on the AFM stage for analysis.

For imaging and Force-Distance measurement, the samples were analysed with a PicoLE Molecular Imaging system operated in contact mode. A microlever MSCT-F (Bruker, Billerica, USA) with nominal stiffness of 0.6 N/m and nominal tip radius of 10 nm was used in all experiments.

Force spectroscopy mapping, consisting of 32×32 approach/retract force-displacement curves was performed in the yeast cell surface. The maximum deflection of the AFM cantilever was set constant yielding a maximum applied force of ≈ 30 nN. The tip-sample approach velocity was about 0.5 µm/s. To this end, an area in the cell was selected so that no bud scars were included, resulting in a total of about 100 approach/retract force-displacement curves from which the median value was kept. The force distance grids were analysed for determination of the apparent Young's modulus which is the quantitative expression of the cell surface elasticity that reflects the stiffness. The Young's modulus was obtained by adjusting the DMT contact model to the approach curves. The force distance grids were processed by home-written software using Wolfram Mathematica (Wolfram Research, Illinois, USA). A total of 32 cantilevers were used and the spring constant of the cantilevers was calibrated using the Sader method [322]. In order to reduce bias due to different cantilevers being used on different cells, each cantilever was used to measured cells at several time points investigated, and the process was randomized. Eventually, however, it was not possible to use the same cantilever at all conditions tested. Results arise from, at least, 3 independent experiments and from the analysis of, at least, 15 cells in each condition.

2.3.4 TRANSCRIPTIONAL ANALYSIS OF CELL WALL BIOSYNTHETIC AND REGULATORY GENES

For gene transcription assays, parental strain cells were harvested during cultivation as described above for AFM and lyticase assays. Total RNA extraction was performed by the hot phenol method[323].

The quantitative real-time Reverse Transcription–PCR (qRT-PCR) protocol used to determine the mRNA levels from *RLM1*, *FKS1*, *FKS2*, *BGL2*, *CHS3*, *CRH1*, *GAS1* and *PRM5* genes followed the manufacturer's instructions. Primer Express software V3.0 (Applied Biosystems) was used for primer design for the amplification of each target cDNA (**Table 2.1**).

Table 2.1 - Primers used for qRT-PCR analysis.

Primer	Sequence (5'-3')
ACT1	fw: CTCCACCACTGCTGAAAGAGAA rev: CCAAGGCGACGTAACATAGTTTT
CHS3	fw: TCACCTGGATGTTTTACCATCAAG rev: CCACTCCGACGAGTTGCAT
FKS1	fw: CATGCTGCTCTGGTCCCTTATT rev:CACCGTGGGCAATTCCA
FKS2	fw: GCTCATGTCGTTGGAGCAGTT rev: CCAATGGCATTACGGAAAAGA
RLM1	fw: CTTTTTTCTGCAACACAGCCATA rev: CGCCAGGAATATTCGATGGT
GAS1	fw: AACCGCTGCTGCTTTTTTTG rev: CTTCAATCGCTGGAACATCGT
CRH1	fw: CGCGGCTGCCGAAAG rev: GCA GTGCTAGAAGCTGCAGTTG
BGL2	fw: TTTTGTTATGGCTAACGCGTTCT rev: GAGTAAGAGGCATTTTGCATGGT
PRM5	fw: TTTTCCACACAACATACCCAGTTT rev: TCTTTGGCGGGATAATCCATA

The RT–PCR reaction was conducted in a thermal cycler block (Cleaver GTC965) and the qPCR was conducted in QuantStudio 5 (Applied Biosystems) using NZYSpeedy qPCR Green Master Mix (NZYTech). The *ACT1* mRNA level was used as the internal control (**Table 2.1**). The relative value obtained for each target gene at the initial time-point at 0 hours under unstressed conditions was set as 1 and the remaining values are relative to this reference value.

2.3.5 ASSESSMENT OF CELL WALL POLYSACCHARIDES CONTENT BY FLUORESCENCE MICROSCOPY

The methodology for the staining of the cell wall polysaccharides was adapted from Pradhan *et al*[324]. Concanavalin A-FITC, Fc-Dectin 1-Alexa 633 and Calcofluor White were used as staining compounds that bind mannans, glucans and chitin, respectively. Yeast cells were harvested at adequate time-points during cultivation as described above. Cells at an OD_{600nm} of 0.5 were incubated with 0.75 µg/mL Fc-Dectin 1 (Sino Biological, China) dissolved in FACS buffer for 45 minutes on ice. After centrifugation at 4300 *g* for 5 minutes at 4ºC, the pellet was washed with 200 µL FACS buffer. Cells were then incubated for 30 minutes with 1:200 of anti-human IgG conjugated with Alexa Fluor 633 (ThermoFisher Scientific, Waltham, USA), 50 µg/mL Calcofluor White (Fluka) and 50 µg/mL Concanavalin A conjugated with Fluorescein (Sigma). After centrifugation at 4300 *g* for 5 minutes at 4ºC, the pellet was washed with 200 µL FACS buffer. Stained cells were resuspended in 100 µL FACS buffer and were immobilized in 2.2% agarose in PBS 1x mounted on a gene frame 1.0x1.0 cm (ThermoFisher Scientific).

All measurements were performed using a Leica TCS SP5 (Leica Microsystems CMS GmbH, Mannheim, Germany) inverted confocal microscope (DMI6000). A 63× apochromatic water immersion objective with a NA of 1.2 (Zeiss, Jena Germany) was used for all experiments. Images were collected at 2048x2048 resolution. For the Concanavalin A-FITC channel, confocal microscopy measurements were carried out with an Argon laser for excitation at 488 nm. Emission was collected between 495-580 nm. For the FC-Dectin1-Alexa 633 channel, confocal microscopy was employed as well, and excitation was carried out through a He-Ne laser line at 633 nm. Fluorescence emission was collected in this channel between 640-770 nm. Finally, 2-photon excitation microscopy was used for the Calcofluor White channel with excitation from a Spectra-Physics Mai Tai BB laser set at 780 nm. Calcofluor white emission was collected between 390-480 nm. Data analysis was carried out using ImageJ. Regions of interest (ROI) corresponding to the cellular surface were defined and average pixel fluorescence

intensities within these ROIs were determined for each channel and average background fluorescence was always subtracted to the calculated values.

The staining of the cells was carried out to ensure that the analysis of mannans, β-glucans and chitin refers to the same cell population. Results of the median intensity fluorescence were obtained from the analysis of at least 34 cells obtained from two independent experiments.

2.4 RESULTS

2.4.1 YEAST ADAPTATION TO ACETIC ACID INVOLVES INCREASED CELL WALL RESISTANCE TO LYTICASE ACTIVITY

The alterations occurring in yeast cell wall architecture during cultivation in MM4 pH 4.0, either or not supplemented with 60 mM acetic acid, were monitored based on cell wall susceptibility to lyticase activity. This technique was previously shown to be valuable to monitor alterations in the cell wall in response to various environmental stresses [253,272,273]. The maximum specific lysis rate for a given timepoint was calculated as the slope of the straight line that best fits the semi-logarithmic plot of the time-course decrease of cell suspension OD_{600nm} after the addition of lyticase (**Figure 2.1, a and b**). Values were plotted during the growth curve (**Figure 2.1, c and d**). The maximum specific lysis rate exhibits similar values during exponential growth (0-5 hours) in the absence of acetic acid (**Figure 2.1, a and c**). However, for cells cultivated in medium supplemented with 60 mM acetic acid (pH 4.0), a rapid and marked significant reduction of the maximum specific lysis rate occurs at 3 hours of acetic acid-induced latency, compared to the initial time-point (from 1.27 to 0.55 $\Delta[OD_{600nm}(\%)]$ min^{-1}; $p<0.00001$; one-way-ANOVA) (**Figure 2.1, b and d**). The lowest maximum specific lysis rate was attained after 7 hours of growth latency (**Figure 2.1, b and d**). Moreover, acetic acid-adapted exponentially-growing cells exhibit a significantly lower susceptibility to lyticase compared with exponentially-growing unstressed cells ($p=0.002$; one-way ANOVA; **Figure 2.1, c and d**). These results indicate that adaptation to acetic acid leads to significant cell wall architecture alterations, resulting in higher resistance to lyticase activity.

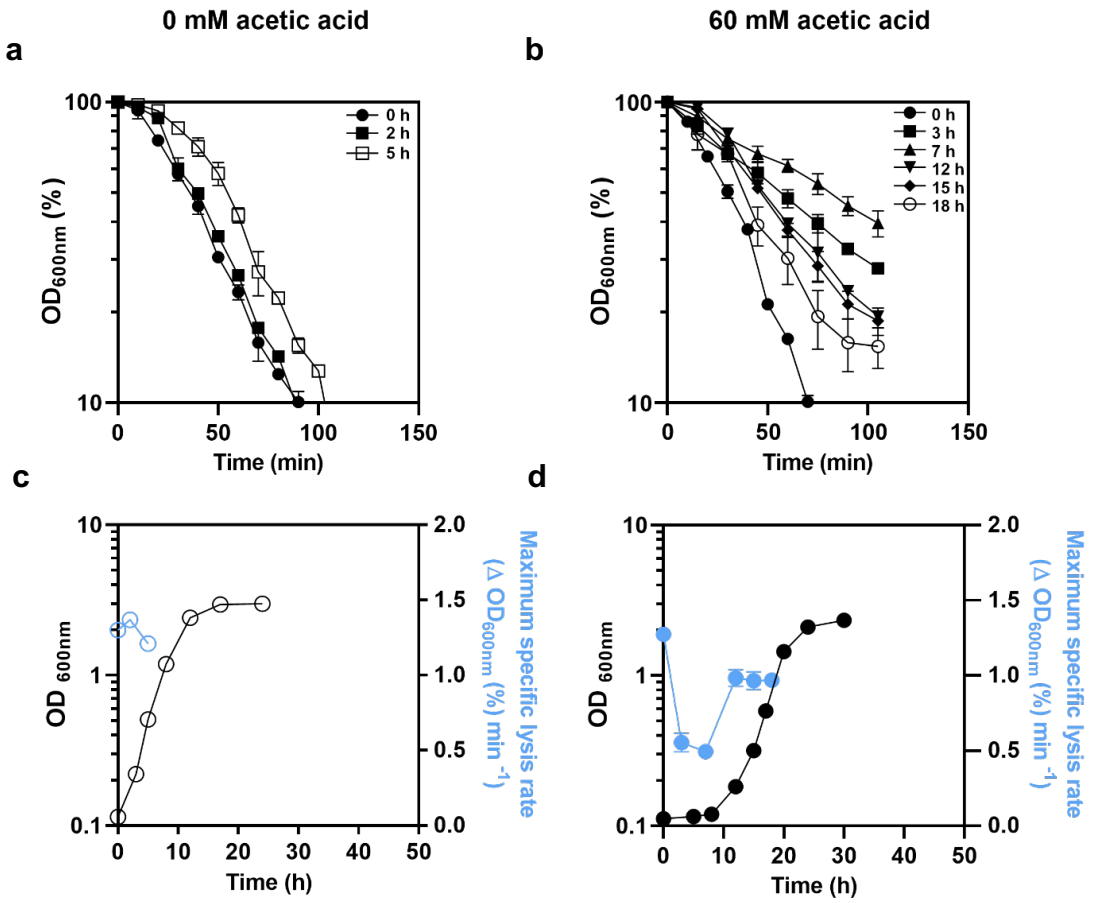

*Figure 2.1 - Effect of lyticase in yeast cell wall during the growth curve in the absence or presence of acetic acid stress. Decrease of the OD_{600nm} of S. cerevisiae BY4741 cell suspensions (in %) following the addition of lyticase, as described in M&M. Cells were harvested at 0 h (filled circle), 2 h (filled square), 5 h (open square) of cultivation in MM4 pH 4.0 not supplemented with acetic acid (**a**) and at 0 h (filled circle), 3 h (filled square), 7 h (filled triangle), 12 h (filled down pointing triangle), 15 h (filled rhombus) and 18 h (open circle) of cultivation in the presence of 60 mM acetic acid at 4.0 (**b**).The maximum specific lysis rates, determined based on data in (**a**) and (**b**) for yeast cells harvested at the selected time-points, are shown during cultivation in the absence (**c**) or presence of acetic acid (**d**). The initial biomass concentration used corresponded to culture OD_{600nm} of 0.1 ± 0.01. Data are means from at least three independent experiments and bars represent standard deviation.*

2.4.2 YEAST CELL ADAPTATION TO ACETIC ACID STRESS INVOLVES THE INCREASE OF YEAST CELL WALL STIFFNESS

The biophysical properties of the yeast cell surface during adaptation to acetic acid stress were examined by AFM to assess the Young's modulus, which is the quantitative expression of the cell surface elasticity that reflects cell wall stiffness. The Young's modulus was not found to vary significantly during the first 5 hours of exponential growth

in MM4 pH 4.0 (**Figure 2.2, a**). Exposure to 60 mM of acetic acid (pH 4.0) was found to lead to the increase of the Young's modulus value during the induced growth latency; after 7 hours of incubation, the cell population exhibits a significantly higher Young's modulus than unstressed cells (**Figure 2.2**; *p*=0.006; one-tailed Mann-Whitney U test). The Young's modulus of exponentially-growing adapted cells in the presence of acetic acid (142 MPa) is also significantly higher than the Young's modulus of the corresponding unstressed cells (**Figure 2.2**; *p*=0.002; one-tailed Mann-Whitney U test). These results indicate that adaptation to acetic acid-induced stress involves the increase in yeast cell wall stiffness.

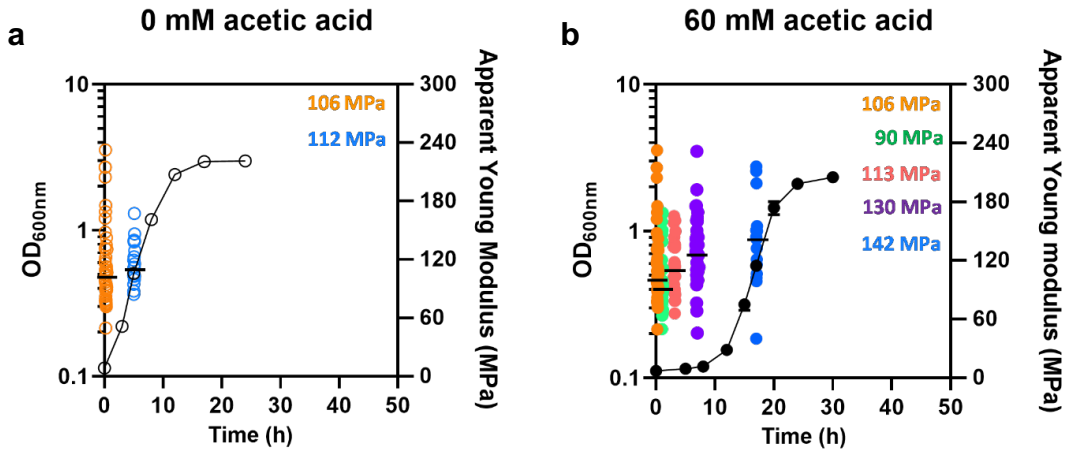

*Figure 2.2 - Apparent Young's modulus of yeast cells during the growth curve in the absence or presence of acetic acid stress. Time-course analysis of the yeast cell surface stiffness, assessed by atomic force microscopy in BY4741 cells cultivated in the absence (**a**) or presence of acetic acid (**b**), as described for Figure 2.1. The stiffness of the cell surface is represented as the apparent Young's modulus. Each point in the graph corresponds to the median value of about 100 curves over a single cell. For each condition, at least, 15 cells were analyzed from, at least, 3 independent experiments.*

2.4.3 TRANSCRIPTIONAL PROFILES OF CELL WALL BIOSYNTHESIS-RELATED GENES IN RESPONSE TO ACETIC ACID

Quantitative real-time Reverse Transcription–PCR (qRT-PCR) was performed to assess the levels of transcription from several cell wall biosynthesis-related genes during cultivation in the absence or presence of 60 mM acetic acid, at pH 4.0. The chosen genes were: *RLM1* (encoding a transcription factor responsible for the transcriptional activation of the majority of genes induced in response to cell wall stress through the CWI pathway [325]), *FKS1* and *FKS2* (encoding β-1-3-glucan synthases [326]), *BGL2* (encoding endo-beta-1,3-glucanase [85]), *CHS3* (encoding a major chitin synthase [327]), *CRH1* (encoding a chitin transglycosylase [87]), *GAS1* (encoding a β-1,3-glucanosyltransferase, an important enzyme for cell wall remodeling involving elongation of (1->3)-β-D-glucan chains and branching [63,85]), and *PRM5* (a Rlm1 target and the hallmark of CWI pathway activation [325]). The mRNA levels from these genes were not found to vary significantly during the first 5 hours of exponential growth in MM4, at pH 4.0 (**Figure 2.3**). However, when the medium was supplemented with acetic acid, the mRNA levels from all genes tested, except for *GAS1* and *FKS1,* were found to decrease markedly during growth latency. The mRNA levels from *FKS1* were similar during cultivation in the presence of acetic acid but the transcript levels from *GAS1* moderately increased to around 1.5-fold higher than the levels of expression in unstressed cells, throughout the timepoints tested (**Figure 2.3**). The maximum activation was obtained after 7 hours of cultivation in the presence of acetic acid (1.54-fold the levels of exponentially-growing unstressed cells; p=0.001421; one-way ANOVA).

Collectively, our results show that cultivation in the presence of acetic acid leads to the down-regulation of transcription from several cell wall biosynthesis-related genes, while the β-1,3-glucanosyltransferase encoding gene *GAS1* was up-regulated.

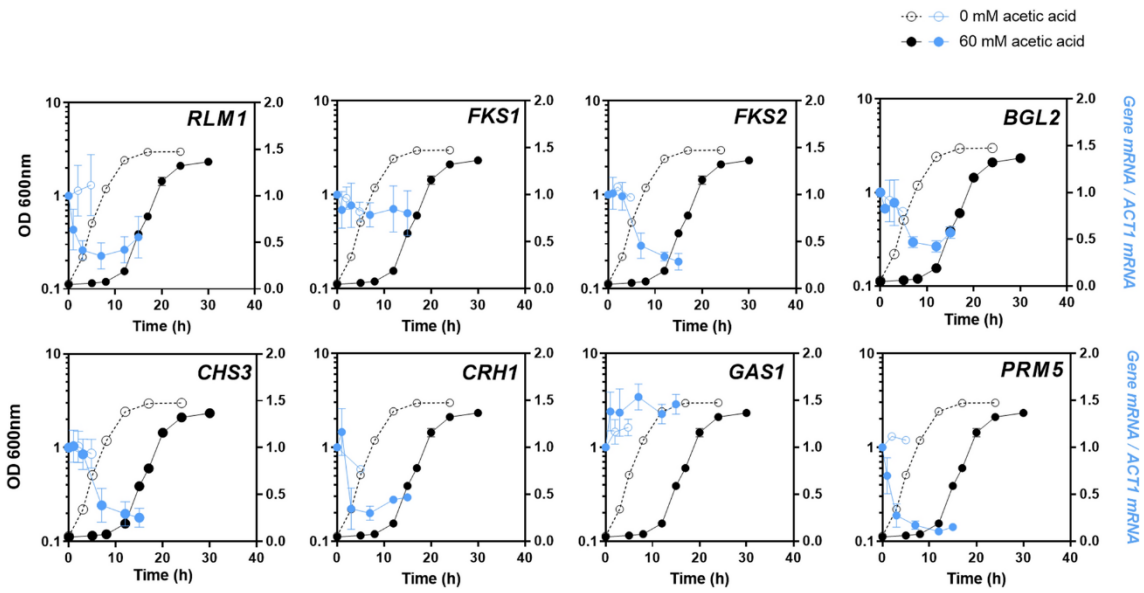

Figure 2.3 - Levels of mRNA from cell wall biosynthesis-related genes during yeast cultivation in the absence or presence of acetic acid. Time-course of the mRNA levels from RLM1, FKS1, FKS2, BGL2, CHS3, CRH1, GAS1 and PRM5 genes during cultivation of BY4741 in absence (open circle) or presence (filled circle) of acetic acid, as described for Figure 2.1. The transcriptional levels (in blue) from the indicated genes were assessed by qRT-PCR, using ACT1 as the internal control. The relative value obtained for each target gene at the initial time-point under unstressed conditions was set as 1. Results are means of, at least, three biological replicates and error bars represent standard deviation. Primers were designed using Primer Express V3.0 (Applied Biosystems).

2.4.4 YEAST CELL ADAPTATION TO ACETIC ACID STRESS INVOLVES THE INCREASE OF CELL WALL β-GLUCANS, AS ASSESSED BY FLUORESCENCE MICROSCOPY

Possible changes in the polysaccharide content of cell wall during cultivation in the presence of an identical acetic acid stress were assessed based on their quantitative analysis by fluorescence microscopy. Images illustrating those obtained by fluorescence microscopy are shown in **Figure 2.4.**

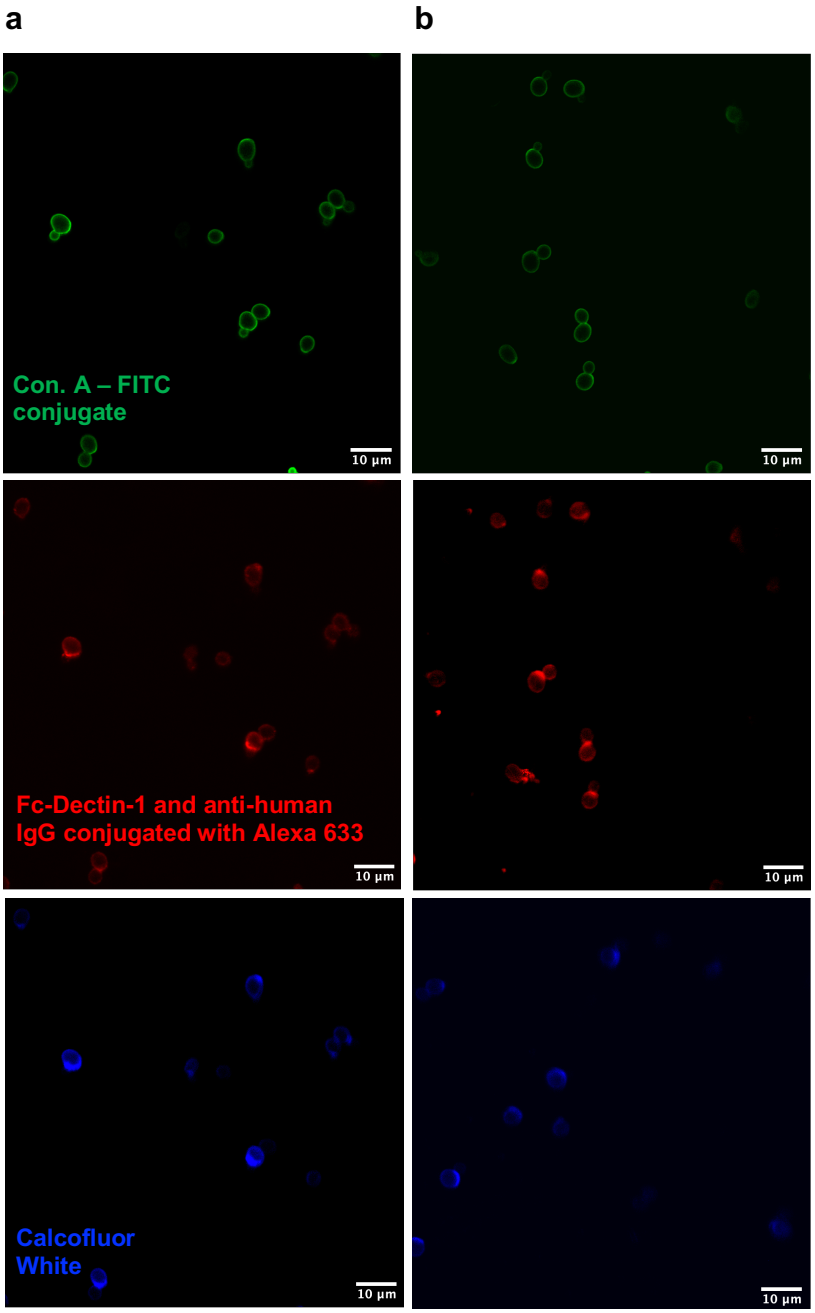

Figure 2.4 - Fluorescence microscopy images of fluorescence-stained cell wall polysaccharides. Illustrative images of stained BY4741 cells were taken at 0 hours (a) and 7 hours (b) of exposure to acetic acid, corresponding to the end of the acetic acid-induced latency. Images were used to quantify cell wall mannans and β-glucans, using confocal microscopy, and chitin, using 2-photon excitation microscopy.

To make it easier to visualize and analyze the results, **Figure 2.5** shows only the median values obtained for cells harvested during stressed and unstressed cultivations. Individual cell measurements are shown in **Figure 2.6**.

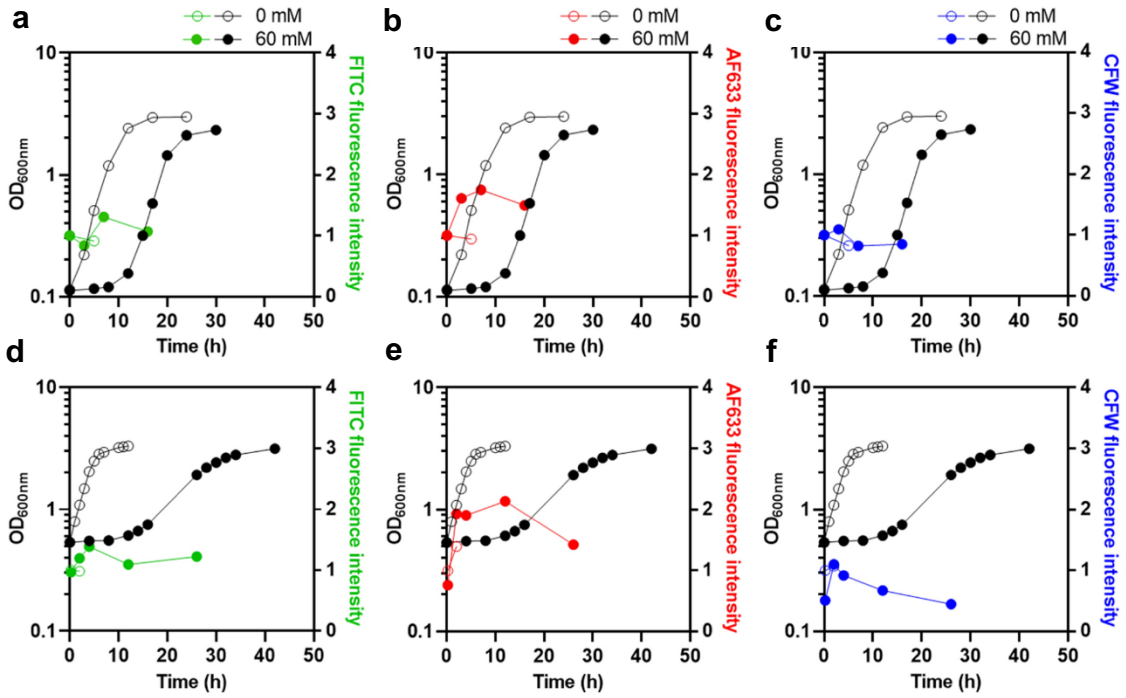

Figure 2.5 - Content of cell wall polysaccharides in yeast cells during adaptation to acetic acid stress by fluorescence microscopy. Quantitative analysis of the cell wall polysaccharides during cultivation of S. cerevisiae BY4741 in the absence (open circle) or presence (filled circle) of acetic acid, as described for Figures 2.1, 2.2, 2.3 (a–c). Results of an independent experiment using a higher level of acetic acid stress and a higher initial biomass concentration (corresponding to initial OD_{600nm} of 0.5 ± 0.05) are also shown (d–f). The cell wall components mannans (green), β-glucans (red) and chitin (blue) were stained with Concanavalin A conjugated with Fluorescein (FITC), Fc-Dectin 1 conjugated with Alexa Fluor 633 (AF633) and Calcofluor White (CFW), respectively. Quantification of the fluorescence intensity was performed with a Leica TCS SP5 (Leica Microsystems CMS GmbH, Manheim, Germany) inverted confocal microscope (DMI600). Median fluorescence intensity values result from the analysis of, at least, 34 cells obtained from two replicates. Individual cell measurements can be consulted in Figure 2.6.

Results show that in growth medium supplemented with 60 mM acetic acid (pH 4.0), the cell wall content in β-glucans increased, as quantified by the fluorescence intensity of Alexa Fluor 633 conjugated anti-human IgG with FC-Dectin1 (**Figure 2.5, b**). Yeast exposure to acetic acid leads to a significant increase in fluorescence intensity at 3 hours of cultivation (1.6-fold higher than the level of the exponentially-growing unstressed cells; $p<0.00001$; one-tailed Mann-Whitney U test) (**Figure 2.5, b**). The levels of β-glucans attained were maintained significantly higher than those in unstressed cells ($p<0.00001$; one-tailed Mann-Whitney U test) even when acetic acid-grown cells reached exponential phase of growth (**Figure 2.5, b**). The increase of the glucan content in yeast cells adapting and adapted to acetic acid was confirmed using a higher level of stress (**Figure 2.5, e**).

Concerning the content in mannans and chitin, quantified by fluorescence intensity of Concanavalin A-fluorescein (FITC) and Calcofluor White (CFW), respectively, its consistent alteration during the whole period of cultivation in the presence of acetic acid, when compared with unstressed cultivation, was not so clear for the two tested conditions (**Figure 2.5, a, d and c, f**). However, the mannan content of yeast cells adapting or growing in conditions leading to higher acetic acid stress was found to be significantly higher compared with unstressed cells (**Figure 2.5, d** and **Figure 2.6, d**). For the chitin content, considering the time-course profile obtained and the fact that calcofluor white can also stain some beta-glucans, possibly interfering with chitin quantification, no definitive conclusions could be taken.

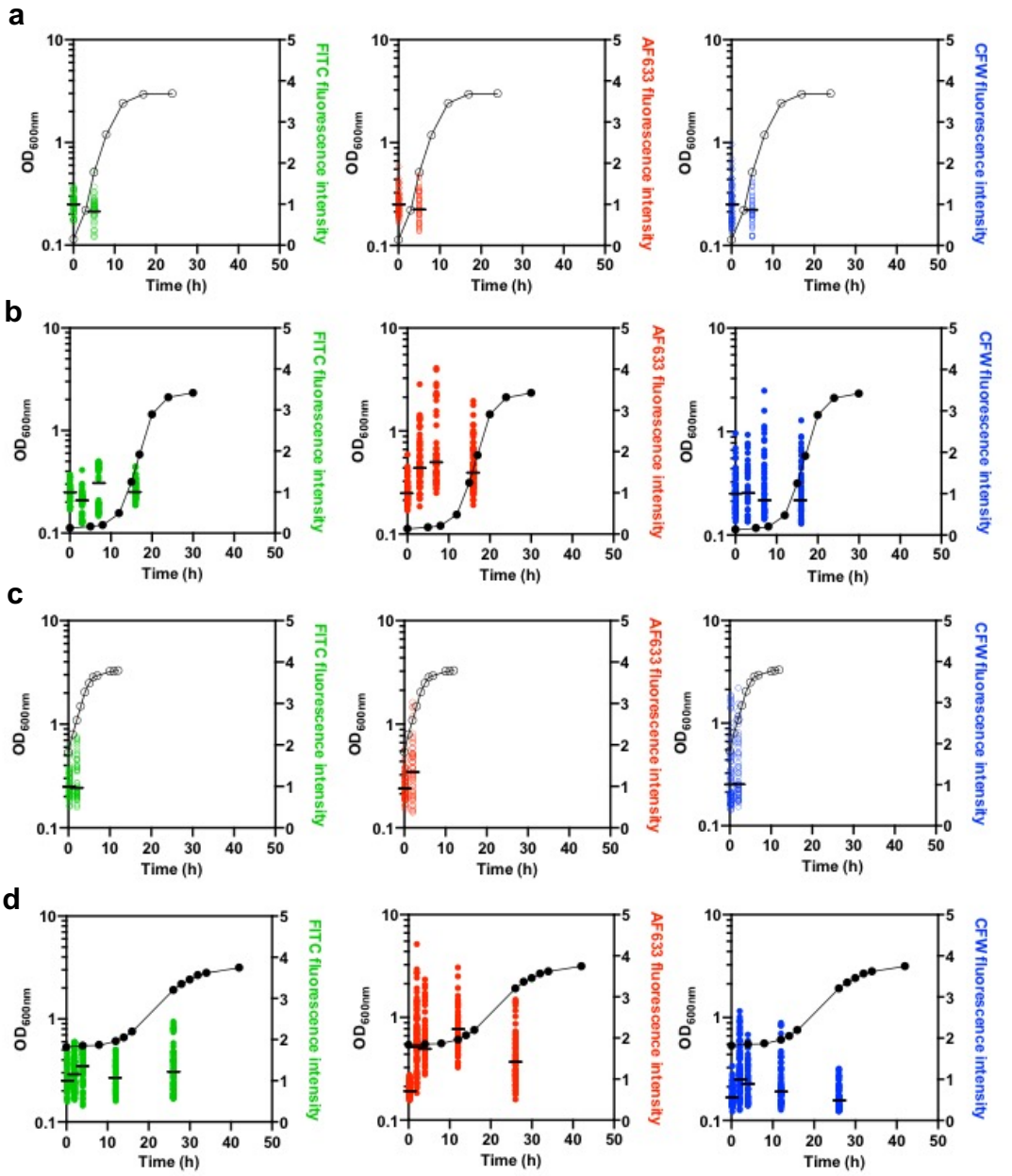

Figure 2.6- Content of cell wall polysaccharides in yeast cells during adaptation to acetic acid stress by fluorescence microscopy. Quantitative analysis of the cell wall polysaccharides was performed during cultivation of BY4741 cells in the absence (○; a and c) or presence (●; b and d) of 60 mM acetic acid (pH 4.0) at an initial OD $_{600nm}$ of 0.1 (a-b) or 0.5 (c-d). The cell wall components mannans (green), β-glucans (red) and chitin (blue) were stained with Concanavalin A conjugated with Fluorescein (FITC), Fc-Dectin 1 conjugated with Alexa Fluor 633 (AF633), and Calcofluor White (CFW), respectively. Quantification of the fluorescence intensity was performed with a Leica TCS SP5 (Leica Microsystems CMS GmbH, Manheim, Germany) inverted confocal microscope (DMI600). Median fluorescence intensity is indicated by a dash and results from the analysis of, at least, 34 cells obtained from two independent experiments.

2.5 DISCUSSION

Among the mechanisms that have been proposed to underlie *Saccharomyces cerevisiae* adaptation and tolerance to acetic acid is the remodeling of the cell envelope [7]. They are considered essential to limit the diffusional re-entry of the acid form of the weak acid following the induced active expulsion of acetate from the cell interior, thus counteracting this futile cycle [7,24,259]. The present work describes, for the first time, a coordinated and comprehensive view on the time-course of the alterations occurring at the level of the cell wall in a yeast cell population during adaptation to sudden exposure to a sub-lethal stress induced by acetic acid. The coordinated alteration of cell wall susceptibility to the action of lyticase activity, cell wall polysaccharide composition and nanomechanical properties, and of the transcription levels of key cell wall-biosynthesis-related genes, following yeast exposure to acetic acid was systematically examined. Collectively, results reinforce the notion that the adaptive yeast response to acetic acid stress involves a coordinate alteration of the cell wall at the biophysical and molecular levels.

The increased resistance to lyticase observed in this study for yeast cells during adaptation to acetic acid was reported before by our laboratory for other weak acids [273]. The marked increase in cell wall resistance to lyticase (containing β-(1→3)-glucan laminaripentaohydrolase with additional β-(1→3)-glucanase, protease, and mannanase activities), during the first hours of exposure to acetic acid strongly suggests that changes occurred in the cell wall composition and/or cross-linking between cell wall macromolecules. Mechanisms involving the synthesis and reorganization of cell wall constituents have been proposed to be relevant to maintain cell wall integrity and to decrease cell permeability to weak acids and thus to limit their contact with plasma membrane, therefore reducing membrane damage and intracellular acidification [7,22,110,259,263,273,328]. In fact, it was demonstrated that pre-exposure to acetic acid or sorbic acid causes a decrease in the rate of intracellular acidification, most likely as a result of changes occurring in plasma membrane and cell wall [24]. The mechanism to reduce intracellular acidification based on the active extrusion of protons and of the acid counter-ion is not, by itself, efficient unless the cell is able to restrict the diffusional re-entry of the weak acid [7,24,259]. Plasma membrane permeability was found to increase upon exposure to acetic acid stress and to decrease following yeast cell adaptation to the stress and growth resumption in its presence, although maintaining permeability levels above those in the corresponding unstressed cells [23]. Remarkably, the minimum value of cell susceptibility to lyticase was found during the latency phase, while the susceptibility to lyticase of cells exponentially growing in the presence of the acid was higher but below

the values for unstressed cells. Results are consistent with the concept that cell wall remodeling is crucial during acetic acid-induced latency when the permeabilization of stressed yeast cells is maximal, thus limiting the deleterious effects of the acid.

The present work provides, for the first time, a time course characterization of the nanomechanical properties of the cell wall by assessing cell surface stiffness of the yeast population following sudden exposure to stress during the full length of the cultivation in medium supplemented with a sub-lethal concentration of acetic acid stress. Due to the plastic nature of the cell wall, stress-induced mechanisms that include changes in composition and/or remodeling of the cell wall may result in changes of its biophysical properties[319]. According to the data of the present study, cell surface stiffness increases during adaptation to acetic acid stress. The stiffness of the cell wall is apparently not linked to changes in a specific component of the cell wall, but rather related with its molecular architecture, involving the cross-link between cell wall components, in particular the cross-link between β-glucans and chitin that seem to have a prominent role in cell wall stiffness [69,70]. The observed correlation between the time-course profiles of the increase of *GAS1* transcription level and the content of glucan in the cell wall suggests that cell wall remodeling under acetic acid stress is, at least partially, due to the β-1,3-glucanosyltransferase activity of Gas1 involved in glucan elongation and also in branching [63,85], while no support for the *de novo* synthesis of more glucan was obtained. Consistent with these results, *GAS1* is a demonstrated determinant of acetic acid tolerance [264] as well as for other environmental stresses such as those induced by H_2O_2 treatment [329], low pH [247] and ethanol [121]. On the contrary, the transcription levels from *FKS2*, *BGL2*, *CHS3*, *CRH1* and *PRM5* genes suffer a continuous reduction during the whole period of stressed cultivation. The mRNA levels from *RLM1*, encoding the major transcriptional regulator of the CWI pathway effectors, were also reduced by cultivation in the presence of this weak acid, consistent with the down-regulation of Rlm1-target genes observed herein. Consistent with previous transcriptomic analysis of cells exposed to sub-lethal and severe acetic acid stress , the observed down-regulation of *RLM1* also suggests that the CWI pathway may not be the major key player in acetic acid stress response [265,317]. However, since the CWI pathway gene *GAS1* was found to be upregulated under acetic acid stress, it is not possible, at this time, to put aside the possible role of this pathway in the adaptive response. Moreover, the expression of *FKS1* does not exhibit the same down-regulation pattern under acetic acid stress of other CWI pathway genes, *FKS1* mRNA levels being similar to those in unstressed cells. The maintenance of at least one of the glucan synthases encoded by the paralogue genes *FKS1* and *FKS2* in acetic acid stressed cells was found to be required [110]. Although other

enzymes, responsible for glucan remodeling and/or glucan/chitin cross-linking or other alterations of cell wall structural composition may also be involved in cell wall remodeling under acetic acid stress, no evidence for this was obtained here.

The present work examined the involvement of the cell wall, at the molecular and biophysical levels, in *S. cerevisiae* adaptation to a sub-lethal concentration of acetic acid. Collectively, our results suggest that this adaptation involves changes in yeast cell wall composition and structure that result in a stiffer and more robust cell wall essential to limit the futile cycle associated to the re-entry of the toxic acid form, after the active expulsion of acetate from the cell interior.

3. Crosstalk between yeast cell plasma membrane ergosterol content and cell wall stiffness under acetic acid stress involving Pdr18

This chapter is based on the published article :

Ribeiro RA, Godinho CP, Vitorino MV, Robalo TT, Fernandes F, Rodrigues MS, Sá-Correia I. *Crosstalk between Yeast Cell Plasma Membrane Ergosterol Content and Cell Wall Stiffness under Acetic Acid Stress Involving Pdr18*. ***Journal of Fungi***. Jan 21. 2022;8(2):103. doi:10.3390/jof8020103

3.1 ABSTRACT

Acetic acid is a major inhibitory compound in several industrial bioprocesses, in lignocellulosic yeast biorefineries. Cell envelope remodeling, involving c plasma membrane composition, structure and function, is among the mechanisms behind yeast adaptation and tolerance to stress. Pdr18 is a plasma membrane ABC transporter of the pleiotropic drug resistance family and a reported determinant of acetic acid tolerance mediating ergosterol transport. This study provides evidence for the impact of Pdr18 expression in yeast cell wall during adaptation to acetic acid stress. The time-course of acetic acid-induced transcriptional activation of cell wall biosynthetic genes (*FKS1*, *BGL2*, *CHS3*, *GAS1*) and of increased cell wall stiffness and cell wall polysaccharide content in cells with the *PDR18* deleted, compared to parental cells, is reported. Despite the robust and more intense adaptive response of the *pdr18*Δ population, stress induced increase of cell wall resistance to lyticase activity was below parental strain levels and the duration of the period required for intracellular pH recovery from acidification and growth resumption was higher in the less tolerant *pdr18*Δ population. The ergosterol content, critical for plasma membrane stabilization, suffered a drastic reduction in the first hour of cultivation under acetic acid stress, especially in *pdr18*Δ cells. Results revealed a crosstalk between plasma membrane ergosterol content and cell wall biophysical properties and suggest a coordinated response to counteract acetic acid deleterious effects.

3.2 INTRODUCTION

Acetic acid is a major inhibitory compound in industrial bioprocesses, in particular in lignocellulosic biorefineries. For this reason, a better understanding of the mechanisms underlying yeast adaptation and tolerance to this stress is essential for the rational improvement of yeast cell robustness [7–9,330]. At a pH below acetic acid pK_a (4.75 at 25 °C)[261], the undissociated form of the acid (CH_3COOH) is able to cross plasma membrane lipid bilayer [7,9,262]. Once inside the cell interior and in the neutral cytosol, acetic acid dissociates leading to the release of protons (H^+) and consequent intracellular acidification and to the accumulation of the counter-ion (CH_3COO^-). Ultimately, acetic acid dissociation causes the inhibition of growth kinetics and metabolism as the result of multiple toxicity mechanisms [7,9].

Several genome-wide approaches allowed a more thorough understanding of the mechanisms involved in yeast response and adaptation to acetic acid stress [7,263–269,280,316,317]. Among these mechanisms is the alteration of the molecular composition and biophysical properties of the cell envelope, acting as the first barrier of protection against challenging environmental conditions [7,21–23,25,61,319,331]. The cell wall is a layered structure, with an inner layer mainly composed by β-glucans and chitin, and an outer layer constituted by highly glycosylated cell wall proteins (CWP). The biochemical composition and the organization of the cell wall are dynamic and were reported to change during adaption to different environmental conditions impacting cell endurance and survival [25,69–71,319]. Changes in cell wall biophysical properties such as the stiffness of the cell surface, mostly dependent on the cross-linking between β-glucans and chitin [69], were reported to occur in response to industrially-relevant stresses [25,70,71]. The time-course of the alterations occurring at the level of the cell wall during adaptation of a yeast cell population to sudden exposure to a sub-lethal stress induced by acetic acid was recently reported. The induced increase of cell wall resistance to lyticase activity, stiffness and content in β-glucans was described [25]. Also, different studies have shown that several genes coding for proteins required for cell wall polysaccharide synthesis and regulation are transcriptionally responsive to acetic acid stress and/or determinants of acetic acid stress tolerance [25,263–266,317]. These responses guarantee a robust adaptive response, essential to limit the futile cycle associated to the re-entry of the acid form after the active expulsion of acetate from the cell interior [25].

Plasma membrane remodeling is also essential for acetic acid tolerance in yeast. The adequate incorporation of sphingolipids and ergosterol in the plasma membrane were found to decrease plasma membrane non-specific permeabilization under acetic acid stress, both in *Saccharomyces cerevisiae* and in the highly tolerant food spoilage yeast

species *Zygosaccharomyces bailii* [21–23,332]. The content of ergosterol is critical for plasma membrane stabilization and adequate selective permeability, limiting the passive diffusion of lipophilic toxic compounds into the cell [333–335]. Ergosterol is also essential for the formation of lipid-raft microdomains that modulate the activity of membrane-embedded proteins [333,335]. Lipid-rafts microdomains are also involved in the proper localization and trafficking of several mannoproteins, in particular glycosyl-phosphatidylinositol (GPI)-anchored proteins, associated with cell wall integrity [336–340]. Cell wall polysaccharides are synthetized at the plasma membrane level and some proteins involved in cell wall integrity sensing or cell wall remodeling exhibit domains that interact with plasma membrane [16,72,101,331,341], reinforcing the importance of plasma membrane homeostasis. The plasma membrane is the home for multidrug/multixenobiotic resistance (MDR/MXR) transporters of the ATP-binding cassette (ABC) Superfamily and of the Major Facilitator Superfamily (MFS), required for yeast resistance to multiple stresses [342–344]. These transporters are considered to contribute to decrease the intracellular concentration of specific toxic compounds, either by actively pumping them out of the cell and/or by promoting physical or chemical changes in the plasma membrane, thus affecting their partition. The MFS transporters Tpo2, Tpo3, and Aqr1, were implicated in the active expulsion of acetate from the cell interior, thus reducing the deleterious effects of acetic acid counterion accumulation [343]. However, the expression, functioning and recycling of these and other plasma membrane transporters is energetically expensive to the cell [345,346]. Yeast cell adaptation to acetic acid stress also involves the remodeling of plasma membrane composition and organization to restrict the diffusional entry of the liposoluble acid form into the cell. An identified player in this process is the plasma membrane ABC transporter Pdr18 [23]. The expression of this pleiotropic drug resistance (PDR) transporter confers resistance to a wide range of chemical and physical stresses, including acetic acid stress [23,26–29]. Pdr18 was proposed to mediate ergosterol transport at the plasma membrane, maintaining adequate plasma membrane physical properties under stress induced by acetic acid that was found to lead to the reduction of ergosterol content [23]. Through its action, Pdr18 allows the maintenance of ergosterol content and plasma membrane order in cells growing exponentially in the presence of acetic acid [23]. The transcriptional up-regulation of *PDR18* observed during the latency period induced by acetic acid stress was found to be essential to counteract the induced increase of plasma membrane non-specific permeability and dissipation of transmembrane electrochemical potential [23]. Overall, Pdr18 is considered essential for adaptation and tolerance to acetic acid stress by promoting the adequate essential physiological function of yeast plasma membrane [23,28].

In the present work, the influence of the expression of *PDR18* in yeast cell wall properties at the molecular and biophysical levels, under acetic acid-induced stress, was examined. The time-course effect of *PDR18* expression in counteracting the decrease of ergosterol concentration and of intracellular pH, and in cell wall resistance to lyticase activity, cell wall stiffness (by atomic force microscopy-AFM), and cell wall polysaccharide composition (based on fluorescence microscopy analysis), were examined. Collectively, the results obtained provide evidence for the crosstalk between yeast plasma membrane ergosterol content, involving Pdr18 activity, and cell wall biophysical properties, suggesting a coordinated and robust yeast response to counteract the deleterious effects of acetic acid stress.

3.3 MATERIALS AND METHODS

3.3.1 YEAST STRAINS AND GROWTH CONDITIONS

The parental strain *Saccharomyces cerevisiae* BY4741 (*MATa, his3*Δ*1, leu2*Δ*10, met15*Δ*0, ura3*Δ*0*) and the derived deletion mutant *pdr18*Δ strain, obtained from the EUROSCARF collection, were used in this study. The strains were maintained at −80°C in YPD media supplemented with 30% (v/v) glycerol. Cells were cultivated at 30°C, with orbital agitation (250 rpm), in minimal growth medium supplemented with amino acids and uracil (MM4). MM4 contains 1.7 g/L yeast nitrogen base without amino acids and ammonium sulphate (Difco, Michigan, USA), 20 g/L glucose (Merck, Darmstadt, Germany), 2.65 g/L $(NH_4)_2SO_4$ (Panreac AppliChem, Connecticut, USA), 20 mg/L methionine, 20 mg/L histidine, 60 mg/L leucine and 20 mg/L uracil (all from Sigma, Missouri, USA). The medium pH was adjusted to 4.0 with HCl. When stated, MM4 medium was supplemented with 60 mM acetic acid (Fluka, Waltham, USA) using a solution of 5 M acetic acid, set to pH 4.0 with NaOH. For all the growth experiments, MM4, supplemented or not with 60 mM acetic acid, was inoculated to an initial optical density at 600_{nm} (OD_{600nm}) of 0.1 ± 0.05 using mid-exponential cells harvested by filtration (Whatman, Maidstone, UK) from cultivation in fresh MM4 medium (pH 4.0) without acetic acid supplementation. Growth was followed by measuring culture OD_{600nm}.

3.3.2 ERGOSTEROL QUANTIFICATION

At adequate time-points during parental and *pdr18*Δ strains cultivation in either MM4 or MM4 supplemented with 60 mM acetic acid (pH 4.0), cells corresponding to 100 OD_{600nm} units were harvested (7,000 *g*, 5 minutes) for ergosterol quantification. Total cellular ergosterol was quantified based on a spectrophotometric method [347]. The absorbance of the sterol-containing layer was traced between 200 and 300 nm to confirm the success of the extraction by visualization of a characteristic four-peaked curve [348]. Optical density at 281.5 and at 230 nm were used to determine ergosterol content by the equations reported before, normalized to the wet weight of the sample [348]. Results were obtained from two independent biological replicates, each arising from three quantification replicates. Results arise from at least three independent experiments and statistically significant differences were identified using one-way analysis of variance (ANOVA).

3.3.3 DETERMINATION OF INTRACELLULAR pH (pHi) BY FLOW CYTOMETRY

For the determination of the intracellular pH values (pHi), both parental and *pdr18*Δ strains were cultivated in either MM4 or MM4 supplemented with 60 mM acetic acid (pH 4.0). At adequate time-points, 0.5 OD_{600nm} units were harvested by filtration using a pore size of 0.2 µm (Whatman, Maidstone, UK), and eluted in 250 µL of citrate-phosphate buffer to which was added SNARF-4F-5-(and-6)-carboxylic acid, acetoxymethyl ester, acetate (Invitrogen, Waltham, USA), at a final concentration of 20 µM. After an incubation period of 20 minutes (at 30°C with 250 rpm orbital agitation), cells were washed, resuspended in 250 µL of citrate-phosphate buffer pH 3.0 without the probe, and analyzed immediately. Flow cytometric analysis was performed on a BD Accuri C6 Plus flow cytometer (Becton, Dickinson and Company, Franklin Lakes, USA). The probe fluorescence emission was collected via FL2 585/40 and FL4 675/25 filters. All experiments were repeated using, at least, two independent biological replicates. A fixed total of 40,000 events per sample were acquired using a slow flow rate (14 µL/min). The FL2/FL4 ratio of fluorescence emission was calculated for every population.

The fluorescence emission collected in both channels from non-labeled exponentially-growing parental and *pdr18*Δ cell suspensions was also measured to subtract the background cell fluorescence from each channel emission signal. The determination of intracellular pH was assessed by fitting the ratios of fluorescence in the equations obtained from calibration curves for parental and *pdr18*Δ populations, that converts the fluorescence ratio to pHi values. For that, the cells were incubated in 250 µL of citrate-phosphate buffer supplemented with SNARF-4F-5-(and-6)-carboxylic acid, acetoxymethyl ester, acetate at defined pH from 5.5 to 8.0 for 1 h at 30°C. The calibration curves (**Figure 3.1**) were obtained by plotting the fluorescence ratio of the different samples as a function of the pH of the buffer in which they were incubated and fitted with a second-order polynomial function.

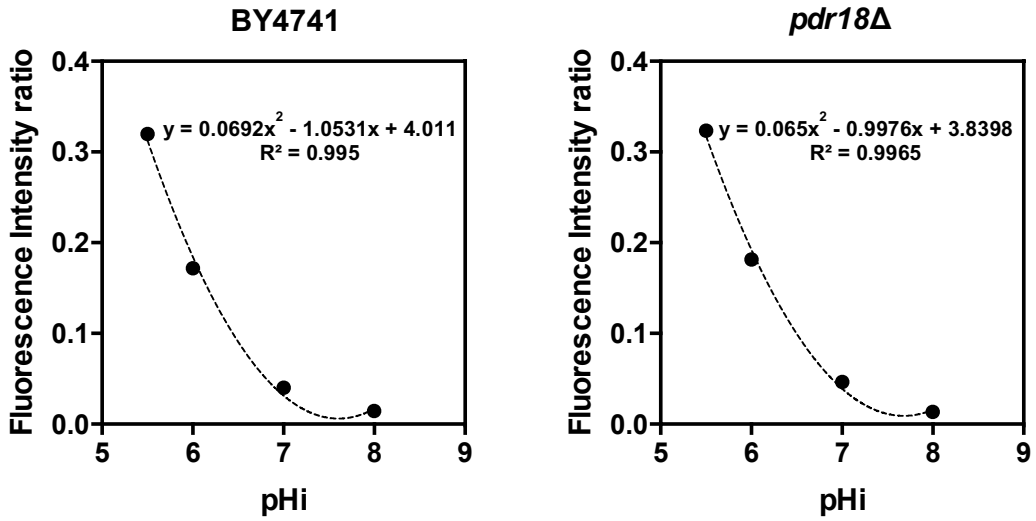

Figure 3.1 - Calibration curves of SNARF-4F-5-(and-6)-carboxylic acid, acetoxymethyl ester, acetate. The calibration curves for parental and pdr18Δ populations convert the fluorescence ratio to pHi values. They were obtained by plotting the fluorescence ratio of the different samples as a function of the pH of the buffer, with a defined pH, in which they were incubated. The equations obtained result from the fitting of a second-order polynomial function.

3.3.4 YEAST CELL WALL SUSCEPTIBILITY TO LYTICASE

To monitor yeast cell wall structural alterations during cultivation in MM4 supplemented or not with 60 mM acetic acid, a lyticase susceptibility assay was conducted as described before [273]. The lyticase used (β-1,3-glucanase from *Arthrobacter luteus*; Sigma; batch 0000109539), contained β-(1→3)-glucan laminaripentaohydrolase, β-(1→3)-glucanase, protease, and mannanase activities. Briefly, cells of the parental and *pdr18*Δ strains were cultivated in MM4 liquid medium, either supplemented or not with 60 mM acetic acid, at pH 4.0, and were harvested by filtration at adequate time-points during cultivation. Cells were washed with bidistilled and deionized water and used to inoculate 100 mL erlenmeyer flasks containing 50 mL of 0.1 mM phosphate buffer (pH 6.6), to a final OD_{600nm} of 0.5. After the addition of 18,000 U/mL of lyticase, cell lysis was followed by measuring the decrease of OD_{600nm} for each cell suspension. Values were converted to the percentage of the initial OD_{600nm} value. Cells susceptibility to lyticase is represented as the maximum specific lysis rate, defined as the absolute value of the slope of the trend line that best fits the linear part of the semi-logarithmic plot of the lysis curve. Results arise from, at least, two independent experiments and statistically significant differences were identified using one-way ANOVA.

3.3.5 MEASUREMENT OF THE YOUNG'S MODULUS OF THE CELL SURFACE BY ATOMIC FORCE MICROSCOPY (AFM)

For the determination of cell wall Young's modulus, which is the quantitative expression of the cell surface elasticity that reflects the stiffness, cells of the *pdr18*Δ strain were grown and harvested at adequate time-points during cultivation, as described above for the quantification of the ergosterol content, pHi, and susceptibility to lyticase assays. The immobilization of the *pdr18*Δ cells for AFM analysis and the Force-Distance measurements for determination of the apparent Young's modulus was performed as previously described [25]. Briefly, cell suspensions of OD_{600nm} of 0.2 in 5 mL of bidistilled deionized water were prepared and filtered through a polycarbonate membrane (Whatman) with a pore size of 3 μm, close to the yeast cell longitudinal size. The filters were washed once with 15 mL of bidistilled deionized water. AFM images and Force-Distance measurements were analyzed with a PicoLE Molecular Imaging system recorded in contact mode, using a microlever MSCT-F (Bruker, Billerica, USA) with nominal stiffness of 0.6 N/m and nominal tip radius of 10 nm in all experiments. Force spectroscopy mapping, consisting of 32 × 32 approach/retract force–displacement (FD) curves, was performed in the cell surface. The maximum deflection of the AFM cantilever was set constant yielding a maximum applied force of ≈ 30 nN and the tip-sample approach velocity was about 0.5 μm/s. An area in the cell was selected so that bud scars were excluded and a total of 100 approach/retract force–displacement curves were obtained, approximately, from which the median value was kept. A total of 32 cantilevers were used and the spring constant of the cantilevers was calibrated using the Sader method [322]. Each cantilever was used to measure cells at numerous time-points, and the order in which cells of different conditions were measured was randomized to reduce bias. However, it was not possible to use the same cantilever for all the conditions tested. The force distance grids were analyzed for the determination of the apparent Young's modulus, obtained by adjusting the Derjaguin-Muller-Toporov (DMT) contact model to the approach curves. The force distance grids were processed by in-house software developed using Wolfram Mathematica (Wolfram Research, Illinois, USA). Results arise from, at least, three independent experiments and from the analysis of, at least, 14 cells for each condition. Statistical significance of differences among conditions was evaluated by applying one-tailed Mann–Whitney U test.

3.3.6 TRANSCRIPTIONAL ANALYSIS OF CELL WALL BIOSYNTHETIC AND REGULATORY GENES

For the transcriptional analysis of cell wall biosynthetic and regulatory genes, cells from *pdr18*Δ deletion mutant strain were harvested during cultivation as described above for other assays. Exponentially growing cells from the parental strain cultivated in unstressed conditions were also harvested, to be used as reference value in transcript quantification. Total RNA extraction was performed by the hot phenol method [323]. The quantitative real-time Reverse Transcription–PCR (qRT-PCR) protocol was used to determine the mRNA levels from *RLM1*, *FKS1*, *FKS2*, *BGL2*, *CHS3*, *CRH1*, *GAS1* and *PRM5* genes following the manufacturer's instructions. Primers were designed using Primer Express software V3.0 (Applied Biosystems) and are listed in **Table 3.1**.

Table 3. 1 - Primers used for qRT-PCR analysis.

Primer	Sequence (5'-3')
ACT1	fw: CTCCACCACTGCTGAAAGAGAA rev: CCAAGGCGACGTAACATAGTTTT
CHS3	fw: TCACCTGGATGTTTTACCATCAAG rev: CCACTCCGACGAGTTGCAT
FKS1	fw: CATGCTGCTCTGGTCCCTTATT rev:CACCGTGGGCAATTCCA
FKS2	fw: GCTCATGTCGTTGGAGCAGTT rev: CCAATGGCATTACGGAAAAGA
RLM1	fw: CTTTTTTCTGCAACACAGCCATA rev: CGCCAGGAATATTCGATGGT
GAS1	fw: AACCGCTGCTGCTTTTTTTG rev: CTTCAATCGCTGGAACATCGT
CRH1	fw: CGCGGCTGCCGAAAG rev: GCA GTGCTAGAAGCTGCAGTTG
BGL2	fw: TTTTGTTATGGCTAACGCGTTCT rev: GAGTAAGAGGCATTTTGCATGGT
PRM5	fw: TTTTCCACACAACATACCCAGTTT rev: TCTTTGGCGGGATAATCCATA

The RT–PCR reaction was conducted in a thermal cycler block (Cleaver GTC965) and the qPCR was conducted in QuantStudio 5 (Applied Biosystems) using NZYSpeedy qPCR Green Master Mix (NZYTech). The *ACT1* mRNA level was used as the internal control. The value obtained for each target gene at the initial time-point (0 h) for the parental strain cells cultivated under unstressed conditions was set as 1 and the remaining values are relative to this reference value. Results arise from at least three independent experiments and statistically significant differences were tested using one-way ANOVA.

3.3.7 ASSESSMENT OF CELL WALL POLYSACCHARIDES CONTENT BY FLUORESCENCE MICROSCOPY

The staining methodology to assess cell wall main polysaccharides content was adapted from that described by Pradhan *et al*. [324] as described before [25]. Fc-Dectin 1-Alexa 633, Calcofluor White and Concanavalin A-FITC were used as staining compounds that bind glucans, chitin and mannans, respectively. Cells of *pdr18*Δ strain population were harvested during cultivation as described above. Cells suspensions of OD_{600nm} of 0.5 were incubated with 0.75 µg/mL Fc-Dectin 1 (Sino Biological, China) in FACS buffer for 45 min on ice. After centrifugation at 4,300 *g* for 5 min at 4 °C, the pellet was washed with 200 µL FACS buffer. Then, cells were incubated for 30 min with 1:200 of anti-human IgG conjugated with Alexa Fluor 633 (ThermoFisher Scientific, Waltham, USA), 50 µg/mL Calcofluor White (Fluka), and 50 µg/mL Concanavalin A conjugated with Fluorescein (Sigma). After centrifugation at 4,300 *g* for 5 min at 4 °C, the pellet was washed with 200 µL FACS buffer and resuspended in 100 µL FACS buffer. These cell suspensions were immobilized in 2.2% agarose in PBS 1× mounted on a gene frame 1.0 × 1.0 cm (ThermoFisher Scientific). All samples were examined using a Leica TCS SP5 (Leica Microsystems CMS GmbH, Mannheim, Germany) inverted confocal microscope (DMI6000). A 63× apochromatic water immersion objective with a numerical aperture (NA) of 1.2 (Zeiss, Jena, Germany) was used for all experiments. Confocal microscopy was used for the FC-Dectin1-Alexa 633 channel and excitation was carried out through a He–Ne laser line at 633 nm. Fluorescence emission was collected in this channel between 640 and 770 nm. For the Calcofluor White channel, 2-photon excitation microscopy measurements were carried out with a Spectra-Physics Mai Tai BB laser set for excitation at 780 nm. Calcofluor white emission was retrieved between 390 and 480 nm. Confocal microscopy was employed for the Concanavalin A-FITC channel and excitation set at 488 nm was carried out using a Argon laser. Emission was collected

between 495 and 580 nm. The fluorescence microscopy images for data analysis were collected at 2048 × 2048 resolution and processed using ImageJ. The regions of interest (ROI) corresponding to the cellular surface were defined and average pixel fluorescence intensities within these ROIs were determined for each channel. The average background fluorescence was subtracted to the calculated values.

The triple staining of the cells was carried out to guarantee that the analysis of β-glucans, chitin and mannans refers to the same cell population. Results of the median intensity fluorescence were obtained from the analysis of at least 33 cells from two independent cultivation experiments. Statistical significance of differences among conditions was evaluated by applying one-tailed Mann–Whitney U test.

3.4 RESULTS

3.4.1 EXPRESSION OF *PDR18* IS REQUIRED TO COUNTERACT THE DECREASE OF ERGOSTEROL CONTENT AND TO REDUCE THE TIME FOR INTRACELLULAR pH (pHi) RECOVERY FOLLOWING ACIDIFICATION INDUCED BY ACETIC ACID STRESS

The deletion of *PDR18* leads to a more extended period of latency in the presence of 60 mM of acetic acid at pH 4.0, compared with the parental strain (50 h versus 13 h) (**Figure 3.2**), consistent with a former observation [23]. In the previous article, Pdr18 was found to be essential for the maintenance of plasma membrane ergosterol content in unstressed cells and in adapted cells exponentially growing in the presence of acetic acid [23]. In the present work, cellular ergosterol was also assessed during the latency phase induced by acetic acid, in cells expressing or not *PDR18* (**Figure 3.2**). Cells expressing *PDR18* exhibit a higher ergosterol content than *pdr18*Δ cells, when cultivated in unstressed conditions ($p < 0.05$; one-way-ANOVA) (**Figure 3.2, a**). Results demonstrate, for the first time, that exposure of yeast cells to the referred acetic acid stress leads to a dramatic reduction in the ergosterol content after only 1 hour of cultivation (decreases from 0.2% to 0.09% of the cell's wet weight for the parental strain and from 0.15% to 0.04% of the cell's wet weight for the *pdr18*Δ strain) (**Figure 3.2, b**). This reduction was found to be statistically significant ($p < 0.05$; one-way-ANOVA). The average levels of ergosterol in *pdr18*Δ cells, after 7 hours of cultivation in the presence of acetic acid, are still low (0.04% of the cell's wet weight). However, after 24 hours of cultivation, these levels increased to 0.06% of the cell's wet weight, this value being close to the value quantified after 50 hours of cultivation, during the exponential growth phase (**Figure 3.2, b**) and the values obtained before for acetic acid adapted cells [23].

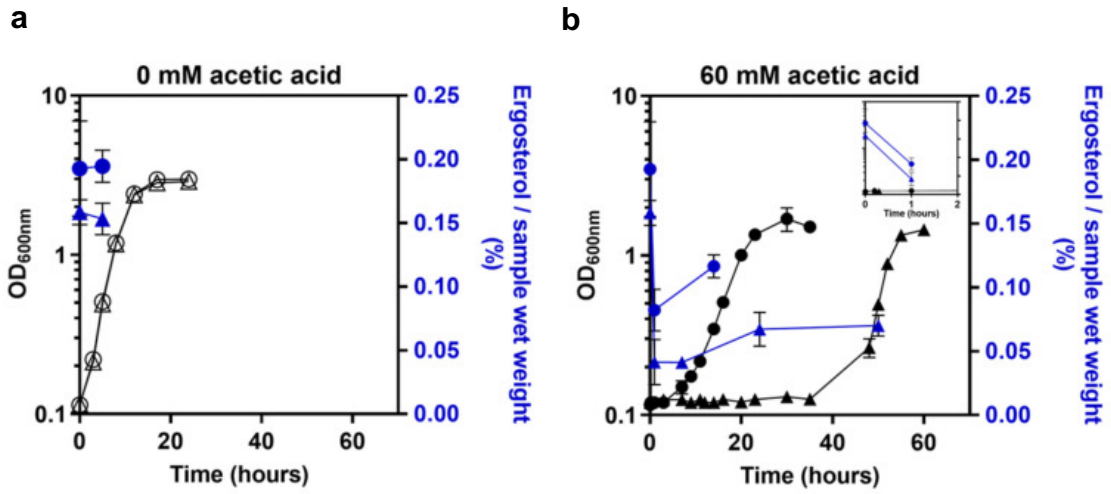

*Figure 3.2 - Alteration of the cellular ergosterol content of populations of the parental strain, S. cerevisiae BY4741 (circles), and of this strain with the PDR18 gene deleted (triangles) during cultivation in the absence or presence of acetic acid stress. Cells of the parental and pdr18Δ strains were harvested during cultivation in MM4 (**a**) or MM4 supplemented with 60 mM acetic acid (**b**). A zoomed view of the first 2 h of cultivation in the presence of the acid is shown in (**b**), for which the left and right axis were maintained. Values are the means of at least two independent experiments, and error bars indicate standard deviations.*

During exponential growth in unstressed conditions, the intracellular pH (pHi), assessed based on flow cytometry using the ratiometric pH indicator SNARF-4F 5-(and-6)-carboxylic acid, acetoxymethyl ester, acetate, exhibits similar values (6.05±0.3 and 6.06±0.2) for both the parental and *pdr18*Δ populations, respectively (**Figure 3.3, a**). However, when the cultivation medium was supplemented with the above referred sub-lethal concentration of acetic acid, the average pHi decreased significantly for both strains during the first 3 h of cultivation (**Figure 3.3, b**). After 7 h of cultivation, the average pHi of the parental strain cell population recovered, accompanying the recovery and exponential growth of the weak acid-adapted cell population, and reached values close to the initial pHi. The deletion of *PDR18* leads to the extension of the duration of the lag phase and to lower pHi values. The recovery of pHi to more physiological values followed the resumption of exponential growth of an adapted population (**Figure 3.3, b**). The average pHi attained for both populations at the late exponential growth phase was below the initial pHi registered for the cells of the inocula (5.92±0.03 and 5.79±1.4, p = 0.0002 and 0.008 for the parental and *pdr18*Δ strains, respectively; one-way-ANOVA; **Figure 3.3, b**), with slightly lower values in the *pdr18*Δ deletion mutant cells. As the stationary phase progressed, the average pHi of both populations decreased, likely due to plasma membrane proton gradient dissipation as the result of ATP depletion [345,349,350].

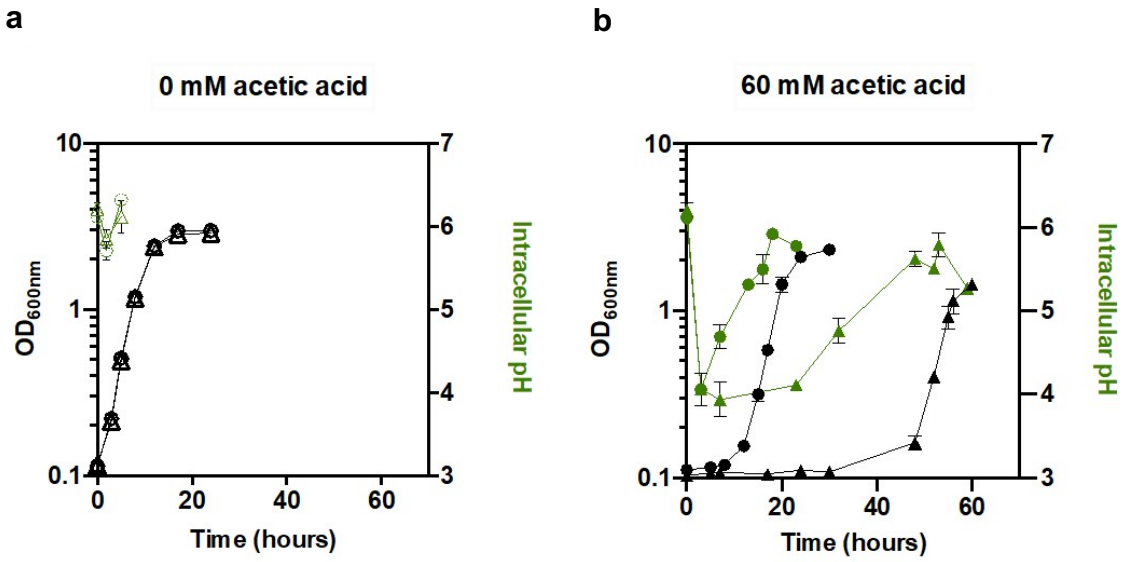

*Figure 3.3- Alteration of the average intracellular pH (pHi) of populations of S. cerevisiae BY4741 (circles) and this strain with the PDR18 gene deleted (triangles) during cultivation in the absence (**a**) or presence (**b**) of acetic acid stress, as described in Figure 3.2 legend. Intracellular pH was assessed by fitting the ratios of fluorescence in the equations obtained from the calibration curves for parental and pdr18Δ strains, which converts the fluorescence ratio to pHi values, as described in M&M. Values are the means of at least two independent experiments and error bars indicate standard deviations.*

3.4.2 EXPRESSION OF *PDR18* IS REQUIRED FOR MAXIMUM RESISTANCE OF YEAST CELLS TO LYTICASE ACTIVITY INDUCED UNDER ACETIC ACID STRESS

The alterations occurring in yeast cell wall architecture during cultivation of the BY4741 strain with the *PDR18* gene deleted, in the presence or absence of 60 mM acetic acid at pH 4.0, the same conditions used above and replicated for all the experiments described this study, were monitored based on cell wall susceptibility to lyticase activity and compared with values of the parental strain (**Figure 3.4**). The maximum specific lysis rate at each time-point during cultivation was determined as the slope of the trend line that best fits the semi-logarithmic plot linear part of the decrease of the OD_{600nm} of the cell suspension following the addition of lyticase (**Figure 3.5**). In the absence of acetic acid stress, the maximum specific lysis rate of the parental and the *pdr18*Δ strains' populations exhibit similar values during exponential growth (**Figure 3.4, a**). When parental strain cells were introduced in the same medium supplemented with acetic acid, a marked reduction of the lysis rate was observed reaching minimum values at 3 h of acetic acid-induced latency (**Figure 3.4, b**). The reduction of the lysis rate for the *pdr18*Δ cell population, during the extended latency phase under acetic acid, was less marked

than observed for the parental strain, and those reduced values were maintained throughout the duration of the more extended lag phase (**Figure 3.4, b**). In the adapted populations of the parental or *pdr18*Δ strains exponentially growing under acetic acid stress, the lysis rate values moderately increased when compared to those registered during latency. However, these values were quite below those registered for unstressed cells, and were lower for the parental strain compared with the *pdr18*Δ mutant (**Figure 3.4, b**). In summary, results indicate that *PDR18* expression is required for the induction of maximum levels of cell wall resistance to lyticase activity during adaptation and growth under acetic acid stress.

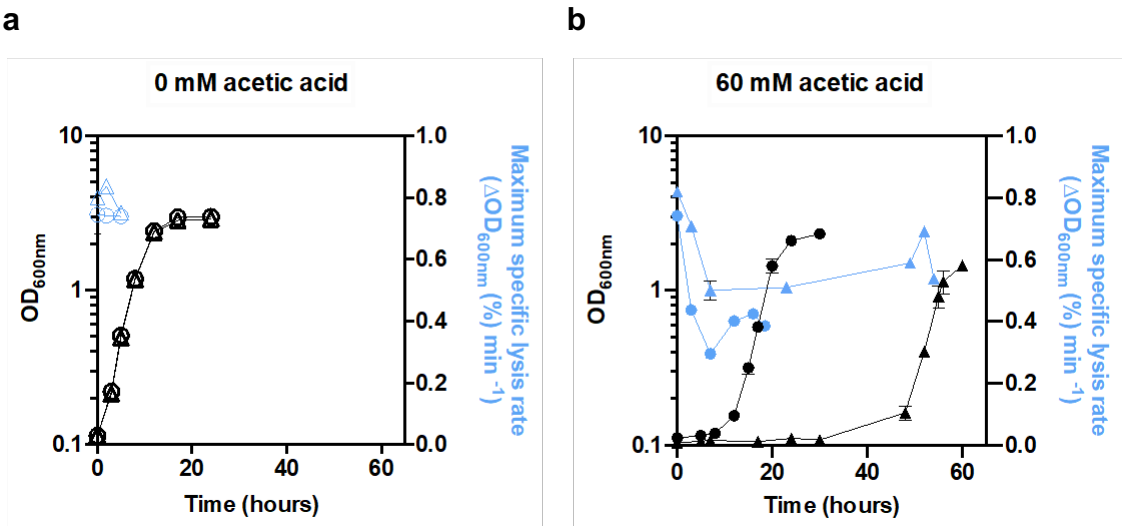

*Figure 3.4 - Yeast cell resistance to lyticase activity during cultivation in the absence or presence of acetic acid stress. Time-course analysis of cell susceptibility to lyticase, associated with the maximum specific lysis rate of the parental strain, S. cerevisiae BY4741 (circles), and this strain with the PDR18 gene deleted (triangles). Cells were harvested from selected time-points during cultivation, in the absence (**a**) or presence (**b**) of acetic acid. The maximum specific lysis rate is defined as the absolute value of the slope of the straight line that best fits the semi-logarithmic plot of the linear part of the lysis curve and was determined based on the decrease of the OD_{600nm} of cell suspensions (in %) following the addition of lyticase, as described in M&M. Results from these lysis experiments are shown in Figure 3.5. Values are means from at least two independent replicates of the lysis experiments, and bars represent standard deviation.*

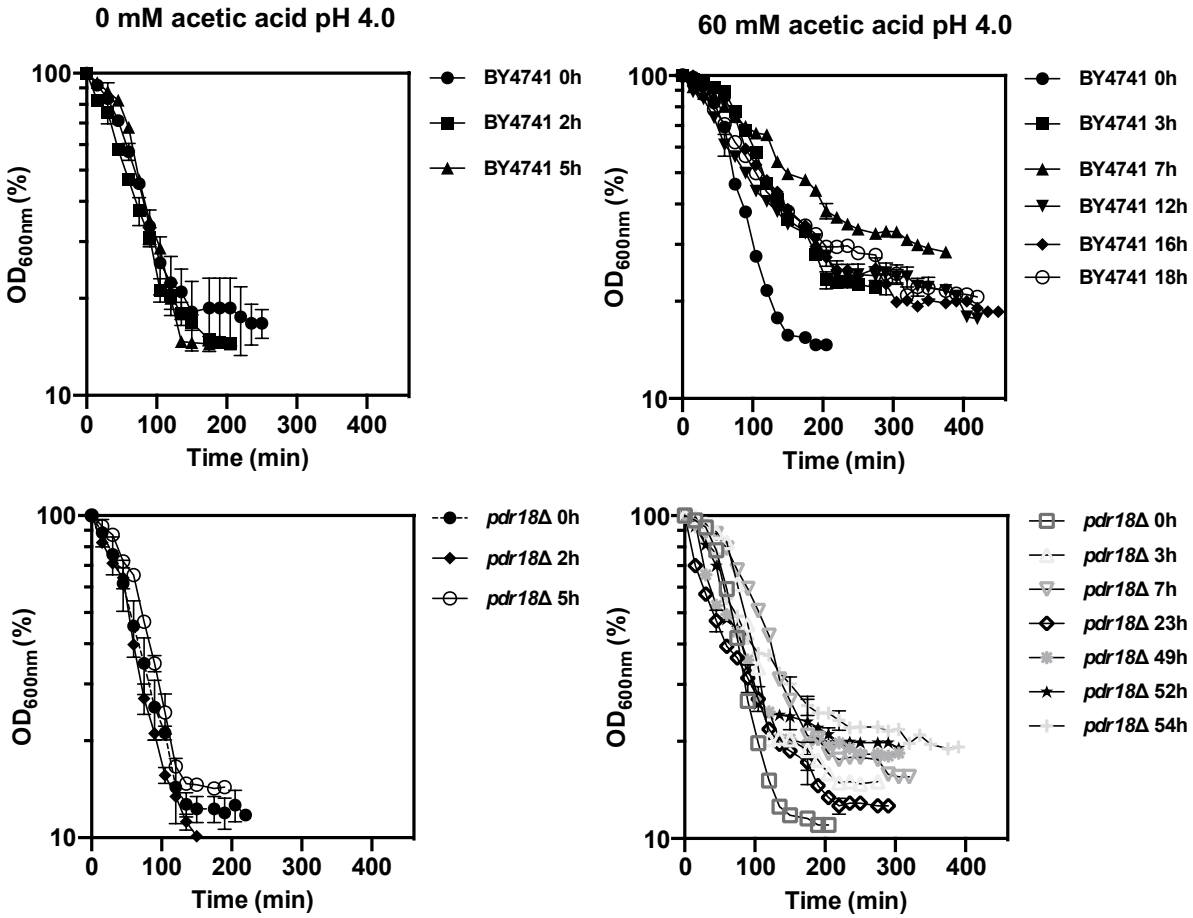

Figure 3.5 - Lyticase susceptibility assays using cells of the parental and pdr18Δ strains harvest during the growth curve in the absence or presence of acetic acid. The decrease of the OD_{600nm} during the incubation time of yeast cell suspensions (in %) following the addition of lyticase is plotted.

3.4.3 THE STIFFNESS OF *pdr18*Δ CELL WALL INCREASES DURING ACETIC ACID-INDUCED LATENCY

The nanomechanical properties of the cell surface of *pdr18*Δ cells during adaptation to acetic acid stress were examined by Atomic Force Microscopy (AFM) to assess the Young's modulus, which reflects cell wall stiffness (**Figure 3.6**). Each experimental result corresponds to the median value of about 100 curves over a single cell and the dispersion of the results for each cultivation time reflects the expected heterogeneity [351] of the yeast populations examined. As previously described for the parental strain [25], the Young's modulus of *pdr18*Δ cells was not found to suffer significant alterations during exponential growth in unstressed conditions (**Figure 3.6, a**). However, when the medium was supplemented with acetic acid using the above-described conditions, the Young's modulus value for *pdr18*Δ cells increased during latency, reaching a maximum value of 148 MPa at 23 hours of cultivation, when compared to the Young's modulus value of 96 MPa of the initial time-point ($p < 0.00001$; one-tailed Mann–Whitney U test; **Figure 3.6, a and b**). Moreover, the Young's modulus of exponentially-growing *pdr18*Δ cells, adapted to acetic acid (128 MPa) was found to be significantly higher than the Young's modulus of the corresponding unstressed cell population (104 MPa; $p = 0.04$; one-tailed Mann–Whitney U test; **Figure 3.6**). Compared with the stiffness values published before for parental strain cells [25], cultivated exactly under the same stress conditions and exhibiting the same growth curves and other parameters (**Figures 3.2-3.4**), the values of cell stiffness obtained for the *pdr18*Δ strain under acetic acid stress were not statistically different from those reported for the parental cells; moreover, the profiles of variation of cell stiffness during the adaptation phase were similar (**Figure 3.6** and Figure 2b from [25]).

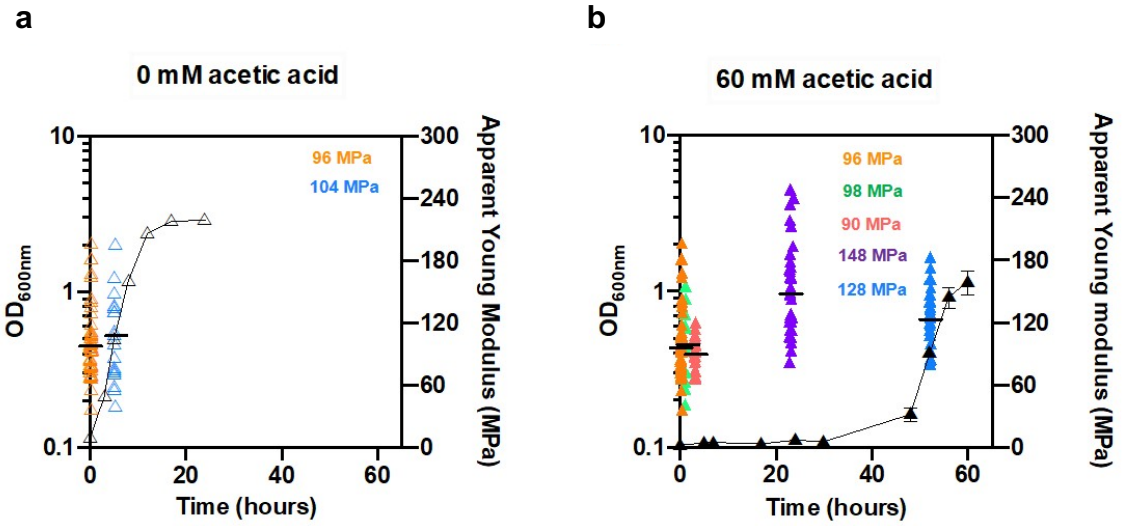

*Figure 3.6 - Apparent Young's modulus of S. cerevisiae BY4741 with the PDR18 gene deleted, cultivated in the absence or presence of acetic acid stress. Time-course analysis of the cell surface elasticity, which reflects cell wall stiffness, represented as the apparent Young's modulus determined by atomic force microscopy, for pdr18Δ cells cultivated in the absence (**a**) or presence (**b**) of acetic acid. Each result in the graph corresponds to the median value of about 100 curves over a single cell. For each condition, at least 13 cells were analyzed from at least 3 independent experiments.*

3.4.4 TRANSCRIPTIONAL PROFILES OF CELL WALL BIOSYNTHETIC GENES IN *pdr18*Δ CELLS' RESPONSE TO ACETIC ACID STRESS

The levels of transcription from several genes related to cell wall biosynthesis were assessed by quantitative real-time Reverse Transcription–PCR (qRT-PCR) during cultivation of the *pdr18*Δ strain in the absence or presence of acetic acid under the above-described conditions. The selected genes, the description of the function of the encoded proteins, and the corresponding bibliographic references are provided in **Table 3.2**.

Table 3.2 - Genes selected for qRT-PCR, description of the function of the encoded proteins, and corresponding references.

Gene	Function of the encoded protein	Bibliographic references
CHS3	Major chitin synthase required for synthesis of the majority of cell wall chitin	Gohlke *et al.*,2017[327]

FKS1	β-1-3-glucan synthase	Douglas, 2001[326]
FKS2	Fks1 paralog, involved in β-1-3-glucan synthesis	
RLM1	Transcription factor responsible for the transcriptional activation of the majority of genes induced in response to cell wall stress through the CWI pathway	Jung *et al*., 2002[325]
GAS1	β-1,3-glucanosyltransferase involved in cell wall remodeling-elongation of (1→3)-β-D-glucan chains and branching	Ram *et al*., 1998[63]; Aimanianda *et al*., 2017[63]
CRH1	Chitin transglycosylase involved in the transfer of chitin to β-1-6 and β-1-3 glucans in the cell wall	Blanco *et al*., 2015[87]
BGL2	Endo-beta-1,3-glucanase involved cell wall remodeling necessary for branching of the β-1-3 glucans in the cell wall	Aimanianda *et al*., 2017[85]
PRM5	Pheromone-regulated protein and a Rlm1 target; a hallmark of CWI pathway activation	Jung *et al*., 2002[325]

During the cultivation of the parental strain BY4741 in unstressed conditions (MM4, pH 4.0) the levels of transcription from all the selected genes, *RLM1, FKS1, FKS2, BGL2, CHS3, CRH1, GAS1* and *PRM5* were found not to vary significantly [25]. The same was

observed herein for the *pdr18*Δ strain, under the same cultivation conditions (**Figure 3.7**).

The alteration of the transcription levels from the selected genes in the parental strain *S. cerevisiae* BY4741, under the same acetic acid stressing conditions used in this work, was previously described [25]. While the mRNA levels from *RLM1*, *FKS2*, *BGL2*, *CHS3*, *CRH1,* and *PRM5* decreased during growth latency in the parental strain, mRNA levels from *FKS1* maintained similar values and mRNA levels from *GAS1* increased moderately throughout cultivation [25]. In the present study, a different transcriptional profile was found for those genes in the strain with the *PDR18* gene deleted. Although a similar pattern of the time-course transcription levels was found herein for all genes, except for *RLM1* and *CRH1*, during the first 12 hours of the latency phase, corresponding to a slight decrease of the mRNA levels during the first hours of response to sudden acetic acid stress, these changes were not considered statistically significant. At 24 hours of cultivation, when the cell population is close to resume growth, the mRNA levels from *FKS1*, *FKS2*, *BGL2*, *CHS3*, *GAS1,* and *PRM5* coordinately increase by 40%, 30%, 37%, 107%, 37%, and 73%, respectively, compared with the 12 hours-time-point values (**Figure 3.7**). The observed increase was statistically significant for *FKS1*, *BGL2, CHS3*, *GAS1*, and *PRM5* ($p < 0.05$; one-way ANOVA), coding for a β-1-3-glucan synthase, a endo-beta-1,3-glucanase, a chitin synthase, a β-1,3-glucanosyltransferase, and a protein of unknown function considered to be a hallmark of CWI pathway activation[325], respectively (**Figure 3.7**). When cells resumed growth, the mRNA levels from those genes did not maintain the peak values attained by the end of the adaptation phase to acetic acid-induced stress. In the case of the *RLM1* gene, coding for a transcription factor responsible for the transcriptional activation of the CWI pathway, and the *CRH1* gene, coding for a chitin transglycosylase, the referred expression pattern was not observed. In fact, the mRNA levels from these genes decreased throughout the time-points tested, down to 50 and 60%, compared to the initial time-point, respectively (**Figure 3.7**).

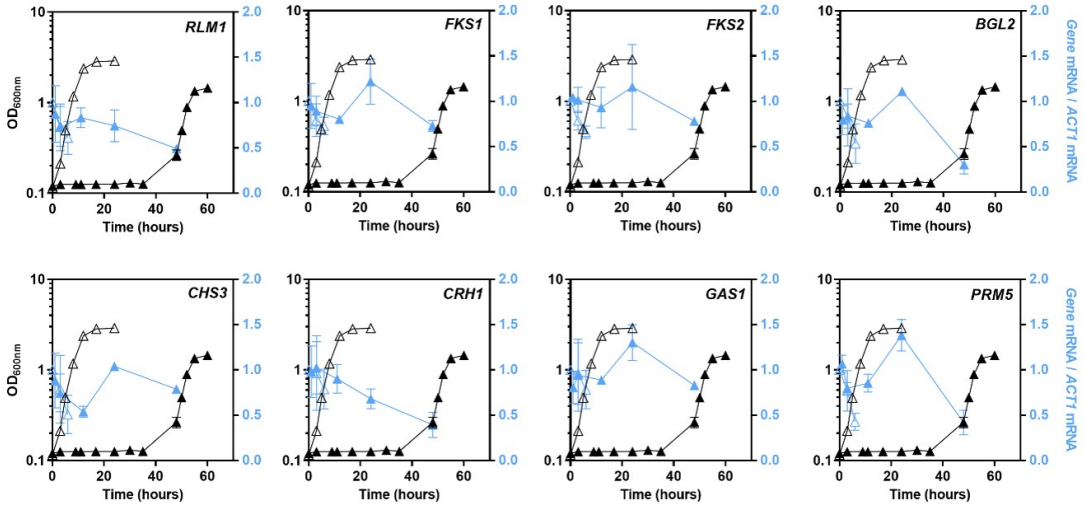

Figure 3.7 - Levels of mRNA from cell wall biosynthesis-related genes during cultivation of S. cerevisiae BY4741 with the PDR18 gene deleted and in the absence or presence of acetic acid. Time-course of the mRNA levels from RLM1, FKS1, FKS2, BGL2, CHS3, CRH1, GAS1, and PRM5 genes during cultivation of pdr18Δ in the absence (open symbols) or presence (filled symbols) of acetic acid. The transcriptional levels (in blue) from the indicated genes were assessed by qRT–PCR, using ACT1 as the internal control. The value obtained for each target gene, at the initial time-point of parental strain cell cultivation under unstressed conditions, was set as 1. Results are means of at least three biological replicates, and error bars represent standard deviation.

3.4.5 THE CONTENT OF THE MAJOR CELL WALL POLYSACCHARIDES INCREASES IN *pdr18*Δ CELLS DURING ACETIC ACID-INDUCED LATENCY

The evolution of the content of cell wall polysaccharides during adaptation and growth in the presence of acetic acid of the more acetic acid susceptible *pdr18*Δ cell population was assessed, as before for the parental strain population [25], using fluorescence microscopy. The cell wall polysaccharides β-glucans, chitin and mannans were stained with Alexa Fluor 633 (AF633), Calcofluor White (CFW), and Concanavalin A conjugated with Fluorescein (FITC), respectively.

Illustrative images acquired by fluorescence microscopy are shown in **Figure 3.8** and the fluorescence intensity median values obtained for the individual *pdr18*Δ cells measurements during cultivation in the absence or presence of acetic acid are shown in **Figure 3.9.**

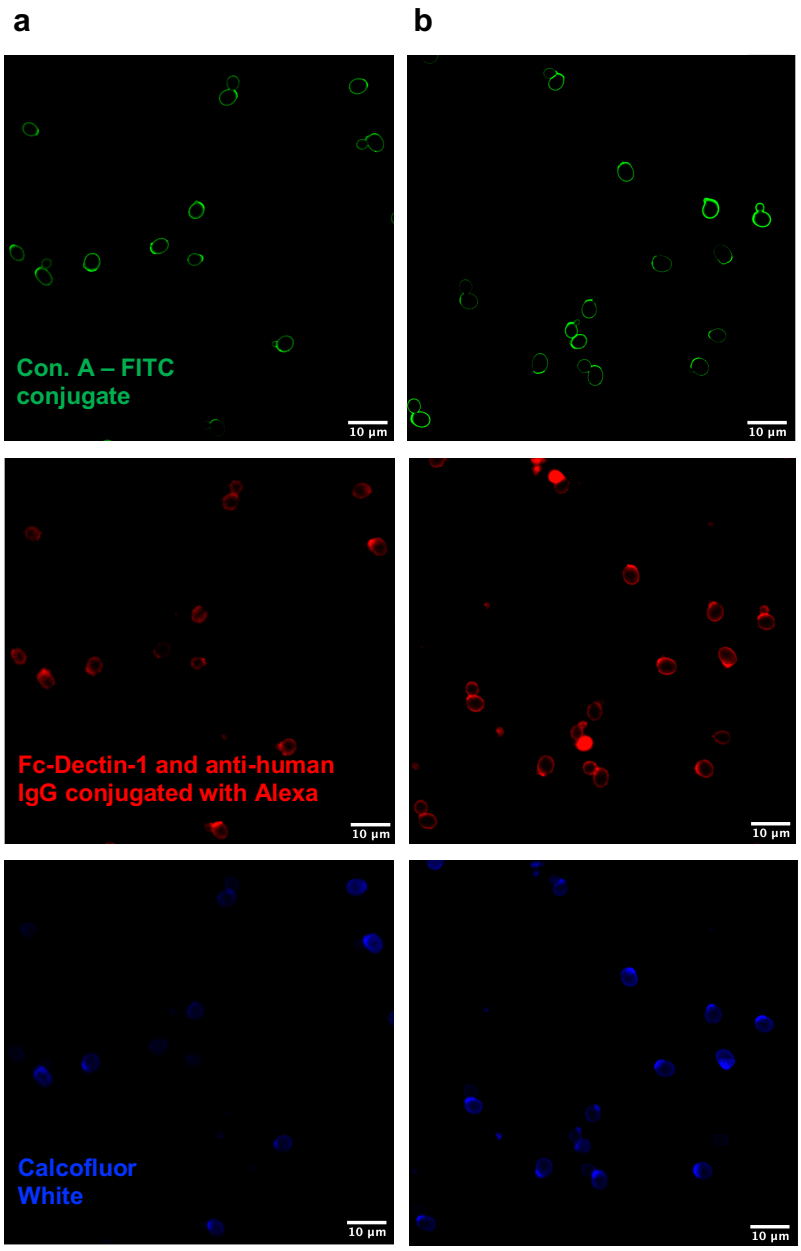

Figure 3.8 - Fluorescence microscopy images of fluorescence-stained cell wall β-glucans, chitin and mannans. Illustrative images of stained pdr18Δ cells were taken after 0 hours (a) and 23 hours (b) of exposure to acetic acid, this corresponding to the middle of the acetic acid- induced latency. Confocal microscopy was used to quantify β-glucans and mannans stained with Fc-Dectin 1-Alexa 633 and Concanavalin A-FITC, respectively, and 2-photon excitation microscopy was used to quantify chitin stained with Calcofluor White.

Each experimental result corresponds to the median value from, at least, 33 cells from two independent cultivation experiments and the dispersion of the results for each cultivation time reflects the expected heterogeneity [351] of the yeast populations examined.

No significant alteration was registered in the content of the cell wall polysaccharides when the parental cells of *S. cerevisiae* BY4741 strain [25] or cells with the *PDR18* gene

deleted were cultivated in the absence of acetic acid stress (**Figure 3.9, a-c**). The study of the alterations occurring in cell wall polysaccharides during adaptation of parental strain cells to the same acetic acid stressing conditions used in the present study was previously published and indicate that the level of β-glucans increases during adaptation and in adapted cells compared to unstressed cells [25]. Under the same stressing conditions, in the case of the more susceptible *pdr18*Δ cell population examined in this study, a statistically significant increase in the content of β-glucans, well above the increase observed before for parental cells [25], also occurred during the adaptation phase. A peak for β-glucans content is reached after 23 hours of cultivation (3.6-fold higher than the initial time-point; $p < 0.00001$; one-tailed Mann–Whitney U test), when the population is about to resume exponential growth (**Figure 3.9, d**). These high values were maintained during exponential growth, and values are 2.2-fold higher when compared to the corresponding unstressed cells ($p < 0.00001$; one-tailed Mann–Whitney U test; **Figure 3.9, a and d**).

The chitin content, associated with the increase of CFW fluorescence intensity, also peaks at 23 h of cultivation (1.9-fold higher than the initial time-point; $p < 0.00001$; one-tailed Mann–Whitney U test) and maintained values 1.5-fold higher than those of unstressed cells $p < 0.00001$; one-tailed Mann–Whitney U test) during exponential growth (**Figure 3.9, b and e**). The chitin content variation profile is similar to the profile of variation of the β-glucans (**Figure 3.9, d and e**). Since CFW can also stain β-glucans at a smaller extent[352], it is not possible to be sure if there is indeed a specific increase of the chitin concentration or if this increase just reflects the increased content of β-glucans (**Figure 3.9, e**).

A statistically significant but slighter increase in the content of mannans, quantified by the fluorescence of Concanavalin A-fluorescein (FITC) (1.6-fold higher than the initial time-point; $p < 0.00001$; one-tailed Mann–Whitney U test), also occurs. However, the peak value was reached earlier in the latency phase, after 3 h of cultivation in the presence of acetic acid (**Figure 3.9, f**). The cell wall content in mannans in acetic acid stressed cells is also slightly higher than the level in cells of the inoculum, which were grown in the absence of acid stress (1.3-fold higher; $p < 0.00001$; one-tailed Mann–Whitney U test; **Figure 3.9, c and f**). In the previous work dedicated to study the parental strain cell wall composition under the same stressing conditions, the increase in the content of mannans in response to acetic acid stress was slighter and not statistically significant, although such increase was already apparent[25].

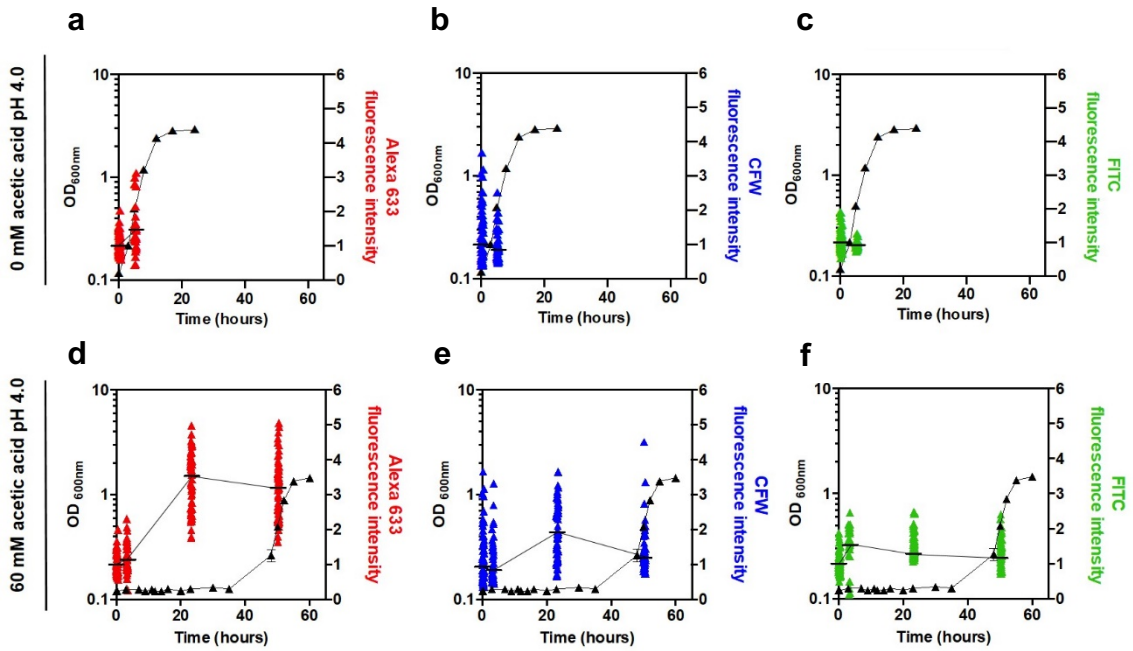

*Figure 3.9 - Content of cell wall polysaccharides in the absence and presence of acetic acid, in S. cerevisiae BY4741 with the PDR18 gene deleted, measured by fluorescence microscopy. The estimated content of cell wall polysaccharides during cultivation of pdr18Δ cells in the absence (**a–c**) or presence (**d–f**) of acetic acid is shown. The cell wall components β-glucans (red), chitin (blue), and mannans (green) were stained with Fc-Dectin 1 conjugated with Alexa Fluor 633, Calcofluor White, and Concanavalin A conjugated with Fluorescein (FITC), respectively. Quantification of the fluorescence intensity was performed with Leica TCS SP5 (Leica Microsystems CMS GmbH, Manheim, Germany) inverted confocal microscope (DMI600). Each experimental result corresponds to the median fluorescence intensity value (indicated by a black dash) from at least 33 cells from two independent cultivation experiments, and the dispersion of the results for each cultivation time reflects the expected heterogeneity of the yeast populations examined. For each channel, the median of the intensity values obtained for pdr18Δ cells at the initial time-point (0 h) was set as 1, and the remaining values are relative to this reference value.*

3.5 DISCUSSION

Among the relevant mechanisms proposed to underlie yeast adaptation to stress imposed by a sub-lethal concentration of acetic acid is the alteration of the composition, organization and properties of the cell envelope [7,21–23,25]. The present work reinforces the concept of the major role played by the cell wall in the global response and tolerance to acetic acid stress in yeast and provides evidence for the crosstalk between the content of plasma membrane in ergosterol, this depending on the activity of the plasma membrane ABC transporter Pdr18, and the biophysical properties of the cell wall under acetic acid stress.

Cell wall remodeling is considered an essential response to limit the futile cycle associated with the re-entry of the toxic form of acetic acid, after the active expulsion of the counter-ion acetate, accumulated in the cell interior, due to a cytosolic pH quite above the pK_a of this weak acid [7,25]. Acetate extrusion was proposed to be mediated by drug-H^+ antiporters (Tpo2, Tpo3, Aqr1) [342–344], while the re-entry of the lipophilic acid form is possible by passive diffusion through plasma membrane lipid bilayer [7,24]. The more active acetate and proton effluxes during adaptation to acetic acid are energy-dependent mechanisms[24], leading to the decrease of the ATP pool under acetic acid stress [24].

In the present work, it was demonstrated that the expression of *PDR18* contributes to counteract the rapid and marked decrease of cellular ergosterol content also registered during acetic acid-induced adaptation phase. The extended period of growth latency observed for the mutant with the *PDR18* gene deleted was correlated with the lower levels of ergosterol present in these cells and the longer duration required for the cell population pHi to recover from the minimum pHi value of approximately 4.0 to more physiological levels, and resume exponential growth. Therefore, the expression of *PDR18* was demonstrated to allow the maintenance of physiological levels of ergosterol, required to overcome the severe deleterious effects of acetic acid-induced stress at the plasma membrane level, such as the coordinated decrease of plasma membrane order, increase of the non-specific plasma membrane permeability, and decrease of transmembrane electrochemical potential [23]. The more extended period of latency during which *pdr18*Δ cells maintain a low pHi is consistent with the reported profile of increased acetic acid-induced permeability in this mutant cell population [23].

Impaired mannosylinositol phosphorylceramide (MIPC) biosynthesis was found to result in increased yeast cell susceptibility to zymolyase [17], an enzyme mixture that, similarly to lyticase, has predominantly β-glucanase activity. This increased susceptibility to zymolyase was partially relieved by overexpression of *ERG9*, encoding an enzyme of the ergosterol biosynthetic pathway [17]. Increased sensitivity to zymolyase was also

reported for the *erg6*Δ mutant [18], highlighting the importance of ergosterol biosynthesis in cell wall integrity. In this study, Pdr18 was found to be required for maximum cell resistance to lyticase activity under adaptation and growth under acetic acid stress reinforcing the idea that plasma membrane lipid composition and homeostasis influence cell wall integrity under stress.

The coordinated increase of yeast cell wall stiffness and content of the major cell wall polysaccharides during adaptation to acetic acid-induced stress, was observed in cells lacking the *PDR18* gene, as reported before for the parental strain [25]. Although the most dramatic increase was observed for β-glucans, apparently, the content of the cell wall in chitin and mannans also increases during acetic acid-induced lag phase and in adapted cells. Since mannoproteins have an important role in controlling the porosity of the cell wall [226,227,353,354], the increased content of mannans in *pdr18*Δ cell wall may contribute to restrict the diffusional entry of acetic acid in the cell. Some glycosyl-phosphatidylinositol (GPI) proteins, which are mannoproteins anchored via a C-terminus GPI anchor remnant in the plasma membrane [11,56,72], are involved in cell wall remodeling and determinants of acetic acid tolerance in *S. cerevisiae* [264]. This is the case of the GPI-protein encoded by *GAS1*, found to be slightly up-regulated during yeast response to acetic acid (Ribeiro *et al*., 2021[25] and this work). Interestingly, plasma membrane lipid composition, in particular concerning the content in ergosterol and complex sphingolipids, determines the proper raft association of Gas1 and sorting to the plasma membrane [355], ultimately influencing its activity. Interestingly, a recent QTL mapping to uncover the genetic basis that confers to the bioethanol industrial strain Pedra-2 (PE-2) tolerance during growth at low pH, identified a non-synonymous mutation (A631G) in *GAS1* prevalent in wild-type isolates and absent in laboratory strains [255]. The chitin content, assessed by the quantification of calcofluor white fluorescence intensity, also increased during acetic acid-induced latency, with a time-course pattern similar to the β-glucans profile. Since this fluorophore also has an affinity for β-glucans, although to a less extent than to chitin [352], chitin quantification may include some contribution from the increase of cell wall β-glucans. However, given that our results also show that under acetic acid stress the transcription level from the *CHS3* gene, encoding the major chitin synthase, increased, as well as cell surface stiffness which is dependent on the cross-linking between chitin and β-glucans [69,70], it is likely that the fluorescence values truly reflect the increase of chitin content in *pdr18*Δ cells cultivated under acetic acid stress.

Cell-to-cell heterogeneity within a population is known to be an important factor contributing to acetic acid tolerance in *S. cerevisiae* [351,356,357]. Cells devoid of *PDR18* were previously found to exhibit a higher cell-to-cell heterogeneity concerning plasma membrane order when exponentially growing in the presence of acetic acid [23]. In the

present study, the cell-to-cell analysis of cell stiffness and polysaccharide content also suggest a higher cell-to-cell heterogeneity for the *pdr18*Δ cell population under acetic acid, stress, compared to the parental strain [25].

The transcription levels from the *FKS1, BGL2, GAS1, CHS3,* and *PRM5* genes were found to significantly increase during the adaptation phase to acetic acid, before growth resumption under stress. The *FKS1, BGL2*, *GAS1* and *CHS3* genes code for a β-1,3-synthase [326], an endo-β-1,3 glucanase involved in β-1,3 branching [85], β-1,3-glucanosyltransferase that leads to elongation of (1→3)-β-D-glucan chains [85], and a chitin synthase[327]. This coordinated gene up-regulation apparently correlates with the higher content of β-glucans and chitin, and the increased stiffness registered for the *pdr18*Δ cell population at the same phase of growth under acetic acid stress, compared with unstressed cells. Consistent with our results and interpretation, it was reported that either yeast mutations or exposure to fluconazole leading to ergosterol decrease results in increased cell wall chitin content [19,20]. The very rapid and dramatic reduction in the ergosterol content occurring during the first hour of cultivation in the presence of the acid for the parental and the *pdr18*Δ populations is here described for the first time. This reduction can be related with acetic acid- induced intracellular acidification which affects biosynthetic enzymes activity. Since ergosterol biosynthesis is an energetically expensive process [358], this process is affected in cells requiring energy for ATP dependent mechanisms of adaption to acetic acid stress (e.g., recovery of pHi, acetate efflux). Results from studies on the reprogramming of genome-wide expression during acetic acid-induced stress include the increased transcription from genes encoding the ergosterol biosynthetic pathway, necessary to counteract acetic acid-induced reduction of the ergosterol content [23,264,265]. Although *pdr18*Δ cells exhibit a lower cellular ergosterol content when compared to the parental strain, during the long-term response to acetic acid stress of the mutant cells the increase of the ergosterol content was also observed, with this response being coordinated in time with more favorable cell wall biophysical alterations. It is hypothesized that yeast cells lacking Pdr18, and therefore containing lower ergosterol levels and a less ordered and more permeable plasma membrane, respond more intensively to the same acetic acid concentration to remodel cell wall composition and organization.

Collectively, our results contribute to a better understanding of the time-course alterations of the cellular envelope occurring during the adaptation of yeast cells to acetic acid stress, in particular in cells lacking Pdr18. Compared to the parental strain, cells devoid of Pdr18 exhibit lower levels of ergosterol, a more extended latency phase during which a low pHi is maintained. As observed for parental cells, adaptation to acetic acid stress in *pdr18*Δ cells requires cell wall remodeling clearly detected in the late phase of

growth latency. Such remodeling is characterized by increased contents of β-glucans, chitin, and mannans in the cell periphery, reflecting the observed up-regulation of transcription from genes involved in β-glucans and chitin synthesis and/or elongation. Altogether, these modifications contribute to an increase of cell stiffness when the *pdr18*Δ cell population was about to resume exponential grow, above the values registered for the parental cell population in the same conditions. However, despite the more intense response of *pdr18*Δ to acetic acid stress, the induced increase of *pdr18*Δ cells resistance to lyticase activity is below the values for parental cells suggesting a role for yeast plasma membrane composition and order in cell wall architecture. The described adaptation profiles involving all the cell envelope parameters examined in this study, correlate with the profile of recovery to more physiological pHi values and growth resumption under acetic acid stress. This study provides relevant new information on how plasma membrane homeostasis engineering can be used for the rational development of superior yeast strains more tolerant to acetic acid. The modulation of plasma membrane ergosterol content and of the expression of ABC transporters involved in lipid homeostasis is pointed out herein as a promising molecular target for the improvement of acetic acid tolerance. This is an essential trait to increase the economic sustainability of lignocellulosic biorefineries.

4. General Discussion and perspectives

An adequate adaptive response and high tolerance of yeasts to industrially-relevant stresses is instrumental for achieving economically sustainable bioprocesses involving this major cell factory, in particular in lignocellulosic biorefineries [48–50,314]. The understanding of the molecular mechanisms underlying adaptation and tolerance to multiple stresses is essential to guide the rational design of more robust yeast strains with improved performance in Industrial Biotechnology, whether this knowledge is applied to the eukaryotic model and cell factory *S. cerevisiae* [3,4], or to non-*Saccharomyces* species that are gaining attention due to its biotechnological potential [5]. Weak acids are often used as food preservatives, preventing food spoilage in large-scale food industries. Weak acids are potential major inhibitory compound in alcoholic-fermentation-based processes [7,8]. Acetic acid, in particular, is one of the major inhibitory compounds present in lignocellulosic hydrolysates used in advanced biorefineries. This thesis work contributes to deepen the knowledge regarding the mechanisms underlying the adaptive response and tolerance to acetic acid focused on the molecular and biophysical alterations occurring at the cell wall level. They involve the identification of changes that occur in the cell wall content in the major polysaccharides β-glucans, mannans and chitin, and in the structure and physical properties of the cell wall during the different phases of the response to sudden acetic acid stress, resulting in a more robust and stiffer cell wall (**Figure 4.1**). This thesis work also contributes to shed light on the interplay between plasma membrane lipid composition and cell wall properties in the yeast cell response to acetic acid stress. The approach used was by studying the impact of the expression of the *PDR18* gene, encoding the pleiotropic drug resistance transporter Pdr18 involved in the transport of ergosterol at the plasma membrane, in the molecular and biophysical properties of the cell wall during cell response and adaptation to acetic acid stress (**Figure 4.1**).

The cell wall, together with the plasma membrane, constitute the first line of defense against multiple stresses, being the cellular response involving the cell wall towards different challenging conditions dynamic, contributing to the maintenance of cell morphology and integrity [12,13]. Results from several genome-wide studies involving transcriptomic [263,265–267,280,316,317],chemogenomic [263,264] and proteomic [268,269] analyses of the yeast response to acetic acid stress, carried out over the years, have suggested that the cell wall may have an important role in the response and tolerance to acetic acid and other environmental challenges [7,25,70,118,120,319]. Together with the dynamic alteration of plasma membrane composition and properties, the alterations occurring in the cell wall, likely implicate a reduction of the diffusion rate of the weak acid toxic form from the cell exterior to the intracellular medium [7,24]. In this way, the re-entry of the toxic acid form,

following the active expulsion of acetate from the cell interior is counteracted, thus limiting the associated futile cycle [7,24]. However, there was no detailed analysis of the time-course of the molecular and biophysical alterations occurring in the cell wall under stress. The findings of the work herein presented provide, for the first time, an integrative and comprehensive view on how the cell wall respond and adapt during acetic acid stress[25].

The remodeling of the cell wall is essential during acetic acid-induced latency, when the permeabilization of stressed yeast cells is maximal. Therefore, responses such as the decrease of cell envelope permeability counteracts plasma membrane damage and intracellular acidification induced by acetic acid stress [25]. The marked decrease of cell wall susceptibility to lyticase activity and the increase of its β-glucan content found to occur during the first hours of exposure to acetic acid stress is part of that global response. When yeast cells resume exponential growth after adaptation to acetic acid, cell wall susceptibility to lyticase activity exhibit higher values but below the values registered for unstressed cells. The content in β-glucans is maintained higher compared with the content in unstressed cells throughout the latency period and exponential growth [25].

The transcriptional downregulation of most of cell wall biosynthesis-related-genes studied in this work during the whole period of stressed cultivation suggest that the CWI signalling pathway is likely not the major key player in acetic acid stress-induced response involving the cell wall [25]. However, the transcription levels from the *GAS1* gene were found to moderately increase under acetic acid stress, correlating with the increase of the cell wall glucan content [25]. It remains to be known if other signalling pathways may play a role in the regulation of cell wall biosynthesis-related genes and in the alterations, at the cell wall level, described in this work during the response and adaptation to acetic acid stress. It is known that other signalling pathways may elicit alterations at the level of cell wall in response to stresses of industrial relevance such as the HOG pathway [15,108,121,154,164,186,191,243] or the Calcineurin pathway [243,257]. Given the different transcriptional expression profile of *GAS1* under acetic acid stress, compared with the other cell wall biosynthesis-related genes analysed in this work, and that its expression was correlated with the content of glucan in the cell wall [25], the increased expression of this gene might be beneficial to enhance tolerance to this weak acid. In fact, the increased tolerance to low pH stress, when induced by a strong acid was reported in result of the overexpression in *S. cerevisiae* of the *GAS1* gene from *S. cerevisiae* (*ScGAS1*) or from *Issatchenkia orientalis* (*IoGAS1*) [359]. Remarkably, the expression of *IoGAS1* in *S. cerevisiae* was reported to lead to enhanced tolerance to acetic acid and

also to formic acid at low pH (2.4 or lower) [308]. This is consistent with the notion of the importance of *GAS1* in the context of the adaptation and tolerance of yeasts to weak acids and with this gene being a promising candidate to be considered to increase yeast robustness by genetic engineering.

The revelation of the impact of plasma membrane ergosterol content on cell wall properties at both molecular and biophysical levels under acetic acid stress was another relevant outcome of this thesis work [30]. The adaptation to acetic acid stress in cells with *PDR18* deleted requires the remodeling of the cell wall, clearly detected in the late phase of acetic acid-induced growth latency, marked by the increased content of major cell wall polysaccharides, β-glucans, chitin, and mannans, reflecting the observed transcriptional up-regulation of genes involved in β-glucan and chitin synthesis and/or elongation [30]. These alterations contribute for the increase of cell stiffness when the cell population is about to resume exponential growth, above the stiffness values of the parental strain in the same conditions [30]. Also, the modifications described during adaptation to acetic acid stress correlate with the profile of pHi recovery and growth resumption under this stress [30]. Pdr18 was described as a determinant of acetic acid tolerance in yeast, being involved in the maintenance of the ergosterol content in plasma membrane under acetic acid stress, thus helping to counteract the acetic acid induced- increase of plasma membrane permeability and the decrease of plasma membrane lipid order and of the transmembrane electrochemical potential [23]. This work reinforces the notion that plasma membrane lipid composition influences cell wall integrity, and further supports the gathered evidence showing that the biological function of ABC transporters required for multidrug/multixenobiotic resistance (MDR/MXR) extends beyond their acknowledged role as drug/xenobiotic exporters [23,360–363]. Other ABC transporters, required for MDR/MXR, are involved in lipid trafficking, impacting membrane lipid composition and properties [342] . This work opens the door to the understanding of the possible role of other ABC transporters in influencing the properties of cell wall during industrially relevant stresses. Moreover, it provides novel insights into the question of how plasma membrane homeostasis engineering may be used for the rational development of superior yeast strains more tolerant to multiple stresses of industrial relevance. This work indicates that cells lacking Pdr18, with a lower ergosterol content and a more permeable plasma membrane [23], respond more intensely for the remodeling of the cell wall in response to acetic acid stress. An interesting topic of research would be to unveil the involved cell sensors that perceive changes in membrane properties, also related with changes in plasma membrane lipid composition, that lead to signal transduction and likely to a more effective response at the level of cell wall under stress.

The general major conclusions of this thesis are gathered in the biochemical model schematized in **Figure 4.1**.

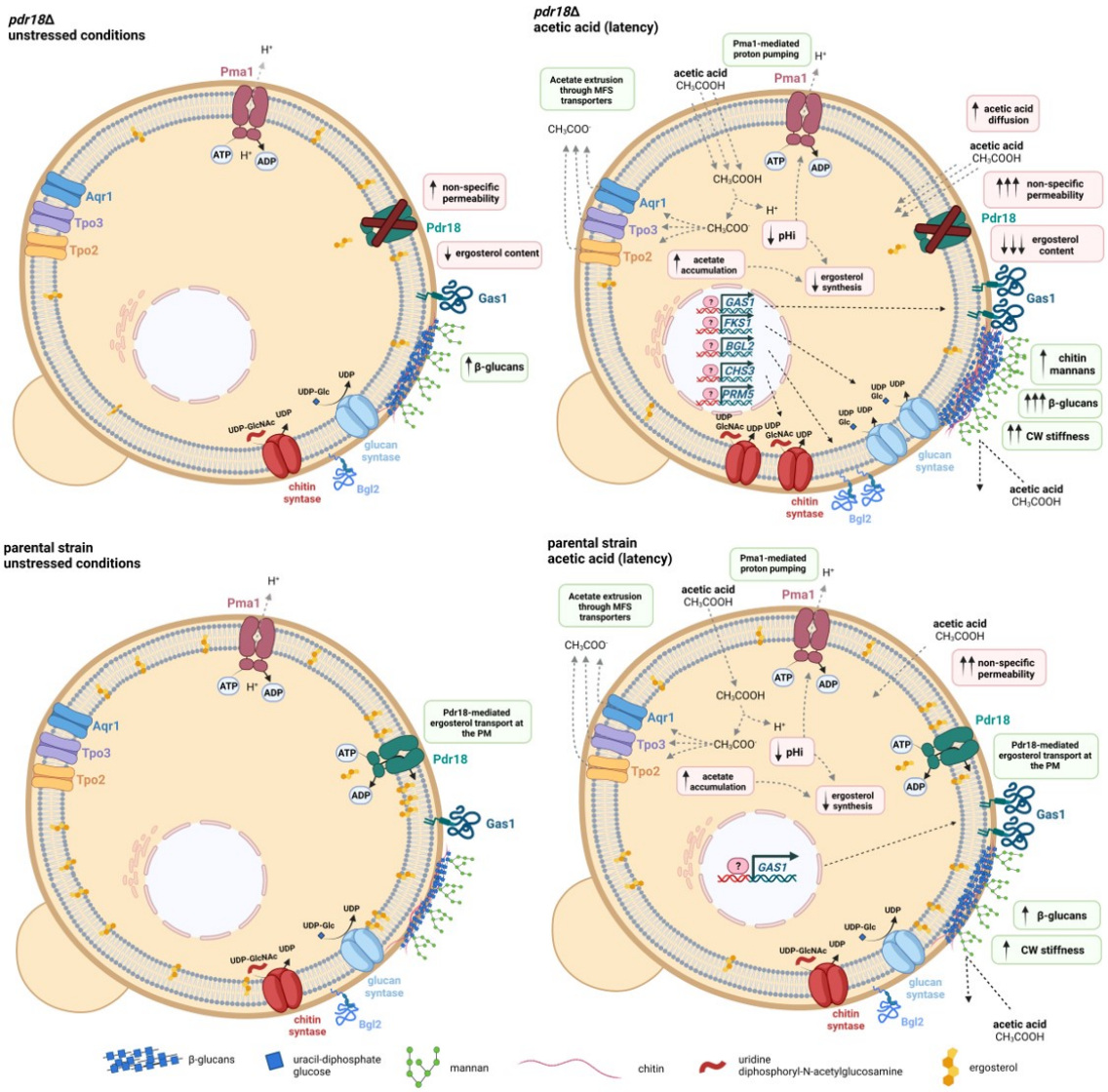

Figure 4.1 - Schematic model for the adaptive response to acetic-acid-induced stress in the parental and pdr18Δ strains, at the level of the cell envelope, suggested by the results presented in chapter 2 and chapter 3 of this thesis. The adaptation mechanisms (described in green boxes) to counteract the deleterious effects of acetic acid stress (described in red boxes) are represented for both strains.

It was also intended to identify possible genes required for the synthesis of cell wall polysaccharides, for cell wall remodeling, cell wall integrity sensing or signalling required for maximum tolerance other more lipophilic monocarboxylic acids with a higher linear carbon chain, in particular butyric acid and octanoic acid. Butyric acid is an intermediate metabolite of the anaerobic digestion of organic residues and is also considered an interesting bioproduct as a precursor for biofuels [364,365]. Octanoic acid is a byproduct of *S. cerevisiae* alcoholic fermentation and its effect as a growth inhibitor is enhanced by low pH and by the presence of ethanol contributing to the fermentation inhibition or even

stoppage [366]. Furthermore, it can be used as antimicrobial agent, surfactant or as a precursor for biofuel production [367–369]. For all those reasons they were considered of interest to extend some of the results described in the thesis.

The susceptibility to acetic, butyric or octanoic acids-induced stress of the parental strain and selected mutants with genes related with cell wall biosynthesis deleted, was compared in YNB medium, at pH 5.0. The selected mutants were: *fks1*Δ and *fks2*Δ (absence of β-1-3-glucan synthases), *chs3*Δ (absence of a major chitin synthase), *gas1*Δ (absence of a β-1,3-glucanosyltransferase involved in (1->3)-β-D-glucan chains elongation and branching), *rlm1*Δ and *swi4*Δ (absence of transcription factors involved in CWI signalling pathway), *slt2*Δ (absence of a MAPK of the CWI signalling pathway), *wsc1*Δ and *wsc2*Δ (absence of CWI signalling pathway sensors), *spi1*Δ (absence of a CWP whose coding gene is upregulated in the presence of different weak acids), and *ygp1*Δ (absence of a CWP whose coding gene contributes for tolerance to weak acids). For the spot assays prepared as described in Chapter 4 of this thesis, the susceptibility phenotypes observed after 72 hours of incubation at 30°C, are shown in **Figure 4.2**.

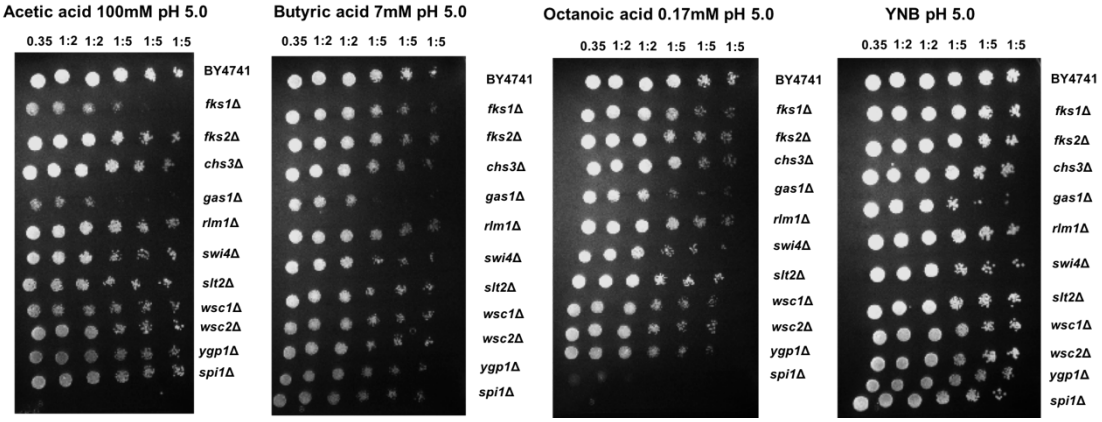

Figure 4.2 – Growth spot assays comparing the susceptibility to acetic acid, butyric acid or octanoic acid, in YPD at pH 5.0 and 30ºC (72h of incubation) of the parental S. cerevisiae strain BY4741 and derived mutants defective in genes required for the biosynthesis, including regulation.

A marked susceptibility phenotype towards 100mM of acetic acid was exhibited by *fks1*Δ and *gas1*Δ deletion mutants while towards 0.17mM of octanoic acid the *spi1*Δ deletion mutant was the more susceptible. These results are consistent with the conclusion that both *FKS1* and *GAS1* genes as determinants of acetic acid tolerance [264]. Also in line with our results *SPI1*-dependent cell wall remodeling was suggested as relevant to reduce plasma membrane damage from the action of more lipophilic weak acids, such as octanoic acid [273]. Interestingly, a susceptibility phenotype in yeast cells devoid of *GAS1* was also exhibited for butyric acid and octanoic acid, suggesting that *GAS1* is a possible

determinant of tolerance to low- and medium- chain fatty acids. The importance of *GAS1* as a determinant of tolerance to acetic and lactic acids was already described [264,291]. However, to our knowledge, genome-wide studies to identify *S. cerevisiae* genes required for butyric acid tolerance were not reported and very few susceptibility assays or transcriptomics studies under octanoic acid stress were reported [370,371], and none suggesting *GAS1* as a possible determinant of tolerance to butyric and octanoic acids. This knowledge is an important starting point for guiding the engineering of strains with increased robustness towards a wide range of weak acids of different liposolubility with *GAS1* as a molecular target.

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6. Thesis publications

Peer-reviewed scientific publications directly related to this thesis:

Ribeiro RA., Bourbon-Melo N., Sá-Correia I. *The Cell Wall and the Response and Tolerance to Stresses of Biotechnological Relevance in Yeasts*. ***Frontiers in Microbiology*** 2022;13:953479. doi:10.3389/fmicb.2022.953479 *(in press)*

Ribeiro RA, Godinho CP, Vitorino MV, Robalo TT, Fernandes F, Rodrigues MS, Sá-Correia I. *Crosstalk between Yeast Cell Plasma Membrane Ergosterol Content and Cell Wall Stiffness under Acetic Acid Stress Involving Pdr18*. ***Journal of Fungi***. 2022; 8(2):103. doi:10.3390/jof8020103

Ribeiro RA, Vitorino MV, Godinho CP, Bourbon-Melo N, Robalo TT, Fernandes F, Rodrigues MS, Sá-Correia I. *Yeast adaptive response to acetic acid stress involves structural alterations and increased stiffness of the cell wall.* ***Scientific Rep*orts** 2021;11(1):12652. doi:10.1038/s41598-021-92069-3.

Oral presentation in <u>international</u> scientific meetings:

Ribeiro RA, Godinho CP, Vitorino MV, Robalo TT, Fernandes F, Rodrigues MS, Sá-Correia I. *Yeast response and tolerance to acetic acid: focus on the cell envelope*; **7th Conference on Physiology of Yeast and Filamentous Fung**i (PYFF7), June 2019 (**Oral presentation**)

Oral presentations in <u>national</u> scientific meetings:

Ribeiro RA, Godinho CP, Vitorino MV, Robalo TT, Fernandes F, Rodrigues MS, Sá-Correia I. *Yeast response and tolerance to acetic acid: focus on cell envelope properties, assessed by AFM*; Congress of Microbiology and Biotechnology 2019 (**Microbiotec'19**), December 2019 (**Oral presentation**)

Ribeiro RA, Vitorino MV, Godinho CP, Bourbon-Melo N, Robalo TT, Fernandes F, Rodrigues MS, Sá-Correia I. *Response to acetic acid on yeast: focus on cell wall remodeling;* Congress of Microbiology and Biotechnology 2021(**Microbiotec'21**), December 2021 (**Short-oral presentation**)

Peer-reviewed scientific publications not included in this thesis:

Monteiro PT, Oliveira J, Pais P, Antunes M, Palma M, Cavalheiro M, Galocha M, Godinho CP, Martins LC, Bourbon N, Mota MN, **Ribeiro RA**, Viana R, Sá-Correia I, Teixeira MC. *YEASTRACT+: a portal for cross-species comparative genomics of transcription regulation in yeasts*. ***Nucleic Acids Research****.* 2020; 48(D1):642–649. doi: 10.1093/nar/gkz859