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HAA1 and *PRS3* overexpression boosts yeast tolerance towards acetic acid improving xylose or glucose consumption: unravelling the underlying mechanisms

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Abstract

Acetic acid tolerance and xylose consumption are desirable traits for yeast strains used in industrial biotechnological processes. In this work, overexpression of a weak acid stress transcriptional activator encoded by the gene *HAA1* and a phosphoribosyl pyrophosphate synthetase encoded by *PRS3* in a recombinant industrial *Saccharomyces cerevisiae* strain containing a xylose metabolic pathway was evaluated in the presence of acetic acid in xylose- or glucose-containing media. *HAA1* or *PRS3* overexpression resulted in superior yeast growth and higher sugar consumption capacities in the presence of 4 g/L acetic acid, and a positive synergistic effect resulted from the simultaneous overexpression of both genes. Overexpressing these genes also improved yeast adaptation to a non-detoxified hardwood hydrolysate with a high acetic acid content. Furthermore, the overexpression of *HAA1* and/or *PRS3* was found to increase the robustness of yeast cell wall when challenged with acetic acid stress, suggesting the involvement of the modulation of the cell wall integrity pathway. This study clearly shows *HAA1* and/or, for the first time, *PRS3* overexpression to play an important role in the improvement of industrial yeast tolerance towards acetic acid. The results expand the molecular toolbox and add to the current understanding of the mechanisms involved in higher acetic acid tolerance, paving the way for the further development of more efficient industrial processes.

Keywords *PRS3* and *HAA1* overexpression · Acetic acid · Xylose consumption · Industrial *Saccharomyces cerevisiae* · Cell wall robustness

Introduction

Over the last years, environmental problems and worldwide economic issues have driven the research interest towards the value of lignocellulosic biomass as a sustainable solution for the production of bioenergy and value-added products (Moyses et al. 2016). Lignocellulosic biomass is a complex structure mainly consisting of lignin, cellulose (composed of glucose monomers) and hemicellulose (containing various hexose and pentose sugars). Pentoses, such as D-xylose, may represent up to 20% of lignocellulose sugar content (Zabed et al. 2016); however, this sugar is not naturally consumed by the yeast *Saccharomyces cerevisiae*. Furthermore, pretreatment and hydrolysis stages, required to obtain fermentable sugars from lignocellulosic material, may result in the release of toxic compounds, such as acetic acid, furfural and hydroxymethylfurfural (HMF). Acetic acid is released mainly from the hemicellulose fraction (Jönsson and Martín 2016) and is a potent inhibitor of microbial growth (Parawira and Tekere 2011). Thus, an economically viable usage of this biomass requires an efficient utilization of its hemicellulosic fraction, which implies the development of genetically modified *S. cerevisiae* strains capable of xylose consumption and with thorough acetic acid tolerance.

Joana T. Cunha and Carlos E. Costa contributed equally to this work.

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Moreover, bacterial contamination poses as a major drawback in yeast-based industrial processes, resulting in decreased yeast growth and viability and lower productivity levels, by competing for nutrition from the medium (Skinner and Leathers 2004; Thomas et al. 2001). Antibiotics, such as penicillin and virginiamycin, are the most commonly used agents for contamination control (Muthaiyan et al. 2011); however, its addition results in the development of drug-resistant strains, and has consequent negative ecological impact (Bischoff et al. 2009). In this sense, the addition/presence of acetic acid may be a valuable tool to prevent bacterial contamination in yeast growth without using antibiotics (Saithong et al. 2009; Tanaka et al. 2012). Nevertheless, the yeast growth ability is also challenged by the presence of high concentrations of this acid, and for this reason, the development of acetic acid-tolerant yeast strains could have the added benefit of more flexible process considerations with respect to the bacterial load of the substrate.

The industrial *S. cerevisiae* PE-2 strain (Amorim et al. 2011) has previously shown inherent robustness, higher fermentation capacities and tolerance to lignocellulosic-derived inhibitors in glucose-based fermentations (Pereira et al. 2011b, 2014a), being capable of readily detoxifying furfural and HMF. Recently, this strain has also shown promising results, when engineered for D-xylose consumption, on fermentations from different non-detoxified hemicellulosic hydrolysates (Costa et al. 2017). Furthermore, it was observed that deletion of the two copies of the *GRE3* gene (to remove the principal native route of xylitol production) in PE-2 strain increased D-xylose consumption rate (Romaní et al. 2015). Despite the natural robustness of industrial strains, such as PE-2, attaining even higher acetic acid tolerance would greatly benefit their industrial applications.

The *HAA1* gene is associated with yeast adaptation and tolerance to acetic acid stress (Fernandes et al. 2005) and is an important regulator of the transcriptome remodelling in response to acetic acid stress. *HAA1* is responsible for the activation of 80% of the acetic acid-responsive genes, several of them determinants of tolerance to this weak acid (Mira et al. 2010b). Consistently, *HAA1* overexpression resulted in increased tolerance towards the presence of acetic acid in distinct *S. cerevisiae* backgrounds and conditions (Inaba et al. 2013; Sakihama et al. 2015; Swinnen et al. 2017; Tanaka et al. 2012). The *PRS3* gene is responsible for the synthesis of phosphoribosyl pyrophosphate (PRPP), which is a precursor for nucleotide, histidine and tryptophan. *PRS3* also presents other functions besides its biosynthetic role: it has been identified as being required for cell integrity (Binley et al. 1999; Wang et al. 2004), cell cycle arrest upon nutrient deprivation, proper organization of the actin cytoskeleton and maintenance of ion homeostasis (Binley et al. 1999). Moreover, *PRS3* has been identified as a key gene in the tolerance towards lignocellulosic-derived inhibitors, being necessary for yeast growth and maximal fermentation rate in wheat straw hydrolysate (Pereira et al. 2011a, 2014b). Overexpression of *PRS3* resulted in improved fermentation from glucose in the presence of lignocellulosic-derived inhibitors in both synthetic or actual lignocellulosic hydrolysates (Cunha et al. 2015) through a suggested increase in regeneration of NADH, a cofactor required for the detoxification of furfural and HMF, while the effect of overexpressing this gene regarding acetic acid tolerance was not elucidated. Both *HAA1* and *PRS3* have been associated with cell wall integrity processes: Haa1p is a potential regulator of cell wall integrity (CWI) pathway genes according to the transcriptional regulatory associations gathered in the Yeastract database (Teixeira et al. 2017) and an extensively reported regulator of cell wall-related proteins (Keller et al. 2001; Mira et al. 2010b; Sakihama et al. 2015; Sugiyama et al. 2014; Tanaka et al. 2012), while *PRS3* was described to play a significant role in the remodelling of the cell wall and to be possibly involved in cell integrity signalling (Wang et al. 2004). The CWI pathway and cell wall organization have been reported to be highly suppressed in response to acetic acid stress (Dong et al. 2017), and specific mutations of the CWI pathway lead to increased rates of survival in presence of lethal concentrations of acetic acid (Rego et al. 2014). However, complete deletion of the glucan synthase, encoded by *FKS1*, leads to lower survival in the presence of acetate (Mollapour et al. 2009) illustrating the complexity of the regulation at work.

Despite the promising results obtained from overexpressing *HAA1* gene, none of the overexpression strategies for tolerance improvement towards acetic acid has been tested in industrial *S. cerevisiae* strains capable of glucose and xylose consumption or combined with *PRS3* overexpression. Therefore, this study aimed to develop new industrial strains overexpressing *HAA1* and/or *PRS3* genes using the proven chassis of the xylose-consuming *S. cerevisiae* PE-2ΔGRE3 (Romaní et al. 2015). Additionally, and considering the putative roles of both *HAA1* and *PRS3* in cell wall-related pathways, this work also aimed to understand the underlying mechanisms responsible for the positive effect of overexpressing these genes on yeast resistance to acetic acid, evaluating their role in cell wall integrity during growth in the presence of this weak acid, both on glucose and xylose media.

Material and methods

Yeast strains and plasmid construction

The diploid industrial *S. cerevisiae* PE-2 (NCYC 3233), that showed improved fermentative performance in lignocellulosic hydrolysate (Pereira et al. 2014a), with both copies of the *GRE3* gene deleted (Romaní et al. 2015), was used as chassis strain in this work. Additionally, this strain was already used

as chassis for modifications for xylose consumption resulting in improved xylose consumption when compared to other industrial *S. cerevisiae* strains (Costa et al. 2017). The plasmid pMEC1049 was used to overexpress *HAA1* and/or *PRS3* genes. This plasmid carries the hphMX4 hygromycin resistance marker and expresses a xylose utilization pathway containing the *XYL1* and *XYL2* genes from *Scheffersomyces stipitis*, and the endogenous *XKS1* and *TAL1* genes, under the control of *S. cerevisiae* promoters *TEF1*, *TDH3*, *PGI1* and *FBA1*, respectively (Romaní et al. 2015). The *XYL1* gene carries the N272D mutation for higher specificity for NADH (Runquist et al. 2010). The *HAA1* and *PRS3* transcriptional units with corresponding native promoter and terminator sequences were amplified from plasmids BHUM1737 and YepJCp, with the primer pairs Hfw/Hrv and Pfw/Prv (Table 1), respectively. The PCR products containing *HAA1* or *PRS3* genes were inserted in the *Xho*I or *Ale*I restriction sites of the pMEC1049 plasmid by in vivo homologous recombination between primer tails and plasmid backbone. The constructed plasmids were extracted and transformed to *Escherichia coli* NZY5α (NZYtech) for propagation and restriction analysis confirmation. The resulting vectors were designated pMEC9001 (expressing *HAA1*), pMEC9002 (expressing *PRS3*) and pMEC9003 (expressing *HAA1* and *PRS3* simultaneously). A detailed description of the vector construction using pydna (Pereira et al. 2015) has been compiled into a series of online Jupyter notebooks hosted in a Github repository (https://goo.gl/TBDKHA). The vectors were introduced in the PE-2ΔGRE3 strain using the lithium acetate method (Gietz et al. 1992), and the PE-2ΔGRE3 containing the pMEC1049 (Romaní et al. 2015) was used as control in the assays (Table 1). Transformants were selected on YPD plates (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose and 20 g/L agar) containing 300 μg/mL of hygromycin and were cultured in YPX medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L D-xylose) until capable of total xylose consumption. The recombinant strains were preserved at 4 °C in YPX plates containing 300 μg/mL of hygromycin to maintain selection for the vectors.

Table 1 Strains, plasmids and primers used in this study. Uppercase sequences correspond to sequences complementary to the template, and lowercase sequences correspond to homologous recombination sites with pMEC1049 plasmid

	Relevant features	Source
S. cerevisiae strains		
PE-2ΔGRE3	PE-2, gre3::natMX4/gre3::kanMX4	Romaní et al. (2015)
PE-Control[a]	PE-2ΔGRE3, pMEC1049	Romaní et al. (2015)
PE-HAA1[a]	PE-2ΔGRE3, pMEC9001	This work
PE-PRS3[a]	PE-2ΔGRE3, pMEC9002	This work
PE-HAA1/PRS3[a]	PE-2ΔGRE3, pMEC9003	This work
Plasmids		
BHUM1737	URA3 marker, containing the *HAA1* gene under the control of its native promoter	Malcher et al. (2011)
YepJCP	URA3 and KanMX marker, containing the *PRS3* gene under the control of its native promoter	Cunha et al. (2015)
pMEC1049	pYPK4-TEF1tp-XR (N272D)-TDH3tp-XYL2-PGI1tp-XKS1-FBA1tp-TAL1-PDC1tp, HphMX4	Romaní et al. (2015)
pMEC9001	pMEC1049 containing the *HAA1* gene with native promoter and terminator	This work
pMEC9002	pMEC1049 containing the *PRS3* gene with native promoter and terminator	This work
pMEC9003	pMEC1049 containing the *HAA1* and *PRS3* genes with native promoter and terminator	This work
Primers	Sequence (5′->3′)	
Hfw	ggttttaccgtgtgcggagatcaggttctgatcccCCATTTCCCCTTTCT	
Hrv	agacaaaccgtgggacgaattcttaagatgctcgaATACCTCATCTCTGCG	
Pfw	taacgatgtagtacagcgtttccgctttttcacccTTATCTTCATCACCGC	
Prv	cataagtacccatccaagagcacgcttattcaccaACAAGAGAAACTTTTG	

[a] *S. cerevisiae* PE-Control, PE-HAA1, PE-PRS3 and PE-HAA1/PRS3 are now deposited in *Micoteca da Universidade do Minho* (MUM, University of Minho, Portugal) with the following codes, respectively: MUM18.12, MUM18.13, MUM18.14 and MUM18.15

Preparation of Paulownia hydrolysate

Fast-growing hardwood, *Paulownia tomentosa*, was used as raw material for the production of hemicellulosic hydrolysate. Paulownia wood was subjected to hydrothermal treatment at 210 °C (or $S_0 = 4.43$) in an 18.75-L stainless stirred reactor (4555, Parr Instrument Company, USA) using a liquid to solid ratio of 6 g of distilled water/g of *P. tomentosa*. Hardness of hydrothermal treatment can be also expressed in terms of severity (S_0) defined as logarithm of severity factor R_0 (Lavoie et al. 2010). Liquor (containing hemicellulose-derived compounds) obtained from hydrothermal treatment was hydrolysed using 1.5% (*w*/*w*) of H_2SO_4 for 45 min at 121 °C in autoclave. Paulownia hydrolysate was neutralized with $CaCO_3$ (pH = 5) and sterilized by filtration (0.2 μm). Chemical composition of *P. tomentosa* and conditions of pretreatment were reported and selected based on previous work (Dominguez et al. 2017).

Inoculum preparation

The yeast strains used for inoculation were grown at 30 °C for 24 h, with orbital agitation (200 rpm), in YPX (10 g/L yeast extract, 20 g/L peptone, 20 g/L D-xylose) or YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L D-glucose) media containing 300 μg/mL of hygromycin. Cells were recovered by centrifugation (15 min, 4000×*g*, 4 °C), and pellets were resuspended in ice-cold 0.9% (*w*/*v*) sodium chloride to obtain an $OD_{600\ nm}$ of 1 (for growth assays) or 200 mg of fresh yeast/mL (for fermentation assays).

Growth assays

Aerobic growth was performed in 24-well microplates or in 100-mL Erlenmeyer flasks (with cotton stoppers) with a working volume of 1 or 30 mL, respectively. Media tested consisted of YPX medium, supplemented or not with 4 g/L acetic acid (pH 4), and of YPD medium (containing 300 μg/mL of hygromycin) supplemented with 4 g/L acetic acid (pH 4). Each well/flask was inoculated to an $OD_{600\ nm}$ of 0.1 and incubated at 30 °C and 200 rpm orbital agitation. The growth was monitored by $OD_{600\ nm}$ measurements (using a BioTek Synergy™ HT reader for microplate assays and a GeneQuant™ 100 spectrophotometer for flask assays) and by sample collection for HPLC analysis.

Fermentation assays

Fermentations were performed in 100-mL Erlenmeyer flasks (with cotton stoppers) with a working volume of 30 mL of Paulownia hydrolysate supplemented with approximately 30 g/L of glucose and 300 μg/mL of hygromycin and inoculated with 5 mg of fresh yeast/mL. The experiments were carried out at 30 °C in an orbital shaker (150 rpm) and were monitored by sample collection for HPLC analysis. Final biomass concentration in the media was measured by dry cell weight in the end of fermentation.

Analytical methods

P. tomentosa wood and pretreated *P. tomentosa* were analysed to determine their composition by quantitative acid hydrolysis (NREL/TP-510-42618), according to Dominguez et al. (2017). The concentrations of glucose, xylose, acetic acid, ethanol, furfural and HMF in the samples from synthetic media and *P. tomentosa* hydrolysates were determined by HPLC using a Bio-Rad Aminex HPX-87H column, operating at 60 °C, with 0.005 M H_2SO_4 and at a flow rate of 0.6 mL/min. The peaks corresponding to glucose, xylose, acetic acid and ethanol were detected using a Knauer-IR refractive index detector, whereas furfural and HMF were detected using a Knauer-UV detector set at 210 nm.

Lyticase sensitivity assay

The analysis of each strain cell wall susceptibility to lyticase was performed after growth, until mid-exponential phase, on xylose- or glucose-containing media with 4 g/L of acetic acid. Yeast cells were collected by centrifugation and washed twice with phosphate buffer 0.1 M, pH 6.6. The cell suspension was diluted to an $OD_{600\ nm}$ of 0.5 in a volume of 30 mL of phosphate buffer in 100-mL Erlenmeyer flasks, and lyticase (Sigma-Aldrich) was added to each flask in a final concentration of 10.22 units/mL. The flasks were incubated at 30 °C and 200 rpm orbital agitation, and the decrease of absorbance was monitored by $OD_{600\ nm}$ measurements in 15-min intervals.

Statistical analyses

GraphPad Prism for Windows version 6.01 was used to carry out the statistical analyses. Differences between the strain profiles in terms of growth, fermentation, sugar consumption and lyticase sensitivity assay were tested by repeated measures two-way ANOVA, followed by Tukey post hoc test. Differences in kinetic parameters were determined using repeated measures one-way ANOVA, followed by Bonferroni post hoc test. Statistical significance was established at $P < 0.05$ for the comparisons and marked by $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Regulatory association analyses

The regulatory associations between genes of interest were determined using Yeastract (Yeast Search for Transcriptional Regulators and Consensus Tracking), an open web tool for the analysis and prediction of transcription regulatory associations

in *S. cerevisiae* (Teixeira et al. 2017). The analysis was performed considering both documented (DNA binding and/or expression evidences) and potential (considering PBM/MITOMI-based motifs) regulations.

Results

Effect of *HAA1* and/or *PRS3* overexpression on the growth performance of *S. cerevisiae* from xylose in the presence of acetic acid

To evaluate the effects of overexpressing *HAA1* and/or *PRS3* genes in yeast tolerance to acetic acid in xylose medium, the control and overexpressing strains were characterized on their ability to grow aerobically in media containing, or not, 4 g/L of acetic acid (Fig. 1). In the absence of acetic acid, overexpression of *HAA1* and/or *PRS3* did not seem to have any noticeable effect on the yeast growth capacity in xylose medium (Fig. 1a). Consistently, the percentage of xylose consumed by the PE-HAA1 and PE-PRS3 strains after 48 h was similar to the control strain (ca. 95%), while PE-HAA1/PRS3 xylose consumption was slightly lower (88%) (Fig. 1b, $P < 0.001$). However, when exposed to 4 g/L of acetic acid, the strains overexpressing *HAA1* and/or *PRS3* exhibited improved growth on xylose, with smaller lag phases than the control strain (Fig. 1c, $P < 0.01$), and significantly higher xylose consumption after 48 h, as the control strain under acetic acid stress was unable to consume this sugar (Fig. 1d, $P < 0.0001$). Furthermore, in the presence of acetic acid, the PE-HAA1/PRS3 strain displayed higher percentage of consumed xylose than the strains overexpressing each gene separately and reached the maximum optical density sooner (Fig. 1c, d, $P < 0.05$).

To further understand the behaviour of these strains in the presence of acetic acid, the concentrations of xylose and acetic acid were monitored during their growth on xylose medium with 4 g/L of acetic acid (Fig. 2). As previously observed, the strains overexpressing *HAA1* and/or *PRS3* exhibited a superior growth performance in comparison with the control strain (Fig. 2a, $P < 0.01$), with the PE-HAA1/PRS3 strain exhibiting the fastest growth profile (Fig. 2a, $P < 0.01$). Accordingly, the PE-HAA1/PRS3 strain showed a faster xylose consumption (Fig. 2b, $P < 0.01$), while the control strain consumed xylose slower than the overexpressing strains (Fig. 2b, $P < 0.05$). The strains overexpressing *HAA1* and/or *PRS3* exhibited a faster removal of this weak acid from the medium compared to the control strain (Fig. 2c, $P < 0.05$). Additionally, it should be noted that, for all strains, xylose consumption only started after acetic acid concentration in the medium has decreased to expressively lower levels (< 2 g/L) (Fig. 2b, c).

Effect of *HAA1* and/or *PRS3* overexpression on the growth performance of *S. cerevisiae* from glucose in the presence of acetic acid

The effect of acetic acid on *S. cerevisiae* PE-2ΔGRE3 transformant strains was also analysed in glucose-containing medium, evaluating growth performance and glucose and acetic acid concentrations (Fig. 3). As observed in xylose-containing medium, the strain PE-HAA1/PRS3 exhibited a higher growth performance, with a smaller lag phase in

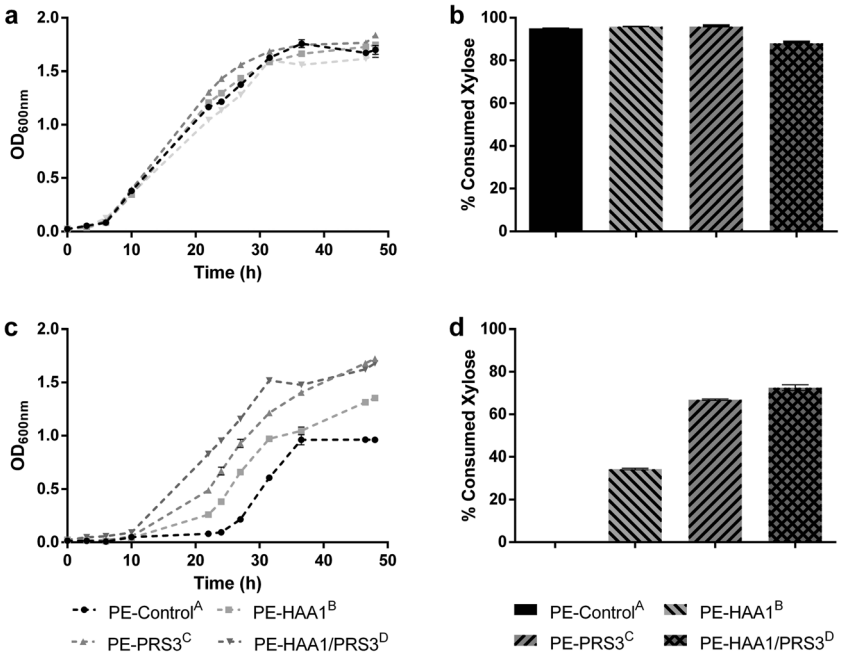

Fig. 1 Growth profiles (**a**, **c**) and xylose consumption percentage (**b**, **d**) after 48 h of *S. cerevisiae* PE-2ΔGRE3 transformants (control and overexpressing *HAA1* and/or *PRS3*) in microplates in: YPX medium (**a**, **b**) and YPX with 4 g/L of acetic acid (**c**, **d**). Data represents the average ± SEM from two biological replicates. **a** A,C**; A,D*; B,C*; B,D*; C,D***. **b** A,D***; B,D***; C,D***. **c** A,B**; A,C****; A,D****; B,C***; B,D***; C,D**. **d** A,B****; A,C****; A,D****; B,C****; B,D****; C,D*

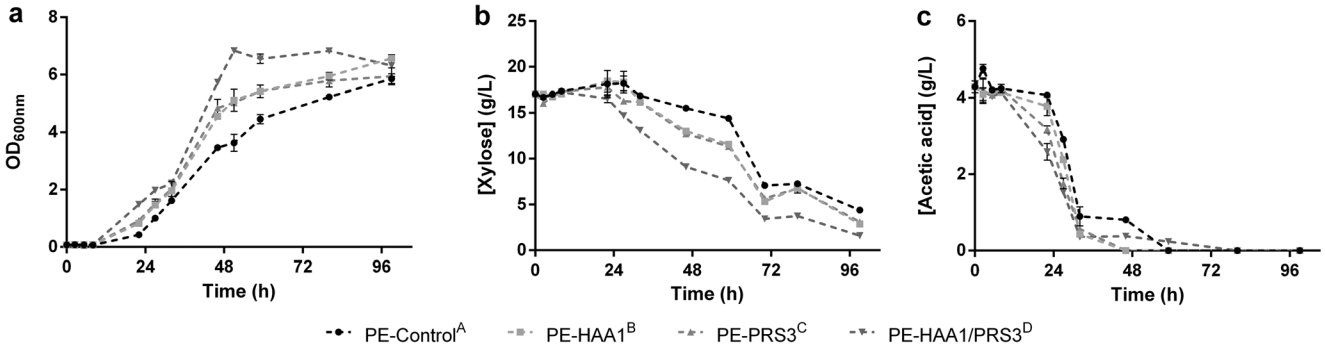

Fig. 2 Growth profile (**a**) and xylose (**b**) and acetic acid (**c**) concentrations of *S. cerevisiae* PE-2ΔGRE3 transformants (control and overexpressing *HAA1* and/or *PRS3*) in YPX medium with 4 g/L of acetic acid in flasks. Data represents the average ± SEM from two biological replicates. **a** A,B**; A,C**; A,D***; B,D**; C,D**. **b** A,B*; A,C*; A,D***; B,D**; C,D**. **c** A,B*; A,C**; A,D**

comparison with the other strains (~ 5 h) and reaching the maximum biomass concentration sooner (Fig. 3a, $P < 0.001$). The strains PE-HAA1 and PE-PRS3 also exhibited faster growth profiles than the control strains (Fig. 3a, $P < 0.05$), with lag phases of ca. 22 h, while the control strain had a lag phase longer than 24 h. Accordingly, the strain overexpressing *HAA1* and *PRS3* showed a faster glucose consumption rate, depleting the sugar in 25 h (Fig. 3b, $P < 0.0001$), while the other strains only started consuming glucose after 25 h (Fig. 3b). The strains PE-HAA1 and PE-PRS3 depleted the sugar in the medium at 46 and 53 h, respectively, significantly faster than the control strain (73 h) (Fig. 3b, $P < 0.001$). Considering the acetic acid, its concentration suffered only slight variations during these growths, reaching ca. 3.4 g/L at 144 h for all the strains growth (Fig. 3c).

Effect of *HAA1* and/or *PRS3* overexpression on the cell wall structural integrity of acetic acid grown cells

Differences in cell wall structural integrity of the strains overexpressing *HAA1* and/or *PRS3* in comparison with the control strain, after their growth under acetic acid stress, were assessed using a lyticase sensitivity assay (Fig. 4). Strains overexpressing *PRS3* and *HAA1* or both were more resistant than the control strain to digestion with lyticase after growth in the presence of acetic acid, both on xylose (Fig. 4a, $P < 0.001$) and on glucose (Fig. 4b, $P < 0.001$) media.

Effect of *HAA1* and/or *PRS3* overexpression on the fermentation performance of *S. cerevisiae* PE-2ΔGRE3 in *P. tomentosa* hydrolysate

The improved sugar consumption of the *S. cerevisiae* strains overexpressing *HAA1* and/or *PRS3* was also demonstrated in terms of fermentation performance in a non-detoxified *P. tomentosa* hydrolysate without nutrient supplementation and containing both glucose and xylose (Fig. 5, Table 2). Paulownia is a fast-growing hardwood. and its hydrolysate, obtained at high severity conditions ($S_0 = 4.43$), was selected due to its high inhibitory load being considered convenient for testing the effect of *HAA1* and/or *PRS3* overexpression in extreme demanding conditions. High harshness condition of hydrothermal treatment was necessary to improve the enzymatic susceptibility of cellulose, yielding a hydrolysate composed by hemicellulose-derived compounds such as xylose, acetic acid, furfural and hydroxymethylfurfural (Dominguez et al. 2017). Chemical composition of solid and liquid (hydrolysate) phases is shown in Table 3. After pretreatment, 94.7 and 99.1% of glucan and Klason lignin were recovered in solid phase, respectively. On the other hand, 48.5% of xylan in

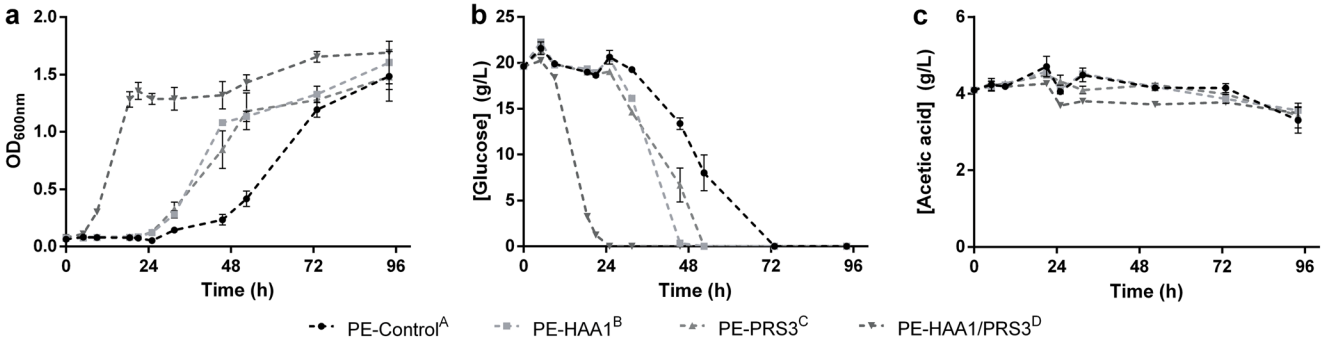

Fig. 3 Growth profile (**a**) and glucose (**b**) and acetic acid (**c**) concentrations of *S. cerevisiae* PE-2ΔGRE3 transformants (control and overexpressing *HAA1* and/or *PRS3*) in YPD medium with 4 g/L of acetic acid in flasks. Data represents the average ± SEM from two biological replicates. **a** A,B**; A,C*; A,D****; B,D***; C,D***. **b** A,B***; A,C***; A,D****; B,D****; C,D****

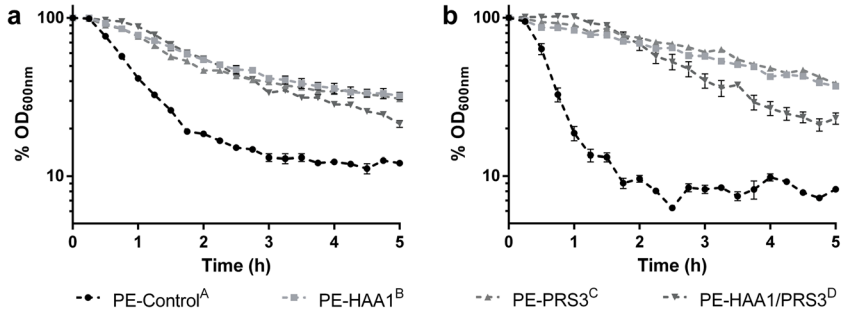

Fig. 4 Profiles of lyticase effect on the cell wall of *S. cerevisiae* PE-2ΔGRE3 transformants (control and overexpressing *HAA1* and/or *PRS3*) after growth on YPX medium with 4 g/L of acetic acid (**a**) and in YPD medium with 4 g/L of acetic acid (**b**). Data represents the average ± SEM from two biological replicates. **a** A,B***; A,C***; A,D***. **b** A,B***; A,C***; A,D***

raw material was solubilized as xylose. As consequence of pretreatment severity, 11.5% of potential xylose in raw material was dehydrated into furfural, achieving a concentration of 1.96 g/L. Moreover, acetyl groups were also hydrolysed into acetic acid increasing the concentration in the hydrolysate up to 5.84 g/L. *P. tomentosa* hydrolysate (Table 3) was used as fermentation media for the assays shown in Fig. 5.

In the first 72 h of fermentation, glucose was almost depleted by the recombinant PE-2ΔGRE3 strains (Fig. 5a, c), while xylose consumption was lower (less than 26%, Fig. 5a, d). It is noteworthy that, in these 72 h, the *HAA1* and/or *PRS3* overexpressing strains exhibited similar profiles (Fig. 5) and fermentation performances were generally superior to the performance of the control strain (Fig. 5). Specifically, the strains overexpressing *HAA1* and/or *PRS3* were capable of glucose depletion (> 97.6%) in 72 h, while the control strain consumed only approximately 85% of the initial glucose at this fermentation time (Fig. 5c, $P < 0.001$). Regarding xylose consumption at this point, the strains overexpressing the *HAA1* gene (PE-HAA1 and PE-HAA1/PRS3) exhibited higher sugar consumption than the control strain (Fig. 5d, $P < 0.05$), while the solely overexpression of *PRS3* showed no significant improvements at this level (Fig. 5d). Additionally, overexpression of *HAA1* and/or *PRS3* significantly improved ethanol production (Fig. 5b, $P < 0.01$). After glucose depletion, xylose was slowly consumed by all strains (at 168 h of fermentation the xylose concentration in the media was 4.1–5.1 g/L, Table 2); nevertheless, the maximum ethanol concentration was achieved at 72 h (for the overexpressing strains) and at 96 h (for the control strain) followed by a gradual decrease until the end of the experimental time frame (Fig. S1 in the supplementary material). Additionally, high levels of glycerol

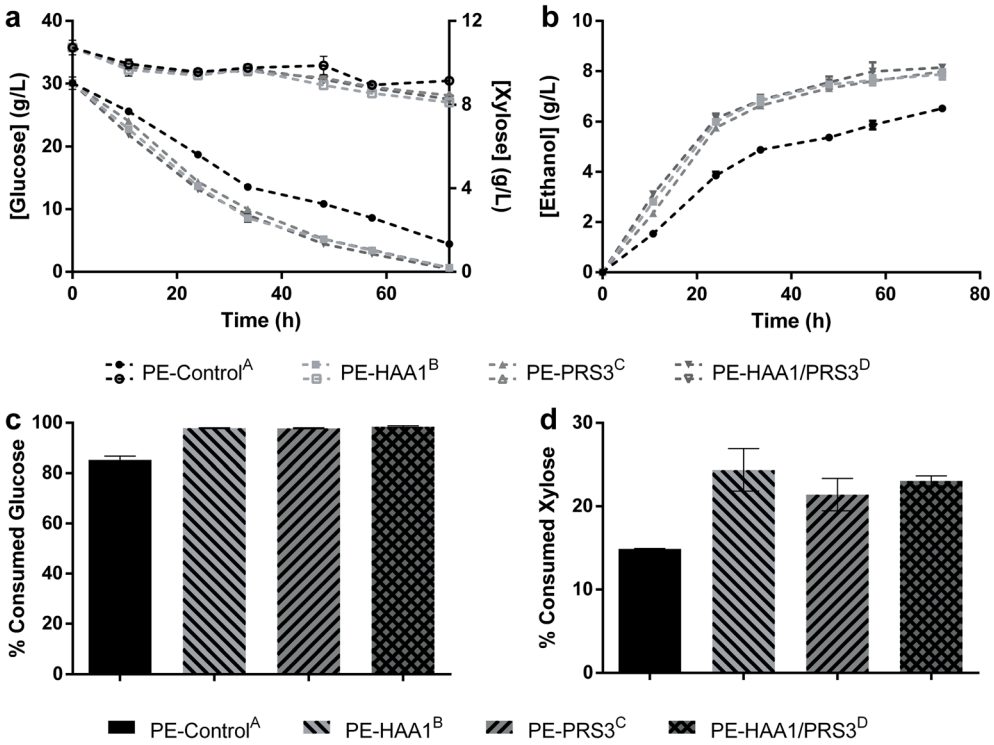

Fig. 5 Fermentation profiles (**a**, **b**) and parameters (**c**, **d**) of *S. cerevisiae* PE-2ΔGRE3 transformants (control and overexpressing *HAA1* and/or *PRS3*) in *P. tomentosa* hydrolysate in flasks with cotton stoppers. Glucose (**a**, filled symbols), xylose (**a**, open symbols) and ethanol (**b**) concentrations (g/L) during the first 72 h of fermentation. Percentage of consumed glucose (**c**) and xylose (**d**) at 72 h of fermentation. **a** Glucose: A,B***; A,C**; A,D***. **b** A,B**; A,C**; A,D**. **c** A,B***; A,C***; A,D***. **d** A,B*; A,D*

Table 2 Main results of fermentation performance of *S. cerevisiae* PE-2ΔGRE3 transformants (control and overexpressing *HAA1* and/or *PRS3*) in *P. tomentosa* hydrolysates. $Xylose_{tf}$ is the xylose concentration at final time (168 h). $Ethanol_{max}$ is the maximal ethanol concentration reached during the experimental timeframe. DCW is the dry cell weight at final time (168 h). $Glycerol_{tf}$ is the glycerol concentration at final time (168 h). $Xylitol_{tf}$ is the xylitol concentration at final time (168 h). $Xylose_{tf}$: A,B**; A,C*; B,D**; C,D**. $Ethanol_{max}$: A,B*; A,C*; A,D*

Strain	$Xylose_{tf}$ (g/L)	$Ethanol_{max}$ (g/L)	DCW (g/L)	$Glycerol_{tf}$ (g/L)	$Xylitol_{tf}$ (g/L)
PE-Control[A]	4.76 ± 0.00	7.06 ± 0.08	3.87 ± 0.02	5.95 ± 0.09	0.45 ± 0.00
PE-HAA1[B]	4.14 ± 0.04	7.88 ± 0.21	4.36 ± 0.18	5.92 ± 0.06	0.52 ± 0.04
PE-PRS3[C]	4.25 ± 0.02	7.98 ± 0.02	4.74 ± 0.22	5.71 ± 0.05	0.50 ± 0.02
PE-HAA1/PRS3[D]	5.09 ± 0.03	8.15 ± 0.08	4.14 ± 0.37	5.58 ± 0.08	0.48 ± 0.03

accumulation and biomass production were detected at the end of the fermentation for all strains, while xylitol concentration was minimal (Table 2).

Discussion

During the last few years, the tolerance mechanisms and responses of *S. cerevisiae* towards the presence of acetic acid have been extensively studied (Dong et al. 2017; Geng et al. 2017; Giannattasio et al. 2013; Lee et al. 2015; Mira et al. 2010a; Sànchez et al. 2013; Swinnen et al. 2017). Nevertheless, genetic engineering strategies to counteract the toxic effects of this weak acid on yeast have been performed mainly on laboratory strains (Hasunuma et al. 2016; Sakihama et al. 2015; Swinnen et al. 2017; Tanaka et al. 2012; Zhang et al. 2015), with only one work with industrial yeast (Inaba et al. 2013). On the other hand, these genetic engineering strategies have been mostly evaluated using glucose as carbon source (Inaba et al. 2013; Swinnen et al. 2017; Tanaka et al. 2012; Zhang et al. 2015), while the works also focusing in xylose consumption used laboratorial background strains (Sakihama et al. 2015; Chen et al. 2016). In this work, the effect of overexpressing *HAA1* and/or *PRS3* in the growth of an industrial *S. cerevisiae* strain was evaluated, both in xylose or glucose media supplemented with acetic acid.

Table 3 Composition of *Paulownia tomentosa* wood concerning raw material, pretreated biomass and hydrolysate (after neutralization with $CaCO_3$)

P. tomentosa composition	g/100 g raw material, oven dry basis
Glucan	39.7 ± 0.9
Xylan	14.7 ± 0.5
Acetyl groups	3.29 ± 0.03
Klason lignin	21.9 ± 0.6
Pretreated *P. tomentosa*	g/100 g raw material, oven dry basis
Glucan	37.6 ± 0.3
Xylan	1.50 ± 0.09
Klason lignin	21.7 ± 0.8
P. tomentosa hydrolysate	g/L
Xylose	11.3 ± 0.3
Acetic acid	5.84 ± 0.01
Furfural	1.96 ± 0.01
HMF	0.719 ± 0.028

The presence of acetic acid (4 g/L at pH 4) in xylose medium severely affected growth capacity in all strains. Nevertheless, the toxic effect of acetic acid was in part overcome by the overexpression of *HAA1* and/or *PRS3*, mainly in the PE-HAA1/PRS3 strain. It was also observed that in the absence of inhibition, the overexpression of *HAA1* and/or *PRS3* is not advantageous to yeast growth from xylose. In fact, the positive effect of *HAA1* overexpression in growth from xylose was previously reported in a laboratorial *S. cerevisiae* strain (Sakihama et al. 2015). Nevertheless, the laboratory strain overexpressing *HAA1* constructed by Sakihama et al. (2015) was unable to grow aerobically in the presence of acetic acid concentrations superior to 2.4 g/L, while the *HAA1*-overexpressing strain described in this work is able to cope with 4 g/L of acetic acid in similar conditions (YPX medium, aerobic, 30 °C and same OD_{600nm} inoculum). Moreover, overexpression of *PRS3* was associated, in this study and for the first time, with an increase in the yeast capacity to utilize xylose in the presence of acetic acid.

Besides their advantage in xylose consumption, the strains overexpressing *HAA1* and/or *PRS3* were also capable of a faster decrease of acetic acid concentration from the medium. In the absence of glucose or other repressive carbon source, acetic acid may be used as carbon source by *S. cerevisiae*: after entering the yeast cell, acetate is converted into acetyl-CoA, which subsequently acts as an intermediator of the anaplerotic glyoxylate cycle and activator of gluconeogenesis (Chen et al. 2016; Gancedo and Gancedo 1986). It must be highlighted that xylose consumption only started after the decrease of acetic acid concentration in the medium. When the strains examined were grown in glucose medium, *HAA1* overexpression resulted in an improved yeast resistance to the presence of acetic acid, as previously reported (Inaba et al. 2013; Swinnen et al. 2017; Tanaka et al. 2012). Additionally, *PRS3* overexpression also led to an increased

yeast tolerance towards this weak acid. Actually, *PRS3* overexpression in *S. cerevisiae* PE-2 strain has already been described to be advantageous for yeast fermentative performance in glucose media containing lignocellulosic-derived inhibitors, including acetic acid, and hypothesized to play a role in NADH regeneration (Cunha et al. 2015). Considering the hurdles involving the cofactor imbalance in yeast expressing the XR/XDH pathway for xylose consumption (Almeida et al. 2011), the overexpression of *PRS3* may, in this way, pose another advantage for yeast growth from this sugar. Furthermore, as observed in xylose medium, the strain PE-HAA1/PRS3 exhibited faster growth and glucose consumption than the other single gene overexpressing strains, highlighting the advantage of simultaneously overexpress *HAA1* and *PRS3* to increase yeast tolerance towards acetic acid. Differently from what was observed in xylose medium, acetic acid concentration remained approximately constant during growth in glucose, most likely due to the catabolite repression mediated by this sugar. In addition, biomass production for all strains was superior in xylose medium than in glucose media, which may be explained by a possible major redirection of carbon to ethanol in glucose media (Crabtree effect) and/or by the fact that in xylose medium the yeast also used acetic acid as carbon source.

Considering the significant role played by *PRS3* in the remodelling of the cell wall (Wang et al. 2004) and the reported effect of *HAA1* as regulator of the expression of cell wall proteins, such as *SPI1* and *YGP1* (Fernandes et al. 2005; Simões et al. 2006), it was hypothesized that the positive effect observed for strains overexpressing these genes in response to acetic acid may be related with an increased cell wall integrity in acetic acid-challenged cells. In fact, the superior resistance of the strains overexpressing *HAA1* and/or *PRS3* towards lyticase, after exposure to acetic acid, clearly indicates that they have less susceptibility or a more efficient response to the effects of this weak acid, displaying lower cell wall defects than the control strain. Essentially, lyticase hydrolyses glucans from the yeast cell wall, specifically poly-β(1→3)-glucoses, meaning that the overexpressing strains were capable of maintaining an higher 1,3-beta-glucan synthase activity, in comparison with the control strain, when exposed to acetic acid. In yeast, the 1,3-beta-glucan synthase activity is performed by a Fks1p and Gsc2p complex, where both *FKS1* and *GSC2* are positively regulated by *RLM1* (Belén Sanz et al. 2012; Garcia et al. 2004; Jung and Levin 1999; Terashima et al. 2000), one of the end points of the CWI pathway. *RLM1* is potentially regulated by *HAA1* (according to the transcriptional regulatory associations gathered in the Yeastract database); additionally, the phosphorylation of Rlm1p is impaired by the deletion of *PRS3* (Wang et al. 2004). Considering this, these results raise the hypothesis that the higher robustness of the cell wall of the overexpressing strains, after exposure to acetic acid, is mediated by activation of the CWI pathway by the overexpression of *HAA1* and/or *PRS3*. These are preliminary steps to understand the mechanisms by which the overexpression of *HAA1* and/or *PRS3* results in a greater acetic acid tolerance. Nevertheless, the simultaneous overexpression of both these genes did not result in an additive protective effect against lyticase, suggesting that the increased resistance of the PE-HAA1/PRS3 strain towards acetic acid (in comparison with the single overexpression of each gene) is not linked to an improved cell wall integrity, and this subject should be focused on further studies.

The effect of overexpressing *HAA1* and/or *PRS3* was validated in fermentation of a non-detoxified hardwood hydrolysate with a high acetic acid content (5.84 g/L) containing both glucose and xylose. It is known that, in mixed sugar fermentations, *S. cerevisiae* will preferentially consume glucose (Kim et al. 2012), and accordingly, in the first 72 h of fermentation, glucose was almost depleted while xylose consumption was slower, with the strains being incapable of completely consuming this sugar during the timeframe of the experiment. This low xylose consumption rate may be due not only to the high inhibitory load of the Paulownia hydrolysate but also to the absence of nutrient supplementation in this media. In fact, previous results with the control strain in less inhibitory xylose-containing hydrolysates and with nutrient supplementation showed 84–93% of xylose consumption within 72 h of fermentation (Costa et al. 2017). Nevertheless, the overexpression of *HAA1* and/or *PRS3* significantly increased glucose and xylose consumption at an initial stage of fermentation, resulting in the attainment of higher concentrations of ethanol. However, after glucose depletion in the medium, ethanol concentration decreased due to the aeration effect. The presence of oxygen leads to an accumulation of glycerol, a by-product of ethanol fermentation from xylose (Matsushika et al. 2013), and also favours biomass production, resulting in lower ethanol yields. On the other hand, xylitol accumulation was minimal. In fact, xylitol production has been associated with two main sources: the unspecific aldose reductase coded by *GRE3* which reduces xylose to xylitol using NADPH as cofactor (Träff et al. 2001) and a cofactor imbalance between the xylose reductase and xylitol dehydrogenase enzymes (Kim et al. 2013). The low xylitol production observed in this work is a clear benefit of the deletion of the *GRE3* gene in the PE-2 strain, as previously reported (Romaní et al. 2015; Costa et al. 2017). Additionally the use of a mutated xylose reductase (N272D, with higher specificity for NADH), is reported to diminish the cofactor imbalance caused in the first two steps of xylose metabolism (Runquist et al. 2010), further contributing to lower xylitol accumulation. Considering these results and despite the non-optimized fermentation conditions, it is clear the benefit of overexpressing *HAA1* simultaneously with *PRS3* in industrial yeast adaptation to non-detoxified lignocellulosic hydrolysates.

Substantial developments have been lately achieved in attaining effective xylose-consuming *S. cerevisiae* strains. The number of genes already overexpressed in xylose consuming yeast has probably hindered the study of tolerance gene overexpression under these conditions. *HAA1* overexpression has been extensively described to have positive effects in yeast resistance to acetic acid. However, to the extent of our knowledge, it is the first time that *PRS3* overexpression has been reported to increase yeast tolerance towards acetic acid, both in glucose and xylose media. Additionally, the overexpressing strains were also found to exhibit a more robust cell wall after exposure to acetic acid, relating *HAA1* and/or *PRS3* expression with the modulation of CWI pathway. The overexpressing results presented hereby together with the elucidation of the role played by *HAA1* and *PRS3* in improving cell wall robustness under acetic acid stress prove that there is room to improve yeast performance in demanding conditions even in industrial strains, paving the way to a more efficient and economically viable usage of *S. cerevisiae* in industrial biotechnological processes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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