Food Microbiology 78 (2019) 201–210

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Chitinases and thaumatin-like proteins in Sauvignon Blanc and Chardonnay musts during alcoholic fermentation

Thulile Ndlovu[a,c], Astrid Buica[b], Florian F. Bauer[a,*]

[a] Institute for Wine Biotechnology, University of Stellenbosch, Matieland, South Africa
[b] Department of Viticulture and Oenology, University of Stellenbosch, Matieland, South Africa
[c] Post-harvest and Agro-Processing Technologies (PHATs), Agricultural Research Council (ARC), Infruitec-Nietvoorbij, Stellenbosch, South Africa

ARTICLE INFO

Keywords:
Chitinases
Thaumatin-like proteins
Alcoholic fermentation
Protein haze

ABSTRACT

Protein precipitation, also referred to as protein instability, may lead to haziness in bottled wines and result in significant commercial losses. To avoid problems of this nature, fining finished wines with clay (bentonite) is the most commonly applied methodology. However, bentonite fining reduces yield and may affect wine quality. Protein haze has been primarily linked to grape pathogenesis-related proteins, in particular chitinases and thaumatin-like proteins. To better understand the persistence of these proteins during fermentation, reverse phase chromatography was used to monitor the evolution of total grape proteins as well as of chitinases and thaumatin-like proteins during alcoholic fermentation. The data confirm a previously reported significant decrease in total protein content during fermentation. This reduction in total protein levels was observed throughout fermentation, and was affected by factors such as fermentation temperature, yeast strain or grape cultivar. However, significant changes in the concentration of free chitinases were observed in a yeast strain-dependent manner. The data thus confirm the correlation between the levels of yeast cell wall chitin and changes in chitinase concentration, and suggest that it is primarily the amount of lateral chitin, and not the chitin in bud scars, that is responsible for this activity.

1. Introduction

Proteins present in wines are primarily derived from the grapes and to a lesser degree from the microorganisms that are present during fermentation. Previous studies have shown that the main fractions of berry-derived proteins in wine belong to two classes of pathogenesis related (PR) proteins, namely chitinases (PR-3) and thaumatin-like (TLP) proteins (PR-5). These proteins have been shown to be resilient to the winemaking process (Ferreira et al., 2002; Falconer et al., 2010; Marangon et al., 2010) and are present in varying concentrations ranging from undetectable levels to over 700 mg/L depending on cultivar, region, vintage, and viticultural and enological practices (Ferreira et al., 2002; Feuillat, 2003; Pocock and Waters, 2006; Waters et al., 2005; Vincenzi et al., 2011). Conversely, yeast proteins have been reported to contribute in only minor ways to total wine proteins (Fukui and Yokotsuka, 2003).

Although highly variable, wine proteins can play important roles in enological processing and can impact the organoleptic quality of the final product. Protein precipitation in finished wines may lead to cloudy or hazy wines, and most finished wines undergo a process of “fining” using a clay called bentonite to reduce protein concentration, an expensive process which can affect organoleptic wine quality negatively. Indeed, wine proteins have been reported to directly affect mouth feel and wine sweetness (Kwon, 2004; Vincenzi et al., 2011), although contributing only minimally to its nutritive value (Batista et al., 2009).

Protein haze has primarily been attributed to grape PR proteins, mainly thaumatin-like proteins (TLPs) and chitinases (Falconer et al., 2010; Marangon et al., 2010; Chagas et al., 2018). Several recent studies strongly indicate that grape chitinases are the major protein family responsible for haze formation (Vincenzi et al., 2005; Marangon et al., 2011; Sauvage et al., 2010; Tian et al., 2017), while TLPs are important contributors to the phenomenon (Esteruelas et al., 2009; Vincenzi et al., 2011; Chagas et al., 2018). Variations between some of these published data sets can likely be explained by the presence of different isomers of these PR proteins (Falconer et al., 2010) and by the fact that haze is modulated by several non-proteinaceous wine components such as metal ions, polysaccharides, and phenolic compounds (Mesquita et al., 2001; Pocock et al., 2007).

Despite extensive research on protein haze formation, there is limited information on the development and fate of grape PR proteins during the winemaking process. Moreover, the few published studies have reported contradictory findings. A decrease in total protein

* Corresponding author.
E-mail address: fb2@sun.ac.za (F.F. Bauer).

https://doi.org/10.1016/j.fm.2018.10.018
Received 11 June 2018; Received in revised form 29 October 2018; Accepted 31 October 2018
Available online 01 November 2018

Table 1
Grape must oenological parameters.

Grape must	Sugar	Yeast Assimilable Nitrogen (YAN)	pH
Sauvignon Blanc	22.6°B	184.8 mg/L	3.51
Chardonnay	20.5°B	274.4 mg/L	3.69

Table 2
Separation gradient.

Time (min)	% B
0	20.2
4.67	43.3
10.67	49
11.33	55
20	66.3
21.33	66.3
21.5	20.2

concentration has been reported by Hsu et al. (1987), Dizy and Bisson (2000) and Manteau et al. (2003), while Bayly and Berg (1967), Fukui and Yokotsuka (2003), and Vincenzi et al. (2011) observed an increase in soluble protein content after alcoholic fermentation. Most of these studies compared must protein content and wine protein content after alcoholic fermentation, but did not evaluate the evolution of protein quantity and protein type during the fermentation process.

In this study, reverse phase high pressure liquid chromatography (RP-HPLC) as described by Van Sluyter et al. (2009) and Marangon et al. (2009), was used to determine grape juice and wine proteins and monitor chitinases and thaumatin-like protein during alcoholic fermentation. RP chromatography takes advantage of the hydrophobicity of proteins promoting separation based on hydrophobic interactions between immobilized hydrophobic ligands (in this case alkyl chains, C8) and nonpolar regions on the surface of proteins. This is the first study to monitor the grape pathogenesis-related proteins during wine making and yeast cell wall chitin changes (including both total and lateral chitin) during wine making.

2. Materials and methods

2.1. Fermentation

Fermentations were carried out in biological quadruplicates at 15 °C and 30 °C in 2 L bottles (1.7 L working volume) without agitation and fitted with air traps. Commercial *S. cerevisiae* wine strains BM45, EC1118 and *S. paradoxus* strains RO88 and P01 146, at an initial 10^6 cells/mL inoculum were used to ferment Sauvignon Blanc and Chardonnay grape musts to dryness (Table 1). These strains were chosen because of their similar fermentation characteristics but differences in cell wall chitin levels (Ndlovu et al., 2018). Residual glucose and fructose concentrations at the end of all fermentations were less than 5 g/L as measured using a D-glucose/D-fructose enzymatic kit (Amersham Biosciences).

2.2. Protein concentration

Total soluble protein in fermenting must was measured on day 0 (2 h post yeast inoculation), 2, 7, and at the end of alcoholic fermentation on day 14. Total protein concentration from the supernatant was quantified using the potassium dodecyl sulphate, KDS protocol described by Fusi et al. (2010), Rowe et al. (2010) and Gazzola et al. (2015). The supernatant was centrifuged at 3250 g for 5 min to remove yeast cells. Sodium dodecyl sulphate (SDS), 10% (w/v) was added to wine samples to give a 0.2% (w/v) final concentration. After heating samples in boiling water bath for 5 min, 2 M KCl was added to reach a final concentration of 400 mM. Samples were mixed and incubated at 4 °C for 45 min. KDS–protein pellets (KDS pellets) were recovered by centrifugation at 16,060 × *g* for 15 min at 4 °C. Total protein content was then determined using the BCA kit (Smith et al., 1985), with BSA as a standard.

2.3. Heat stability test

The heat stability of wine samples was determined as described by Pocock and Waters (2006) with all measurements made in triplicate at the end of alcoholic fermentation. Briefly, the assay was carried out by first centrifuging fermented Chardonnay and Sauvignon Blanc musts at 3250 g for 5 min to remove yeast cells. After taking absorbance readings at 520 nm, the wine samples were heated at 80 °C for 2 h and then cooled to 4 °C for 16 h. Absorbance at 520 nm was measured again after room temperature acclimatization for 30 min and the calculated difference in absorbance before and after heating of the wine sample was regarded as haze.

2.4. Calcofluor White staining and fluorescence microscopy

Fermenting musts were sampled on day 2, 5, 8 and 14 at the end of alcoholic fermentation. About 200 µL of the fermentation samples were centrifuged and the cells washed with phosphate buffered saline (PBS,

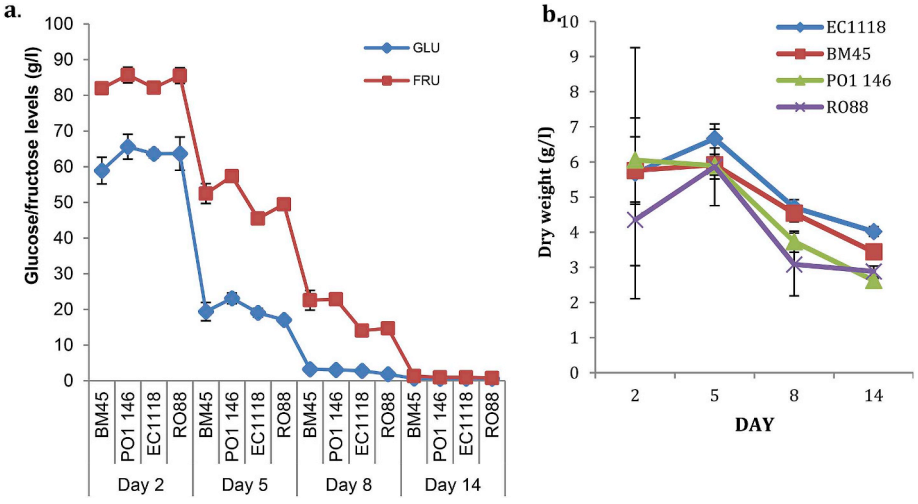


Fig. 1. a. Evolution of glucose and fructose levels during fermentation of S. Blanc wines fermented using EC1118, RO88, P01 146 and BM45 yeast strains b. Dry weight of yeast cells sampled during the alcoholic fermentation.

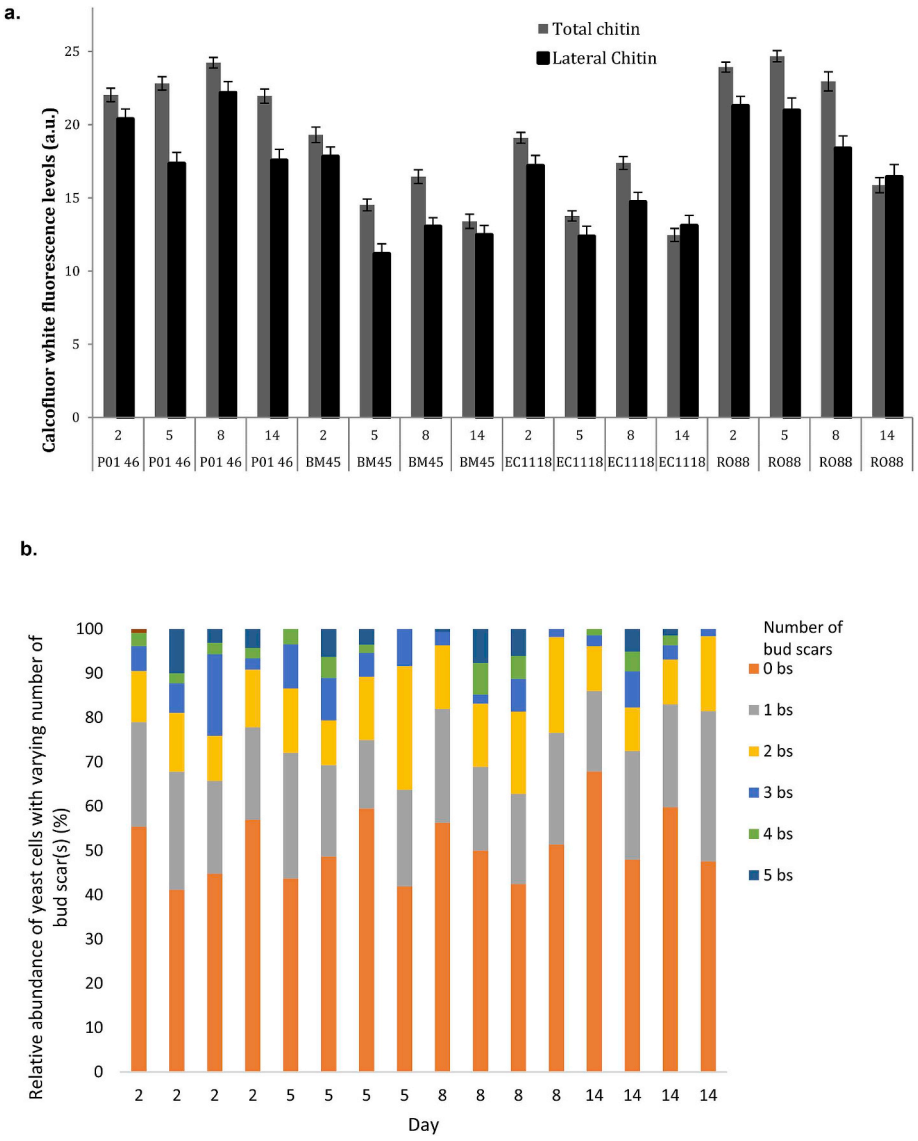


Fig. 2. a: Fluorescence levels quantified from whole yeast cell (total chitin) and lateral chitin levels excluding fluorescence on the bud scar for the days 2, 5, 8 and 14, using Carl Zeiss Confocal LSM 780 Elyra S1 with Super-Resolution Structured Illumination Microscopy (SR-SIM). **b:** Relative abundance of yeast cells with varying number of bud scars expressed as percentage of the total number of cells used in the study for the quantification of both total and lateral chitin sampled during fermentation on day 0, 2, 5, 8 and 14.

pH 7.4; Na_2HPO_4). Cells were then stained with 10 µL Calcofluor White after addition of 10 µL 10% KOH, following the manufacturer's instructions (Sigma-Aldrich). Z sectioning image acquisition was performed on Carl Zeiss Confocal LSM 780 Elyra S1 with Super-Resolution Structured Illumination Microscopy (SR-SIM) platform. The excitation laser used was the violet laser at 407 nm and the emission filter used was the Pacific Blue channel with a 450/40 nm band pass filter. Images were processed and background-subtracted using the Zeiss Zen lite® 2011 software and presented in a maximum intensity projection. For the quantification of chitin levels, Zeiss Zen lite® 2011 software was used to quantify fluorescence from individual cells. Boundaries were traced around each yeast cell (Sheng et al., 2016) and traces were performed in Zeiss Zen lite® 2011 software. Consequently, fluorescence levels were then quantified from whole yeast cell, which is referred to as total chitin in this study.

Lateral chitin levels on the other hand was established by excluding the fluorescence from bud scars achieved by selecting a region of interest (ROI) which excluded the bud scars before quantifying the fluorescence under ROI using Zeiss Zen lite® 2011 software. The background was excluded from the fluorescence of individual cell measured. Images were taken from yeast cells sampled during fermentation on day 2, 5, 8 and 14. From each image taken, all full individual cells were quantified. A minimum of about 1000 yeast cells was analyzed under the microscope for each sampling point. The data obtained from quantifying the fluorescence from individual cells followed a normal distribution curve similar to that observed when flow cytometry is used to quantify chitin fluorescence from a yeast population (images not shown).

2.5. Reverse phase high pressure liquid chromatography (RP-HPLC)

During alcoholic fermentations, 50 mL samples were taken from 2 h post inoculation, day 1, 2, 3, 4, 5, 7, 10 and at the end of fermentation. Samples were centrifuged at 3250 g for 5 min to remove cells after which the supernatant was concentrated using Amicon Ultra-15 Centrifugal Filter columns (Millipore™, Merck, Ireland) with a cut-off of 10 kDa to approximately 2 mL volume. The concentrated samples were freeze dried and re-dissolved in 2 mL solution containing 10% acetonitrile and 0.1% trifluoro-acetic (TFA) acid before being filtered through 0.45 µm filter membrane.

Table 3
a: Total number of cells used to quantify total chitin and lateral chitin levels excluding bud scar fluorescence; **b:** Shows strain effect ($P < 0.05$) on chitin levels; **c:** Effect of day on total chitin levels and lateral chitin levels; **d:** The correlation coefficient between total chitin, lateral chitin and grape chitinase levels at the end of alcoholic fermentation. The P values indicate the level of significance.

Strain	Day	Total chitin (n)	Lateral Chitin (n)
P01 46	2	225	233
P01 46	5	227	261
P01 46	8	363	327
P01 46	14	204	432
BM45	2	170	180
BM45	5	295	335
BM45	8	214	196
BM45	14	205	294
EC1118	2	345	353
EC1118	5	379	427
EC1118	8	246	231
EC1118	14	237	277
RO88	2	396	469
RO88	5	326	179
RO88	8	112	111
RO88	14	179	124

Day	Total chitin	Lateral chitin (mean)
D14	15.78[a]	14.51[a]
D5	18.52[b]	14.85[a]
D8	20.50[c]	16.33[b]
D2	21.40[d]	19.20[c]

Strain	Total chitin	Lateral chitin (mean)
BM45	15.65[a]	13.69[a]
EC1118	15.77[a]	14.36[a]
RO88	22.64[b]	19.41[b]
P01 146	22.98[b]	19.58[b]

	Sauvignon Blanc 15 °C	Sauvignon Blanc 30 °C	Chardonnay 15 °C	Chardonnay 30 °C
Total chitin	−0.80	−0.76	−0.62	−0.74
P value	*0.00*	*0.00*	*0.01*	*0.00*
Lateral Chitin	−0.91	−0.93	−0.82	−0.93
P value	*0.00*	*0.00*	*0.00*	*0.00*
Bud scar chitin	−0.49	−0.39	−0.25	−0.35
P value	*0.06*	*0.08*	*0.24*	*0.10*

Protein composition and concentration of grape and wine fractions was determined by RP-HPLC with a C8 column on an Agilent 1260 system according to a method modified from Marangon et al. (2009, 2011) for higher elution flow rate. Samples (25 μL) were loaded onto a C8 column (4.6 × 250 mm, Vydac 208TP54) fitted with a C8 guard column kit (Vydac 208GK54, 4.6 × 5 mm) held at 35 °C. Mobile phase A consisted of 0.1% TFA in water and mobile phase B 0.1% TFA in acetonitrile. The separation gradient is given in Table 2. Total run time was 25.5 min including re-equilibration for 4 min and flow rate was 1.5 mL/min. Elution was monitored by absorbance at 210, 220 and 280 nm. Protein identity was assigned by comparison of their retention times to those of purified grape PR proteins found in the literature (Van Sluyter et al., 2009). Peaks with a retention time between 9 and 12 min were assigned to the TLP classes, while peaks eluted between 18.5 and 24.5 min were designated to be the chitinases. Protein quantification was through integration of peak area as described by Marangon et al. (2009).

2.6. Yeast cell wall binding assay-using GFP-chitinase

Grape chitinase tagged to GFP was used to carry out yeast cell wall binding assays. The GFP-chitinase construction and the yeast cell wall binding assays used in the study are as described in Ndlovu et al. (2018). The yeast cells were cultured in YPD broth and washed with 1 x PBS buffer (pH 7.4). Subsequently, 100 μL of crude GFP tagged chitinase protein were added to the cells suspended in 200 μL BPS buffer. These were then incubated for 2 h at either room temperature or 37 °C with shacking after which the cells were centrifuged at 3250 g, washed twice with PBS buffer and visualized under a confocal fluorescence microscopy. The excitation laser used was the solid state sapphire laser at wavelength of 488 nm and the emission filters used was the FITC channel, with a 502 long pass and 530/30 band pass filter.

3. Results

3.1. Microscopic monitoring of yeast cells and fermentation kinetics

Chardonnay and Sauvignon Blanc grape must fermentations were carried out using two *S. cerevisiae* commercial wine yeast strains, BM45, EC1118, and two *S. paradoxus* yeast strains that have been isolated from wine and previously described, P01 146 and RO88. Fermentation progress was monitored by following glucose and fructose consumption and yeast biomass measured as dry weight (Fig. 1). All strains fermented with broadly similar speed, and all sugar had been consumed by day 14. The total biomass of all strains was similar at all stages of fermentation.

Fig. 2a shows changes in yeast cell wall chitin quantified using fluorescence confocal microscopic images, while Table 3a shows the statistical analysis and the number of yeast cells that were analyzed for the data shown in the figure. Table 3b and c shows the statistical analysis evaluating the effect of yeast strain and fermentation day on both total and lateral chitin levels. There were significant yeast strain differences ($p < 0.05$) in both total and lateral chitin levels with EC1118 and BM45 having lower chitin levels when compared to RO88 and P01 146 observed for all the sampling time points. In all yeast strains, day 2 of alcoholic fermentation showed the highest total chitin and lateral chitin levels ($p < 0.05$) (Table 3c). Cell wall chitin levels decreased in all strains during alcoholic fermentation, with the lowest chitin levels observed at the end of alcoholic fermentation. Fig. 2b shows the percentage amount of cells that were analyzed for the data shown in Fig. 2a. The calculated percentages are based on the bud scar number displayed per yeast cell used in the study for the quantification of lateral chitin levels. The data shows that more than 63% of cells analyzed in the study had no bud scar or had one bud scar sampled throughout the fermentation while fewer cells (1%) presented more than 5 bud scars on their cell walls.

The data confirm our previous study (Ndlovu et al., 2018) that the *S. paradoxus* strains RO88 and P01 146 contain significantly higher levels of cell wall chitin when compared to *S. cerevisiae* EC1118 and BM45. In the previous study, flow cytometry was used to quantify yeast cell wall chitin. The data in the current study, generated using confocal microscopy, however, showed a smaller difference between the strains. This may be due to inherent limitations of the methodologies. Flow cytometry likely provides a better perspective on total chitin, whereas confocal microscopy allows a better evaluation of chitin distribution. The microscopy likely underestimates total chitin since measurement of chitin in chitin-dense areas such as bud scars reaches saturation.

3.2. Total protein and pathogenesis related (PR) protein evolution during alcoholic fermentation

Total protein was monitored during alcoholic fermentation. Fig. 3 shows that a similar pattern in the evolution of total protein was observed for all the yeast strains and in both grape must cultivars. At the start of fermentation, the Sauvignon Blanc grape must contained about half less total protein than the Chardonnay grape must, with a total protein concentration of 1,10 mg/mL for Sauvignon Blanc and 0,58 mg/mL for Chardonnay. In both juices, protein levels decreased significantly ($p < 0.05$) with a similar rate in both juices, and protein levels were reduced by 82%–90% of total protein at the end of

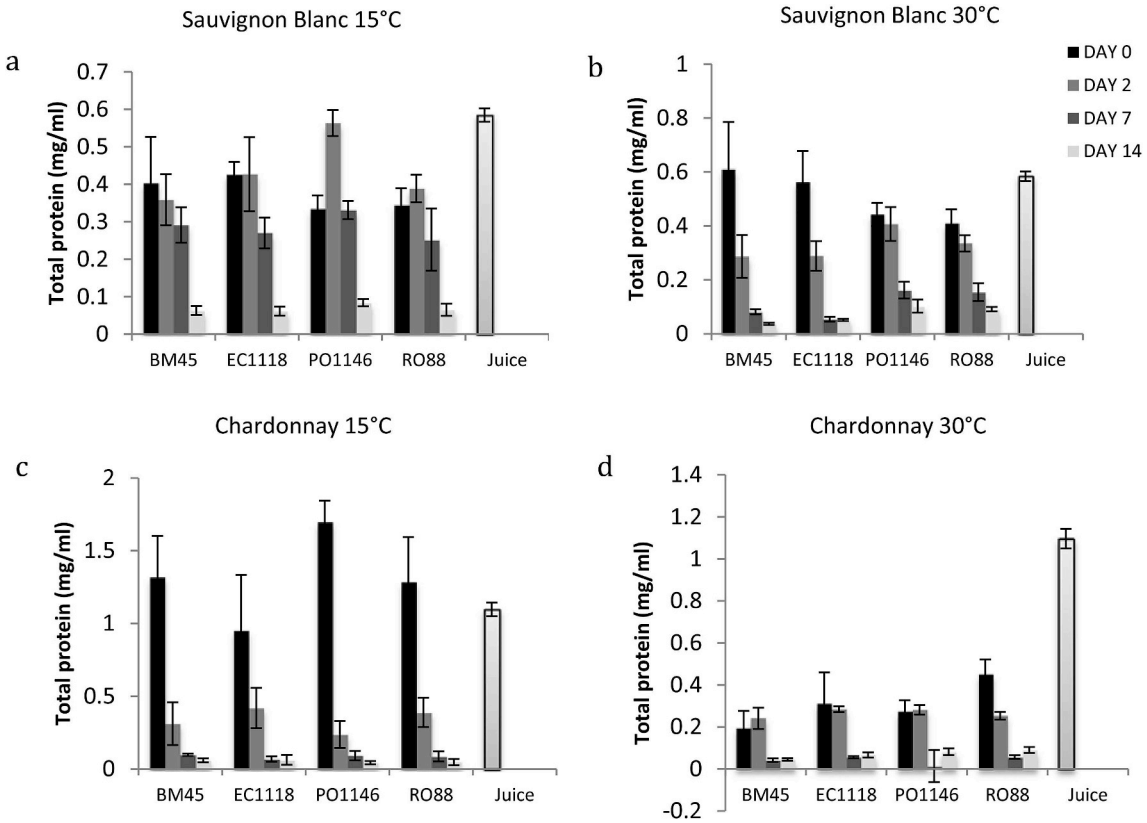


Fig. 3. Total protein levels during alcoholic fermentation quantified using the BCA kit. a. and b. Sauvignon Blanc at 15 °C and 30 °C respectively. c. and d. Chardonnay at 15 °C and 30 °C respectively. Day 14 represents the end of alcoholic fermentation.

fermentation. Temperature had an effect on the amount of quantified soluble protein as lower total protein levels ($P < 0.05$) were observed at 30 °C when compared to 15 °C for both grape cultivars.

Total chitinases and TLP content was three fold higher in Sauvignon Blanc than in Chardonnay grape must. Furthermore, total TLP levels that were significantly higher ($p < 0.05$) than chitinase levels (Figs. 4 and 5).

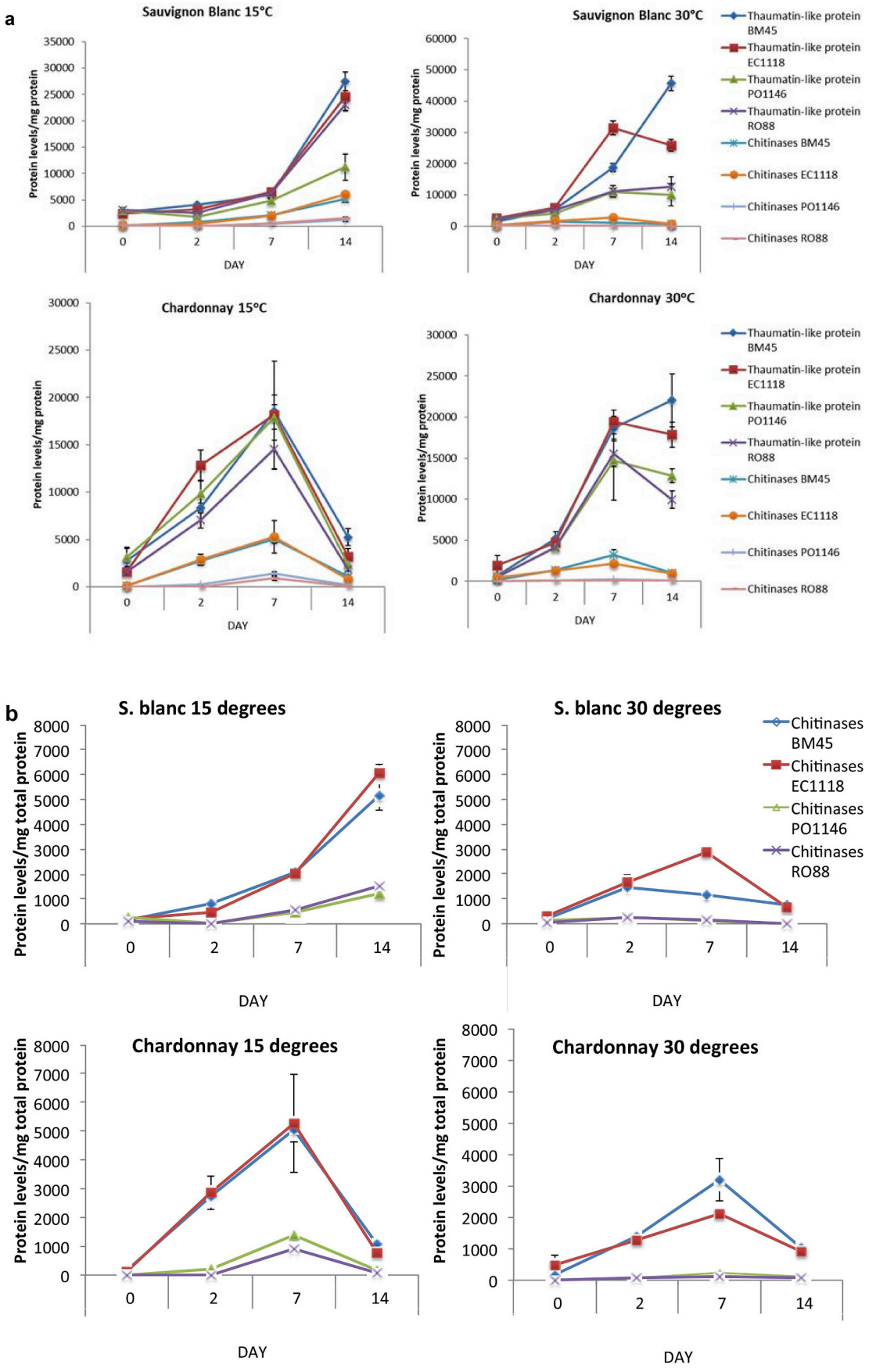
To better represent the evolution of these PR proteins, the data are shown both as a proportion of total protein concentration expressed in Absorbance units (AU 280 nm) HPLC/mg total protein (BCA assay) (Fig. 4) or as absolute concentrations (Fig. 5). As shown in Fig. 4a and b, a significant increase in the proportion of PR proteins in the fermenting musts was observed for both TLPs and chitinases up to day 7, while in 15 °C Sauvignon Blanc the levels remained high even at the end of fermentation. This indeed reflects the relatively higher stability of these protein families. TLPs showed significantly higher enrichment than chitinases.

Chitinase levels (Fig. 4b) in both Sauvignon Blanc and Chardonnay followed similar trends with higher levels being observed at 15 °C when compared to 30 °C. However, significant differences were observed between fermenting yeast strains: Musts inoculated with RO88 and P01 146 yeast strains showed lower levels ($p < 0.05$) of chitinases at the end of alcoholic fermentation, which were about 75–100% less in comparison to Sauvignon blanc and Chardonnay wines fermented with BM45 and EC1118 at both temperatures 15 and 30 °C. Moreover, Sauvignon Blanc must fermented using P01 146 resulted in significantly lower TLPs at 15 and 30 °C compared to other musts fermented using yeast strains.

When the changes in TLP levels during fermentation is expressed per volume (Fig. 5), the levels of TLPs showed minor fluctuations, but did not vary considerably from the initial levels sampled irrespective of the strain used for fermentation. The fluctuations and slight increase in TLP and chitinase concentration in the Sauvignon Blanc fermentations observed in Fig. 5 could be due to TLP and chitinases that are released from aggregates or from yeast cell walls. Such a release might be linked to increasing ethanol concentrations, which would be consistent with previously reported hydrophobic interactions impacting the binding of the TLP and chitinases (Marangon et al., 2010; van Sleuter et al., 2015). In addition, no distinguishable pattern was observed in both Sauvignon Blanc and Chardonnay fermenting grape musts and at both 15 and 30 °C.

When the evolution of chitinase levels during fermentation is expressed per volume (Fig. 5) significant strain differences are observed with around 70% lower chitinase levels for RO88 and P01 146 fermented musts when compared to those fermented with the two *S. cerevisiae* strains. Sauvignon Blanc fermenting grape musts had higher chitinase levels relative to Chardonnay fermenting grape musts at both temperatures, 15 and 30 °C. Remarkably, no temperature impact was observed on the evolution and levels of chitinases.

Moreover, it was observed that the chitinase levels were not significantly correlated with the bud scar chitin levels but with the lateral chitin levels found on the yeast cell wall. The percentage bud scar chitin levels relative to the lateral chitin levels ranged from 8% up to 30% during fermentation while at the end of fermentation the percentage bud scar chitin levels relative to the lateral chitin levels ranged from 0 to 25%. The correlation between wine chitinases and yeast cell wall chitin levels were calculated at the end of alcoholic fermentation for bud scar, lateral and total chitin fluorescence chitin levels as shown in Table 3d for Chardonnay fermented at 15 °C and 30 °C and in Sauvignon Blanc at 15 °C and 30 °C. A significant negative correlation coefficient ($P < 0.05$) was observed for the lateral chitin levels and the grape chitinase levels, indicating the most likely involvement of lateral chitin in haze prevention. Furthermore, fluorescence microscopy of yeast cells with *Escherichia coli*-produced GFP-tagged grape chitinase did not highlight bud scars, but appeared distributed around the cells and in patches (Fig. 7).



(caption on next page)

Fig. 4. a: Effect of temperature on TLP and chitinases in Sauvignon Blanc and Chardonnay must normalized to total protein expressed in Absorbance units (AU 280 nm) HPLC/mg total protein (BCA assay). The KDS protocol was used to extract the protein, which was then quantified using BCA kit from fermenting musts (Fusi et al., 2010; Rowe et al., 2010; Gazzola et al., 2015). **b**: Cultivar and temperature influence on chitinase levels for Chardonnay and Sauvignon Blanc musts under fermentation and wines normalized to total protein levels expressed in Absorbance units (AU 280 nm) HPLC/mg total protein (BCA assay). Total protein was quantified using BCA kit after protein extraction from fermenting musts and wine using the KDS protocol (Fusi et al., 2010; Rowe et al., 2010; Gazzola et al., 2015).

3.3. Protein stability of wines fermented with different yeast strains

To assess whether the differences in protein content, thaumatin-like proteins and chitinases had an impact on haze formation potential at the end of fermentation, the fermented wines were subjected to the standard heat test. Significant differences ($p < 0.05$) were observed between the haze formation potential of the wines fermented with different strains (Fig. 6). Chardonnay wines fermented with RO88 and P01 146 had the least ($p < 0.05$) haze formation potential when compared to *S. cerevisiae* BM45 and EC1118. The yeast strains RO88 and P01 146 reduced wine protein haze by ≈ 60% relative to the Chardonnay wines fermented with BM45 and EC1118. A similar trend was observed in Sauvignon Blanc wines, where a ≈71% difference was observed when wines were fermented with P01 146 and RO88 compared to BM45 and EC1118 yeast strains (data not shown).

4. Discussion

Chitinases and thaumatin-like proteins are important grape PR proteins which persist during fermentation and may influence wine clarity. This study monitored their evolution during wine making and the impact of factors such as fermentation temperature, grape cultivar, and yeast strain on these protein levels.

As we had previously reported that cell wall chitin of yeast can bind grape chitinases and consequently reduce chitinase concentrations in a cell wall chitin dependent manner (Ndlovu et al., 2018), we also evaluated the changes in yeast cell wall chitin levels during wine fermentation using confocal imaging. In line with those previous data, S. paradoxus RO88 and P01 146 had higher chitin levels than *S. cerevisiae* EC1118 and BM45. The new data however show that the cell wall chitin levels changed during fermentation, with a general reduction observed for all the strains throughout the fermentation process ranging from 0.34 to 34% with P01 146, BM45, EC1118 and RO88 showing a 0.34, 30.76, 34.66 and 33.68% reduction, respectively. These values were calculated based on the difference between the day 2 and day 14 total mean chitin levels (Fig. 2a). The reason for this reduction requires further investigation, but suggests that fermenting yeast reduce chitin biosynthesis. This reduction in total chitin levels could also partly be explained by the observation that birth scars containing less chitin than bud scars, expand and fade with age (Powell et al., 2003).

Our study concurs with other research findings showing that chitin levels can vary greatly between different yeast species and within one species, depending on the growth conditions (Aguilar-Uscanga and Francois, 2003). These authors observed that the cell wall polysaccharides content could vary by more than 50% depending on the nature of the carbon source, nitrogen limitation, pH, temperature and aeration, and with the mode of cell cultivation (shake flasks *vs* controlled fermenters).

This is the first study to show differences in yeast cell wall chitin levels (both lateral and total cell wall chitin levels) during fermentations. Differences between total yeast cell wall chitin fluorescence levels and lateral chitin (chitin present on the yeast cell wall excluding bud scars) were observed indicating the levels of chitin present in the bud scars (Powell et al., 2003). Under normal growth conditions, the deposition of chitin in the lateral walls after cytokinesis has been reported to be about 0.1–0.2% excluding the bud scar(s) and up to 2% including the bud scars/dry weight % cell (Shaw et al., 1991). However, it has also been highlighted that chitin synthesis is activated as part of a cell wall salvage mechanism (Liesche et al., 2015) and the levels in the lateral walls of those cells may become as high as 20% of the wall dry weight higher than that of the bud scars proportion (Garcia-Rodriguez et al., 2000). In this study, the proportion of lateral chitin was significantly higher than that of the bud scar chitin. This could be reflective of the different methods used to quantify the lateral and bud scar chin, as in this study Calcofluor white fluorescence was used for quantification, while in previous studies chemical and enzymatic hydrolysis of the cell wall could have resulted in an underestimation of lateral chitin as a result of partial hydrolysis during the extraction procedure (Klis et al., 2002). However, the percentage of the lateral chitin and bud scar chitin to dry cell wall weight were not determined in the current study.

In this study, we used confocal fluorescence microscopy, while several previous studies had used epifluorescence microscopy to quantify chitin levels in fungi (Henry-Stanley et al., 2004; Hoch et al., 2005; Lin and Heitman; 2005; Walker et al., 2008). Liesche et al. (2015) reported that flow cytometry data were consistent with epifluorescence microscopy observations. In our data, the magnitude in differences between the yeast strains are lower than when the Flow cytometer was used (Ndlovu et al., 2018), suggesting that confocal microscopy may be less accurate for absolute quantification. It is also possible that differences in growth medium could be partially responsible for these differences, since grape must was used and not YPD broth.

Total soluble protein levels were observed to decrease during fermentation. Such decreases may be due to denaturation or proteolytic activity (Bayl and Berg, 1967; Murphey et al., 1989; Dambrouck et al., 2003; Manteau et al., 2003; Vincenzi et al., 2011). The findings are in line with data from Ferenczi (1996), Kluba et al. (1978), Lamikanra and Inyang (1988) and Pueyo et al. (1993) who observed a reduction in wine protein levels ranging in the magnitude of 60%–82%. In the current study, reductions in total protein was in the magnitude of 82%–90% from the initial grape must levels. The variation observed in the total protein concentrations (Fig. 3), day 0, post-inoculation could have been a result of the differential beginning of the fermentation process. Moreover, lower protein concentration was observed in wines fermented at 30 °C compared to 15 °C.

Supporting previous findings (Ndlovu et al., 2018), strain differences impacted chitinase levels, with the wines fermented by *S. cerevisiae* showing 75% higher chitinase levels when compared to wines fermented with S. paradoxus strains. On the other hand, thaumatin-like protein levels did not follow the same patterns during fermentation. No significant differences between BM45, EC1118 and RO88 yeast strains were observed while the musts fermented with P01 146 resulted in lower thaumatin-like protein levels. This result of reduced TLP proteins requires further investigation. The results demonstrate that yeast strains impact chitinase but not thaumatin-like protein levels significantly (Fig. 5). We do not have an explanation for the fluctuations in the levels of thaumatin-like protein during the fermentation process, which requires further investigation. It is possible that factors such as ethanol concentrations impact the release of TLPs from aggregates and/or affect hydrophobic interactions. In addition, homogenization of the 2 L fermentation bottle with an air trap is difficult, and might have contributed to the variations observed. However, the thaumatin-like protein levels remained within a reasonable range, and endpoint concentrations are similar to the starting point.

Previous studies had reported on decreases in chitinase levels during fermentation (Van Sluyter et al., Manteau et al. (2003). These authors hypothesized that the grape chitinases were bound to the cell wall of *Saccharomyces cerevisiae* yeast (yeast lees) after alcoholic fermentation and on the cell wall of *Oenococcus oeni* bacteria after malolactic fermentation (Manteau et al., 2003), a hypothesis that is confirmed by our

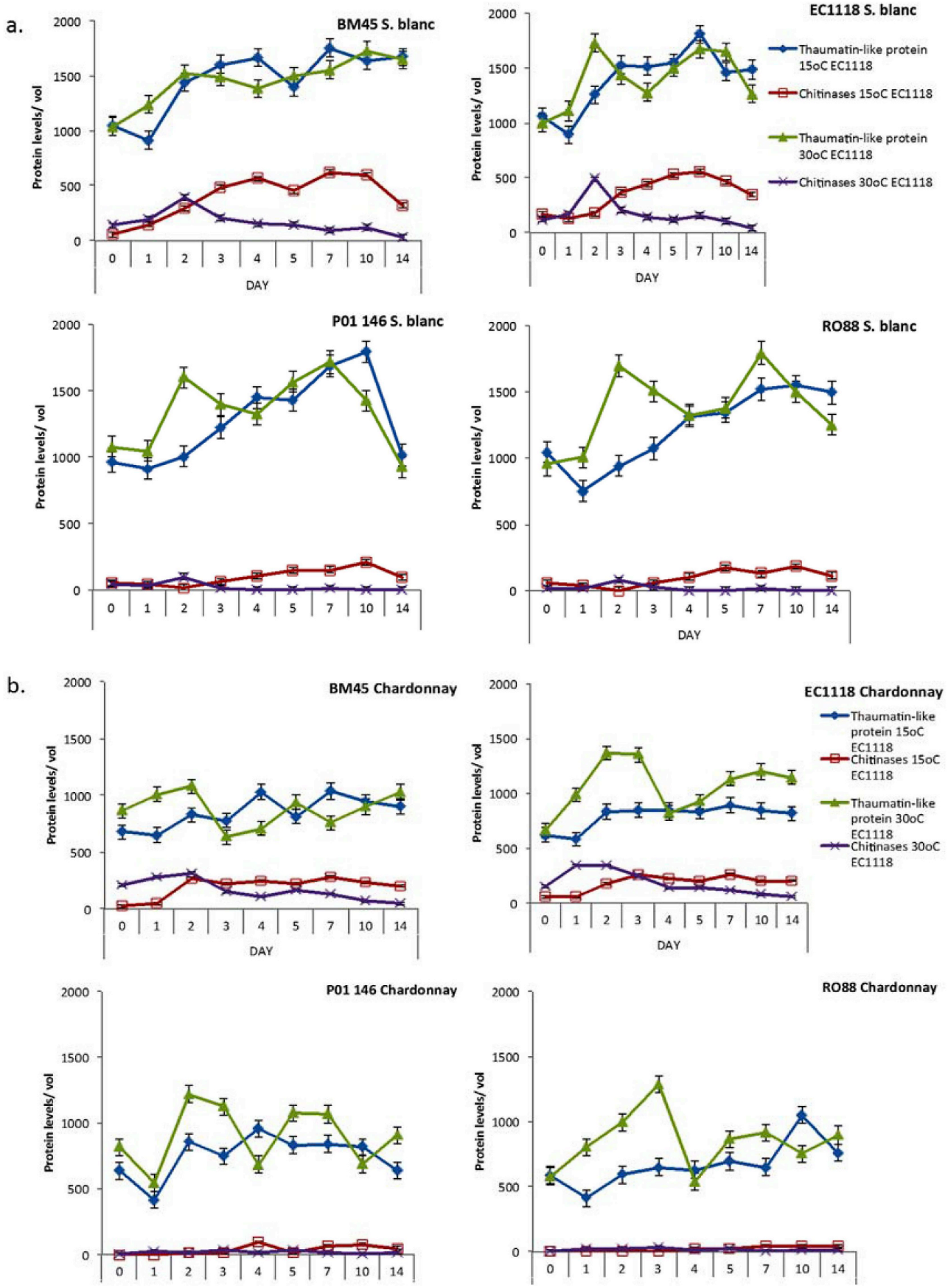


Fig. 5. a: Strain influence on TLP and chitinases protein in Sauvignon Blanc must during and at the end of alcoholic fermentation **5b**: Chitinase and thaumatin-like protein levels during alcoholic fermentation as quantified using Reverse phase-high performance liquid chromatography in chardonnay must at both 15 °C and 30 °C.

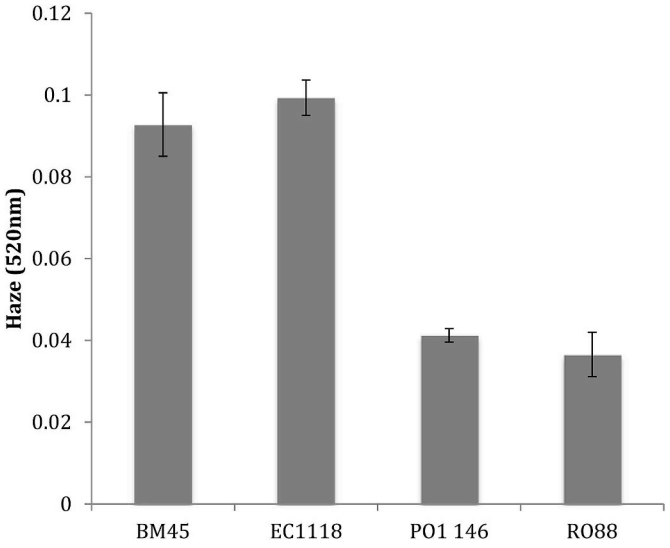


Fig. 6. Haze formation potential in Chardonnay wines fermented at 15 °C measured using the heat test method (Pocock and Waters, 2006).

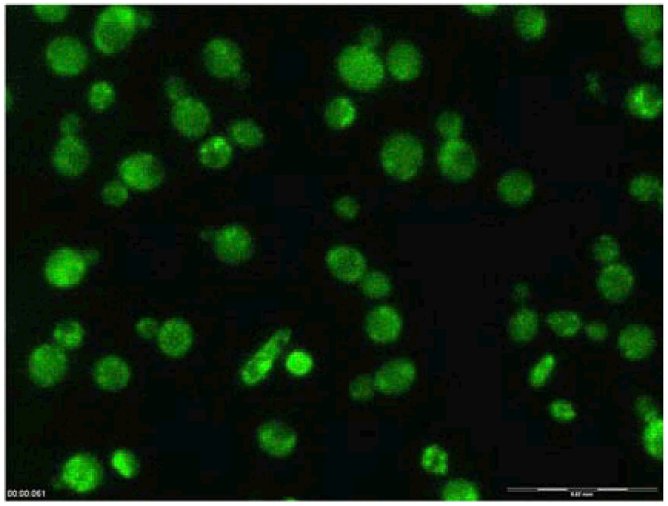


Fig. 7. The image shows the distribution of GFP-chitinase (Ndlovu et al., 2018) around the RO88 yeast strain viewed under a confocal (SR-SIM superresolution) fluorescence microscope. (Scale bar = 10 μm).

observations.

The lateral chitin levels of the strains RO88 and P01 146 were significantly higher than that of the strains BM45 and EC1118, and were associated with the lower chitinase levels observed at the end of alcoholic fermentation. Taken together with the images from fluoresence microscopy of GFP-chitinase labelled cells, the data suggest that grape chitinases primarily bind to lateral cell wall chitin, since no bud scars are highlighted in these images. This would not be surprising since bud scars chitin is tightly packed and may not be accessible to the chitinases.

In addition, the results substantiate previous studies (Falconer et al., 2010; Marangon et al., 2011; Tian et al., 2017) that grape chitinases are primarily responsible for wine haze, and thus removal of such proteins reduces wine protein haze formation efficiently. These studies indeed show the presence of thaumatin-like protein in the insoluble fraction of wines but indicate no measurable impact on turbidity although other studies reports contradictory findings (Falconer et al., 2010; Marangon et al., 2010; Chagas et al., 2018).

Although Sauvignon Blanc grape must contained only about half of the total proteins of the Chardonnay grape must, Sauvignon Blanc chitinases and thaumatin-like proteins were both about 3 times higher than the levels observed in Chardonnay grape must. This study therefore also highlights that there is no correlation between total protein and protein haze formation (Bayly and Berg, 1967), but that protein haze is indeed directly correlated to the presence or levels of PR proteins, and in particular grape chitinases (Tian et al., 2017) since higher haze levels were observed in Sauvignon Blanc fermented wines compared to Chardonnay wines.

5. Conclusion

In summary, our data show variation in total and PR-protein levels during real wine fermentation. The data also suggest that chitin concentrations in yeast cell walls vary during the process, and that yeast cells specifically remove chitinases from fermenting must primarily through binding of chitinases to the lateral chitin in the cell walls. As reported previously (Ndlovu et al., 2018), yeast cells with higher chitin levels are efficient in reducing haze formation. However, commercial *S. cerevisiae* wine yeast strains do not show significant variation in cell wall chitin levels (data not shown), suggesting that yeast selection or breeding programs should consider targeting increased levels of cell wall chitin as a selection criteria.

Acknowledgements

This work was financially supported by the SARChI grant from the National Research Foundation of South Africa. Confocal fluorescence microscopy experiments were carried out at the Central analytical facility (CAF, Stellenbosch University) with the help of Mrs. L. Engelbrecht, Ms. Dumisile Lumkwana and Ms. Rozanne Adams.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2018.10.018.

References

Aguilar-Uscanga, B., François, J.M., 2003. A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. Lett. Appl. Microbiol. 37, 268–274.
Batista, L., Monteiro, S., Loureiro, V.B., Teixeira, A.R., 2009. Ferreira R.B. The complexity of protein haze formation in wines. Food Chem. 112, 169–177.
Bayly, F.C., Berg, H.W., 1967. Grape and wine proteins of white wine varietals. Am. J. Enol. Vitic. 24, 18–32.
Chagas, R., Laia, C.A.T., Ferreira, R.B., Ferreira, L.M., 2018. Sulfur dioxide induced aggregation of wine thaumatin-like proteins: role of disulfide bonds. Food Chem. 259, 166–174.
Dambrouck, T., Marchal-Delahaut, L., Parmentier, M., Maujena, A., Jeandet, P., 2003. Immunodetection of proteins from grapes and yeast in white wine. J. Agric. Food Chem. 51, 2727–2732.
Dizy, M., Bisson, L.F., 2000. Proteolytic activity of yeast strains during grape juice fermentation. Am. J. Enol. Vitic. 51, 155–167.
Esteruelas, M., Poinsaut, P., Sieczkowski, N., Manteau, S., Fort, M.F., Canals, J.M., Zamora, F., 2009. Characterization of natural haze protein in sauvignon white wine. Food Chem. 113, 28–35.
Falconer, R.J., Marangon, M., Van Sluyter, S.C., Neilson, K.A., Chan, C., Waters, E.J., 2010. Thermal stability of thaumatin-like protein, chitinase, and invertase isolated from Sauvignon Blanc and Semillon juice and their role in haze formation in wine. J. Agric. Food Chem. 58, 975–980.
Feuillat, M., 2003. Yeast macromolecules: origin, composition, and enological interest. Am. J. Enol. Vitic. 54, 211–213.
Ferenczi, S., 1996. Etude des proteines et des substances azotées. Leur évolution au cours des traitements oenologiques. Conditions de la stabilité proteique des vins. Bull. OIV 39, 313–336.
Ferreira, R.B., Picarra-Pereira, M.A., Monteiro, S., Loureiro, V.B., Teixeira, A.R., 2002. The wine proteins. Trends Food Sci. Technol. 12, 230–239.
Fukui, M., Yokotsuka, K., 2003. Content and origin of protein in white and red wines: changes during fermentation and maturation. Am. J. Enol. Vitic. 54, 178–188.
Fusi, M., Mainente, F., Rizzi, C., Zoccatelli, G., Simonato, B., 2010. Wine hazing: a predictive assay based on protein and glycoprotein independent recovery and quantification. Food Contr. 21, 830–834.
Garcia-Rodriguez, L.J., Duran, A., Roncero, C., 2000. Calcofluor antifungal action depends on chitin and a functional high-osmolarity glycerol response (HOG) pathway:

evidence for a physiological role of the *Saccharomyces cerevisiae* HOG pathway under noninducing conditions. J. Bacteriol. 182, 2428–2437.
Gazzola, D., Vincenzi, S., Pasini, G., Lomolino, G., Curioni, A., 2015. Advantages of the KDS/BCA assay over the bradford assay for protein quantification in white wine and grape juice. Am. J. Enol. Vitic. 66, 227–233.
Henry-Stanley, M.J., Garni, R.M., Wells, C.L., 2004. Adaptation of FUN-1 and Calcofluor white stains to assess the ability of viable and nonviable yeast to adhere to and be internalized by cultured mammalian cells. J. Microbiol. Methods 59, 289–292.
Hoch, H.C., Galvani, C.D., Szarowski, D.H., Turner, J.N., 2005. Two new fluorescent dyes applicable for visualization of fungal cell walls. Mycologia 97, 580–588.
Hsu, J.C., Heatherbell, D.A., Flores, J.H., Watson, B.T., 1987. Heat-unstable proteins in grape juice and wine. II. Characterization and removal by ultrafiltration. Am. J. Enol. Vitic. 38, 17–22.
Klis, F.M., Mol, P., Hellingwerf, K., Brul, S., 2002. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 26, 239–256.
Kluba, R.M., Mattick, L.R., Hackler, R.L., 1978. Changes in concentration of free and total amino acids of several native American grape cultivars during fermentation. Am. J. Enol. Vitic. 29, 181–186.
Kwon, S.W., 2004. Profiling of soluble proteins in wine by nano-high-performance liquid chromatography/tandem mass spectrometry. J. Agric. Food Chem. 52, 7258–7263.
Lamikanra, O., Inyang, D.I., 1988. Temperature influence on muscadine wine protein characteristics. Am. J. Enol. Vitic. 39, 113–116.
Liesche, J., Marek, M., Günther-Pomorski, T., 2015. Cell wall staining with Trypan blue enables quantitative analysis of morphological changes in yeast cells. Front. Microbiol. 6, 107.
Lin, X., Heitman, J., 2005. Chlamydospore formation during hyphal growth in *Cryptococcus neoformans*. Eukaryot. Cell 4, 1746–1754.
Manteau, S., Lambert, B., Jeandet, P., Legendre, L., 2003. Changes in chitinase and thaumatin-like pathogenesis-related proteins of grape berries during the champagne winemaking process. Am. J. Enol. Vitic. 54, 267–272.
Marangon, M., van Sluyter, S.C., Haynes, P.A., Waters, E.J., 2009. Grape and wine proteins: their fractionation by hydrophobic interaction chromatography and identification by chromatographic and proteomic analysis. J. Agric. Food Chem. 57, 4415–4425.
Marangon, M., Van Sluyter, S.C., Chan, C., Waters, E.J., Falconer, R.J., 2010. The different behaviours of thaumatin-like proteins and chitinases during white wine haze formation. In: Aust. Wine Industry Tech. Conference, Adelaide, Austria.
Marangon, M., Van Sluyter, S.C., Neilson, K.A., Chan, C., Haynes, P.A., Waters, E.J., Falconer, R.J., 2011. Roles of grape thaumatin-like protein and chitinase in white wine haze formation. J. Agric. Food Chem. 57, 733–740.
Mesquita, P.R., Picarra-Pereira, M.A., Monteiro, S., Loureiro, V.B., Teixeira, A.R., Ferreira, R.B., 2001. Effect of wine composition on protein stability. Am. J. Enol. Vitic. 52, 324–330.
Murphey, J.M., Spayd, S.E., Powers, J.R., 1989. Effect of grape maturation on soluble protein characteristics of gewürztraminer and white riesling juice and wine. Am. J. Enol. Vitic. 40, 199–207.
Ndlovu, T., Divol, B., Bauer, F.F., 2018. Yeast cell wall chitin reduces wine haze formation. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.00668-18.
Pocock, K.F., Alexande, G.M., Hayasaka, Y., Jones, P.R., Waters, E.J., 2007. Sulfate a candidate for the missing essential factor that is required for the formation of protein haze in white wine. J. Agric. Food Chem. 55, 1799–1807.
Pocock, K.F., Waters, E.J., 2006. Protein haze in bottled white wines: how well do stability tests and bentonite fining trials predict haze formation during storage and transport? Aust. J. Grape Wine Res. 12, 212–220.
Powell, C.D., Quain, D.E., Smart, K.A., 2003. Chitin scar breaks in aged *Saccharomyces cerevisiae*. Microbiol. 149, 3129–3137.
Pueyo, E., Dizy, M., Polo, M.C., 1993. Varietal differentiation of must and wines by means of protein fraction. Am. J. Enol. Vitic. 44, 255–260.
Rowe, J.D., Harbertson, J.F., Osborne, J.P., Freitag, M., Lim, J., Bakalinsky, A.T., 2010. Systematic identification of yeast proteins extracted into model wine during aging on the yeast lees. J. Agric. Food Chem. 58, 2337–2346.
Sauvage, F.-X., Bach, B., Moutounet, M., Vernhet, A., 2010. Proteins in white wines: thermo-sensitivity and differential adsorption by bentonite. Food Chem. 118, 26–34.
Shaw, J.A., Mol, P.C., Bowers, B., Silverman, S.J., Valdivieso, M.H., Duran, A., Cabib, E., 1991. The function of chitin synthases 2 and 3 in the *Saccharomyces cerevisiae* cell cycle. J. Cell Biol. 114, 111–123.
Sheng, H., Stauffer, W., Lim, H.N., 2016. Systematic and general method for quantifying localization in microscopy images. Biol. Open. 5, 1882–1893.
Smith, P.,K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.
Tian, B., Harrison, R., Morton, J., Jaspers, M., Hodge, S., Grose, C., Trought, M., 2017. Extraction of pathogenesis-related proteins and phenolics in sauvignon blanc as affected by grape harvesting and processing conditions. Molecules 12, 22. https://doi.org/10.3390/molecules22071164. E1164.
Van Sluyter, S.C., Marangon, M., Stranks, S.D., Neilson, K.A., Hayasaka, Y., Haynes, P.A., Menz, R.I., Waters, E.J., 2009. Two-step purification of pathogenesis-related proteins from grape juice and crystallization of thaumatin-like proteins. J. Agric. Food Chem. 57, 11376–11382.
Van Sluyter, S.C., McRae, J.M., Falconer, R.J., Smith, P.A., Bacic, A., Waters, E.J., Marangon, M., 2015. Wine protein haze: mechanisms of formation and advances in prevention. J. Agric. Food Chem. 63, 4020–4030.
Vincenzi, S., Polesani, M., Curioni, A., 2005. Removal of specific protein components by chitin enhances protein stability in a white wine. Am. J. Enol. Vitic. 56, 246–254.
Vincenzi, S., Marangon, M., Tolin, S., Curioni, A., 2011. Protein evolution during the early stages of white winemaking and its relations with wine stability. Aust. J. Grape Wine Res. 17, 20–27.
Walker, L.A., Munro, C.A., de Bruijn, I., Lenardon, M.D., McKinnon, A., Gow, N.A., 2008. Stimulation of chitin synthesis rescues Candida albicans from echinocandins. PLoS Pathog. 4, e1000040.
Waters, E.J., Alexander, G., Muhlack, R., Pocock, K.F., Colby, C., O'Neill, B.K., Høj, P.B., Jones, P., 2005. Preventing protein haze in bottled white wine. Aust. J. Grape Wine Res. 11, 215–225.