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Inactivation of *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae* in dry and sweet wines by high hydrostatic pressure

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ABSTRACT

The aim of the research was to investigate a potential application of high hydrostatic pressure (HHP) for reduction/elimination of *Brettanomyces bruxellensis* and *Saccharomyces cerevisiae* in wines. Dry red wine was inoculated with *B. bruxellensis* and sweet white wine was inoculated with *S. cerevisiae* yeast. Both wines were treated by HHP under 100 and 200 MPa for 1, 3, 5, 15 and 25 min. The culturability was determined immediately after the treatment and again after 30, 60 and 90 days of storage. The phenolic content and chromatic characteristics were evaluated spectrophotometrically immediately after the treatment and after 90 days of storage. The culturability of *B. bruxellensis* was not confirmed immediately after the most invasive treatment (200 MPa for 15 and 25 min). With the same parameters, only a decrease in the culturability of *S. cerevisiae* was observed. During storage, opposing results were observed for two yeasts treated with 200 MPa for 15 and 25 min: there was a complete reduction of *S. cerevisiae* in the wine treated, but the culturability of *B. bruxellensis* completely recovered in all wines, implying that *B. bruxellensis* yeast entered a viable but not culturable (VBNC) state after HHP exposure. Regarding the chemical analyses, applied process parameters induced a slight decrease of anthocyanins in red wine, while changes of total phenolics and total colour difference value were negligible. In conclusion, HHP could potentially be successful for microbial stabilisation of sweet wines and consequently assure a lower use of sulphur dioxide, while inactivation of *B. bruxellensis* could only be successful in the early stages of wine contamination.

KEYWORDS

Brettanomyces bruxellensis, high hydrostatic pressure, *Saccharomyces cerevisiae*, wine

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INTRODUCTION

Wine production is a complex microbiological and chemical process that involves the activity of different microorganisms – primarily yeasts. In order to produce high-quality wines, it is crucial to control the growth of microorganisms during the winemaking process. It is not only important to maximise the fermentative performance of *Saccharomyces cerevisiae* yeast and lactic acid bacteria during alcoholic and malolactic fermentation, but also to manage the growth of undesirable yeasts and bacteria (Fungelsang *et al.*, 2007). Uncontrolled growth of specific wine microorganisms leads to significant wine spoilage, which could occur at several stages of winemaking: during alcoholic and malolactic fermentation, during the storage in barrels prior to packaging, and in the bottled wine (Agnolucci *et al.,* 2017). One of these undesirable microorganisms is *Brettanomyces bruxellensis*, the main spoilage yeast in red wines. Contamination with this yeast negatively affects the sensory properties of wine through production of off-flavours that are often described as "barnyard", "horse sweat" and "medicinal". Moreover, this consequently results in wine not suitable for consumption and, subsequently, in great economic losses (Oelofse *et al.*, 2009; Childs *et al.*, 2015). The compounds most responsible for this off-flavour of red wine are volatile phenols (4-ethylphenol and 4-ethylguaiacol); these are formed by a *B. bruxellensis* metabolism from hydroxycinnamic acids (*p*-coumaric and ferulic acid), which are their precursors naturally present in wine (Kheir *et al.,* 2013). The main challenge in controlling *B. bruxellensis* growth is its strong ability to survive and grow in unfavourable conditions, such as low pH, high alcohol content, anaerobic conditions and very low amounts of fermentable sugars (Fabrizio *et al.*, 2015). In stress conditions, this yeast has an ability to enter a viable but not culturable (VBNC) state, a physiological condition characterised by the inability of cells to divide on culture media, even if they are still alive and maintain metabolic activity (Agnolucci *et al.*, 2010; Serpaggi *et al.*, 2012; Agnolucci *et al.*, 2017). This represents an additional challenge in controlling the *B. bruxellensis* population. Conversely, *S. cerevisiae* is considered the main yeast for alcoholic fermentation, but it could also negatively affect wine quality through refermentation of sweet wines and bottled wines with residual sugars (Loureiro and Malfeito-Ferreira, 2003). In addition to *S. cerevisiae,* refermentation could also be caused by other yeasts such as *Zygosaccharomyces bailii, Schizosaccharomyces pombe* and *Saccharomycodes ludwigi,* but it is *Z. bailii* and *S. cerevisiae* that most commonly cause refermentation in white sweet wines (Martorell *et al.*, 2005; Enrique *et al.*, 2007; Rai and Bridge, 2009; Alonso *et al.*, 2015).

Microbiological stability is fundamental for preserving wine quality. Generally, this may be achieved by the use of chemical additives, physical removal through filtration, various biological procedures or other methods, along with the control of cellar/tank temperatures (Kheir *et al.*, 2013; Lisanti *et al.*, 2019). The proposed biological methods used to avoid yield losses caused by *B. bruxellensis* are related to antimicrobial peptides derived from natural proteins (Enrique *et al.*, 2008), killer toxins produced by non-*Saccharomyces* yeasts (Santos *et al.*, 2009; Mehlomakulu *et al.*, 2014; Mehlomakulu *et al.*, 2015), as well as tailored selected yeast and bacterial strains inoculated to promote alcoholic and malolactic fermentation (Berbegal *et al.*, 2018). Nevertheless, the most popular, inexpensive and effective agent is the chemical preservative sulphur dioxide (SO_2). In terms of antimicrobial activity, molecular SO_2 is the most active form while bisulphite ions show low antimicrobial activity and bound SO_2 exerts only a low antibacterial action. In finished wine, the typical targets for preventing microbial spoilage are at least 0.6 and 0.8 mgL^{-1} of molecular SO_2 for dry and sweet wines, respectively (Waterhouse *et al.*, 2016). However, the level of molecular SO_2 should be kept under its sensory threshold of 2 mgL^{-1} (Waterhouse *et al.*, 2016). Previous investigations reported that a concentration of 1.4 mgL^{-1} of molecular SO_2 induced a VBNC state with up to 29 % of viable cells of *B. bruxellensis*, while viable cells were not detected at a concentration of 2.1 mgL^{-1} of molecular SO_2 (Agnolucci *et al.*, 2013). Meaning that it can be expected that concentrations of total SO_2 in wine are likely to exceed legal limits and potentially also affect human health. According to Fabrizio *et al.* (2015), even if the health aspect is neglected the addition of SO_2 is not effective due to the presence of resistant strains or cross contamination in cellar operations.

Furthermore, the use of SO_2 is being reconsidered due to increasing allergenic

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concerns, and researchers are investigating alternative methods to reduce the concentrations commonly added during winemaking (Santos *et al.*, 2012; Guerrero and Cantos-Villar, 2015), wherein the use of high hydrostatic pressure (HHP) is highlighted as promising. Generally, HHP is a non-thermal batch process that utilises the application of pressures between 100 and 600 MPa to inactivate spoilage microorganisms in beverages and other foods (van Wyk *et al.*, 2018). This is an interesting alternative to traditional food processing and preservation methods due to its limited effects on covalent bonds, and results in minimal modifications in the nutritional and sensory quality of treated products. Previous investigations regarding the effect of HHP on microbial stabilisation of wine are mainly related to controlling the growth of *B. bruxellensis* (González-Arenzana *et al.*, 2016; van Wyk and Silva, 2017a; van Wyk and Silva, 2017b; van Wyk and Silva, 2019) and *S. cerevisiae* yeast (Tonello *et al.*, 1998; Marx *et al.*, 2011). Studies have demonstrated that the effectiveness of HHP on microbial inactivation is strongly dependent on applied pressure and treatment duration (González-Arenzana *et al.*, 2016; van Wyk and Silva, 2017a,b), as well as on the type of wine treated (van Wyk and Silva, 2017b).

The aim of this research was to preliminary investigate the effectiveness of HHP process parameters (pressures and treatment duration) for inactivation of *B. bruxellensis* and *S. cerevisiae* yeasts. Its efficiency was examined in terms of the different types of wine, where dry red wine was used for inoculation by *B. bruxellensis*, and sweet white wine for inoculation by *S. cerevisiae* yeast. The effect of HHP on main quality parameters (total phenolics, total anthocyanins and colour change) was also examined.

MATERIALS AND METHODS

1. Chemicals

Deionized water was produced by a Millipore Milli Q system (Bedford, USA). *Brettanomyces bruxellensis* CBS 2499 was purchased from Westerdijk Fungal Biodiversity Institute (Utrecht, Netherlands), while *S. cerevisiae* DSM 70468 was obtained from Leibniz-Institut DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Yeast extract, glucose, peptone and the Wallerstein Labs Nutrient (WLN) agar were purchased from Biolife (Milano, Italy). Brettanomyces agar was obtained from Laboratorios Conda (Madrid, Spain), glycerol, sodium hydroxide and ethanol absolute from Gram-mol (Zagreb, Croatia) and chloramphenicol from Sigma Aldrich (St. Louis, USA). Folin-Ciocalteu reagent was obtained from Kemika (Zagreb, Croatia), sodium bisulfite from Acros Organics (Geel, Belgium), hydrochloric acid (37 %) from Carlo Erba (Val del Reuil, France) and sodium carbonate anhydrous (99 %) from T.T.T. (Sveta Nedjelja, Croatia).

2. Wines

The wines used in this study were vintage 2016 red wine Cabernet Sauvignon and sweet wine Graševina, obtained from winery Erdutski vinogradi d.o.o. (Erdut, Croatia). The wines were passed through a 0.4-µm pore filter. Next, the physicochemical parameters and microbial population were determined by spreading both wines on various solid mediums (WLN agar, Brettanomyces agar, Sabourand dextrose agar and Lysine agar). After confirming that there were no undesirable microorganisms, each wine was further inoculated with the tested yeast. The average physicochemical parameters of the wines were analysed by FTIR spectrophotometer Bacchus II (Microdom, Saint-Savinien, France) and the results are showed in Table 1.

3. Wine preparation

B. bruxellensis CBS 2499 and *S. cerevisiae* DSM 70468 were used as test microorganisms. These strains were selected because the *B. bruxellensis* CBS 2499 strain has a high tolerance to stress and *S. cerevisiae* DSM 70468 strain has a high tolerance to ethanol, and because they were not previously tested for inactivation by HHP. Moreover, the CBS 2499 strain has a published genome and the DSM 70468 strain has a high-quality Illumina sequence read dataset, which should facilitate future studies at the transcriptome, proteome, and metabolome levels, hopefully leading to a complete understanding of HHP effects on these yeasts. Both cultures were stored at -80 °C in 20 % glycerol (v/v). Yeast peptone dextrose (YPD) medium (10 gL^{-1} yeast extract, 20 gL^{-1} peptone and 20 gL^{-1} glucose) was used for growing *B. bruxellensis* and *S. cerevisiae* inoculum. YPD medium was acidified to pH 3.5 with phosphoric acid.

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TABLE 1. Physicochemical properties of dry red wine Cabernet-Sauvignon and sweet white wine Graševina.

	Cabernet-Sauvignon	Graševina
Alcohol (vol %)	13.0±0.1	11.7±0.1
Total acidity (gL^{-1} of tartaric acid)	6.4±0.1	5.5±0.2
Volatile acidity ($gL^{-1}$of acetic acid)	0.6±0.0	0.4±0.0
pH	3.5±0.0	3.3±0.0
Reducing sugars (gL^{-1})	3.7±0.1	53.7±0.5
Malic acid (gL^{-1})	0.5±0.0	1.3±0.1
Lactic acid (gL^{-1})	1.5±0.1	0.3±0.0
Free SO_2 (mgL^{-1})	12±1.1	24±1.3
Total SO_2 (mgL^{-1})	48±1.2	80±2.2

* Concentrations expressed as mean ± standard deviation (N=3).

The inocula were prepared as described by Delsart *et al.* (2016) and Križanović *et al.* (2019) in a four-step procedure, to ensure yeast adaptation to wine conditions. Both yeast cultures, *B. bruxellensis* and *S. cerevisiae,* were grown in separate YPD media and then in YPD media with 4, 8 and 12 % (v/v) ethanol, at 28 ºC without shaking. When the yeast population entered the stationary phase in the first medium, another medium was used and inoculated with 10 %, v/v of culture from the previous medium. The growth of both yeasts during the inoculum preparation in YPD media without or with ethanol was followed by measuring the absorbance at 600 nm and by plate counts (Table 2), but the cell population of both yeast in wines was only measured by plate counts.

Cells of *B. bruxellensis* and *S. cerevisiae* yeast were harvested by centrifugation at 4000 rpm for 10 min and inoculated into wine samples at approximately 6 and 5 log ($CFUmL^{-1}$) for red and sweet wine, respectively. The initial cell populations of *B. bruxellensis* and *S. cerevisiae* yeast in wine samples were then confirmed by plate counts. The inoculation of wine samples was performed 24 h before HHP treatment and these were incubated at 20 ± 2 °C. The cell population of both yeasts in the wine samples immediately after inoculation and after 24 h, i.e. before HHP treatment, was identical.

4. High hydrostatic pressure processing

The experiment was performed in three biological replicates for each yeast. Each biological replicate was treated with high hydrostatic pressure system FPG7100 (Stansed Fluid Power, Harlow, UK) in triplicate for each set of processing parameters and then analysed. After inoculation with tested yeast, 100 mL of wine was poured into a plastic bottle, packaged in an individual plastic bag, vacuum sealed, and placed in a working vessel of high hydrostatic pressure system. To assess the possible effects of HHP treatment, an experimental test was conducted with various pressures (100 and 200 MPa) and processing times (1, 3, 5, 15 and 25 min). A control sample represented the wine sample inoculated with *B. bruxellensis* or *S. cerevisiae* and not exposed to HHP treatment. The control sample and the samples obtained after HHP treatment were placed in sterile bottles and stored for 90 days at 20 ± 2 °C, to assess the long-term effects of applied treatment.

5. Culturability analysis

Brettanomyces agar (100 mgL^{-1} chloramphenicol, 10 mgL^{-1} cycloheximide and 100 mgL^{-1} coumaric acid) was used to analyse *B. bruxellensis* cell culturability, and WLN agar with 100 mgL^{-1} chloramphenicol was used to analyse the *S. cerevisiae* cell culturability.

Inoculated wines were analysed before and immediately after HHP treatments as well as after storage for 30, 60 and 90 days. The samples were decimally diluted in physiological saline solution. The serial decimal dilutions and the undiluted sample of inoculated red wines were plated onto Brettanomyces agar in triplicate and incubated at 28 ºC for up to 7 days, and the samples of inoculated sweet wines were plated onto WLN agar (with added chloramphenicol) in triplicate, and incubated at 25 ºC for up to 2 days. The number of colony-forming units (CFU) was calculated according to the following formula (Eqn. 1):

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TABLE 2. Growth of *B. bruxellensis* CBS 2499 and *S. cerevisiae* DSM 70468 yeasts expressed as log (CFU mL^{-1}) and optical density (OD) during inoculum preparation.

Medium	*B. bruxellensis*		*S. cerevisiae*	
	log ($CFUmL^{-1}$)	OD_{600nm}	log ($CFUmL^{-1}$)	OD_{600nm}
YPD	8.25±0.11	7.86±0.25	7.28±0.00	4.64±0.10
YPD with 4 % (v/v) ethanol	8.19±0.20	7.35±0.10	7.19±0.12	4.49±0.00
YPD with 8 % (v/v) ethanol	8.21±0.08	7.52±0.10	7.11±0.10	3.48±0.20
YPD with 12 % (v/v) ethanol	7.65±0.25	4.76±0.15	6.33±0.10	3.35±0.10

* Data expressed as mean ± standard deviation (N=3).

$$CFU = \frac{number\ of\ formed\ colonies}{sample\ volume\ used} \times reciprocal\ value\ of\ decimal\ dilution$$

6. Spectrophotometric and chromatic analyses

The spectrophotometric analyses were carried out using a Specord 50 Plus spectrophotometer (AnalytikJena, Jena, Germany). Total phenolics (TP) content was determined by the Folin-Ciocalteu method as described in detail by Singleton and Rossi (1965). The results were expressed as mgL^{-1} of gallic acid equivalents (mg $GAEL^{-1}$). The total anthocyanins (TA) content was determined using the SO_2 bleaching method as previously described by Ribéreau-Gayon and Stonestreet (1965). All spectrophotometric analyses were conducted in triplicate.

The chromatic characteristics of the wine samples were measured with a Specord 50 Plus AnalytikJena spectrophotometer (Jena, Germany) using the CIELab space (OIV, 2006). Chromatic characteristics were expressed as CIE coordinates of L (lightness), a (redness/greenness) and b (yellowness/blueness), with illuminant D65 and observer 10° standardisation, using the software WinASPECT PLUS (Jena, Germany). All measurements were performed in triplicate and the results presented as average value of total colour difference. The total colour difference value (ΔE) between the control and treated wine samples was calculated with the following formula (Eqn. 2):

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

7. Data analysis

Significant differences between samples for chemical and microbiological analyses were determined by one-way analysis of variance (ANOVA) using the Statistica V.10 software (StatSoft Inc., Tulsa, USA). Tukey's honestly significant difference (HSD) test ($p<0.05$) was used for comparison when samples differed significantly after ANOVA.

RESULTS

1. Short-term effect of HHP

Figure 1 shows the reduction of *B. bruxellensis* and *S. cerevisiae* yeasts in wines subjected to pressures of 100 and 200 MPa for 1, 3, 5, 15 and 25 min. The initial cell population of *B. bruxellensis* yeast in wine before HHP treatment was 5.5 log ($CFUmL^{-1}$) and *S. cerevisiae* yeast was 4.4 log ($CFUmL^{-1}$). With 100 MPa HHP treatment for all treatment durations, no reduction in cell culturability was observed for the yeasts. Conversely, with 200 MPa HHP treatment a reduction in cell culturability was observed for both yeasts even for the shortest treatment duration of 1 min.

However, at 200 MPa the treatment duration had a significantly different impact between the two yeasts. After treatment for 1 min, a similar reduction in culturability was observed for the yeasts. At longer treatment durations (3 and 5 min), the observed culturability reduction was much higher for *B. bruxellensis* and the difference increased with treatment duration. In *S. cerevisiae* the largest culturability reduction was 1.5 log ($CFUmL^{-1}$), observed after the 15- and 25-min treatments (the reduction was 1.0 log ($CFUmL^{-1}$) after the 1-min treatment). In *B. bruxellensis* the cell culturability decreased rapidly, achieving complete reduction after the 15-min treatment, with the observed culturability reduction of 5.5 log ($CFUmL^{-1}$).

2. Long-term effect of HHP

The long-term effect of HHP was investigated during 90 days of storage at three different sampling points (after 30, 60 and 90 days), and

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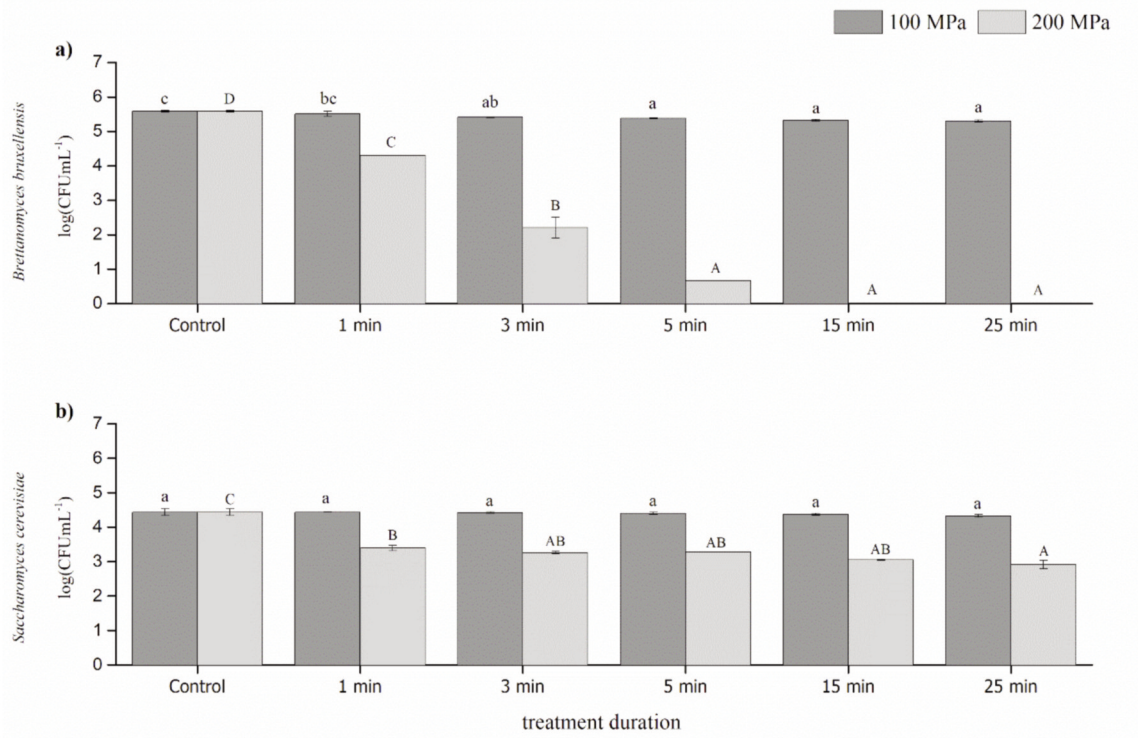


FIGURE 1. Cell culturability of (a) *B. bruxellensis* CBS 2499 and (b) *S. cerevisiae* DSM 70468 in wine samples before and immediately after HHP treatments of 100 and 200 MPa.

the results are presented in Figure 2. In terms of culturability, the opposing results can be observed for both yeasts when comparing the long-term effects of HHP at 100 MPa to those at 200 MPa, and when comparing the long- and short-term effects of HHP at 200 MPa.

For dry red and sweet white wines inoculated with *B. bruxellensis* or *S. cerevisiae* yeast treated using 100 MPa, no change in cell culturability was observed for either yeast in treated and control (untreated) samples after storing for 30 and 60 days. With further storage (after 90 days) an increase in culturability was observed for *S. cerevisiae* in both treated and control samples, while cell culturability for *B. bruxellensis* remained the same as in earlier sampling points for the control sample but decreased in almost all treated samples.

Monitoring wines treated with 200 MPa during the 90-day storage showed a significant reduction in *S. cerevisiae* cell culturability, while *B. bruxellensis* culturability completely recovered over the period. After 30 days of storage for dry red wine treated with 200 MPa, the cell culturability of *B. bruxellensis* differed in samples treated for 5, 15 and 25 min compared to the control sample. After 60 days, culturability in the control sample was higher than in the treated samples (for all treatment durations). After 90 days, the *B. bruxellensis* cell culturability was higher in almost all treated samples compared to the control sample.

While monitoring sweet white wine treated with 200 MPa during storage, the cell culturability of *S. cerevisiae* was observed only in samples treated for 1, 3 and 5 min, but not in samples treated for 15 and 25 min. A gradual increase in cell culturability during storage was observed in samples treated for 1, 3 and 5 min. However, after 30 and 60 days, cell culturability in the control sample was higher than in samples treated for up to 5 min, while after 90 days the culturability for those samples was roughly equal to the control sample.

3. Changes in wine phenolics and chromatic characteristics

To evaluate the potential effect of HHP on wine quality, total phenolics and total anthocyanins were determined in all samples, and the results are shown in Table 3. In the red wine, immediately after treatment the concentrations of both total phenolics and total anthocyanins were significantly different in treated and untreated

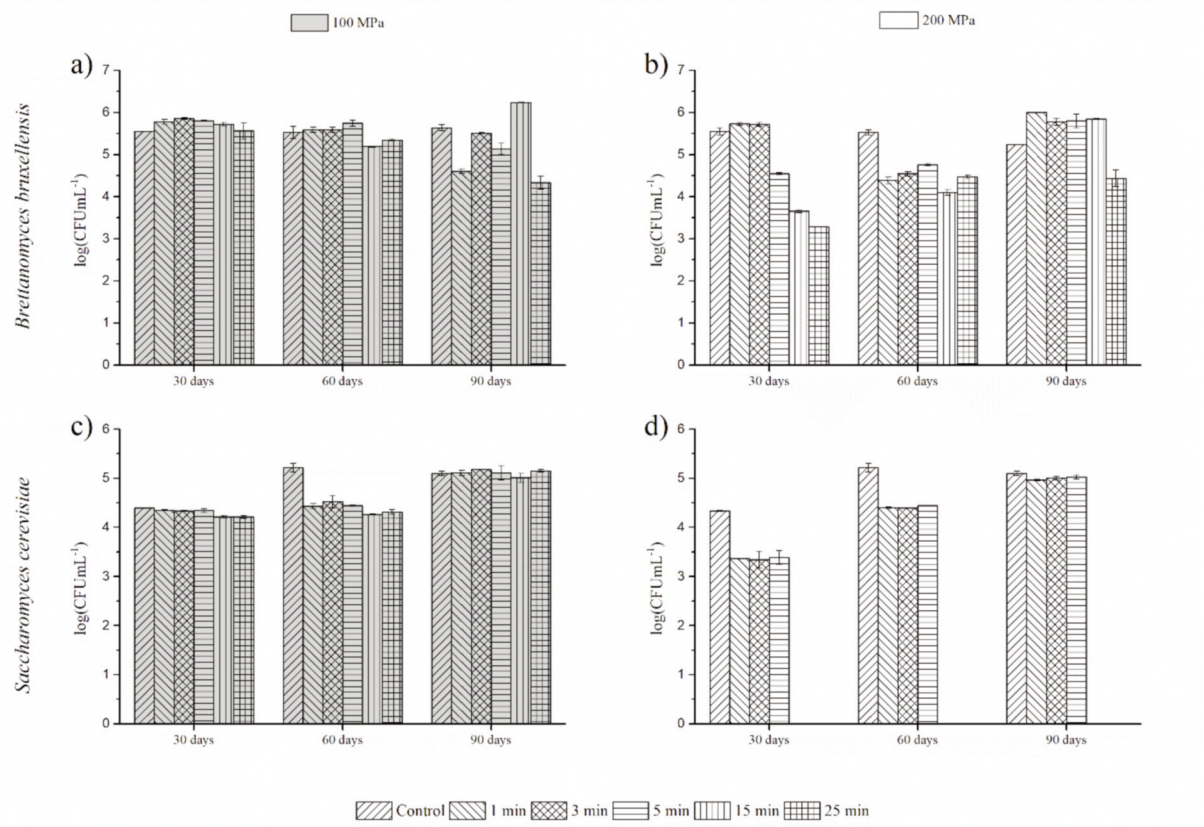


FIGURE 2. Cell culturability of (a and b) *B. bruxellensis* CBS 2499 and (c and d) *S. cerevisiae* DSM 70468 in wine samples treated by HHP during storage (30, 60 and 90 days).

wines, but those differences were not significant. The greatest differences were observed for total anthocyanins in wines treated with 200 MPa for 15 and 25 min, but the decrease in these compounds was less than 10 % in comparison to control wine. During the storage period, the difference in the concentration of total phenolics were also statistically significant, but rather small between the control and treated wines. Conversely, the differences in the concentration of total anthocyanins were statistically significant between the samples treated with different pressures, but not statistically different between the samples treated with the same pressure concentrations. Herein, the lower concentrations were observed in the treated samples, with a larger decline determined in the samples treated with 200 MPa. Moreover, longer treatment durations resulted in a larger decrease of these compounds. For example, the most invasive treatment – 200 MPa for 25 min – resulted in a more than 26 % lower concentration of total anthocyanins after 90 days of storage. In terms of total phenolics sweet wine demonstrated a similar trend to that observed in red wine, where the differences in concentration of total phenolics in the control and treated wines were statistically significant, but also quite small at less than 5 %. After 90 days of storage a decrease was observed for all analysed parameters, but there were no significant differences in the concentrations of total phenolics between treated and control samples.

In addition, the total colour difference value (ΔE) between the control and treated wine samples was calculated and the results are also shown presented in Table 2. In red wine, significant values of ΔE could be observed immediately after the HHP treatment but all were below 2, which is considered a limit value. Similar results were obtained in sweet wine, except for 200 MPa treatment for 1, 3 and 5 min, where the values were 2.0, 2.9 and 3.4, respectively. During the storage period, these values increased in the red wines to above 3.0, especially in samples treated with 100 MPa and after longer treatment with 200 MPa (15 and 25 min). This trend could not be observed in sweet wine samples, where a slight increase, or

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TABLE 3. Total phenolics, total anthocyanins and total colour difference value (ΔE) immediately after HHP treatment and after 90 days of storage.

Days	Red wine									
	Total phenolics		Total anthocyanins		ΔE		Total phenolics		ΔE	
	0	90	0	90	0	90	0	90	0	90
HHP – 100 MPa										
Control	2703.8±39.8^{bc}	2454.3±22.1^{c}	155.8±1.4^{b}	76.5±0.1^{c}	-	-	216.6±0.4^{b}	185.1±2.9^{a}	-	-
1 min	2686.0±34.7^{cd}	2432.0±26.3^{c}	149.6±0.7^{a}	74.9±1.4^{c}	0.9±0.00^{bcd}	3.2±0.04^{de}	213.3±6.0^{bc}	182.5±0.5^{a}	1.2±0.44^{f}	0.9±0.10^{b}
3 min	2676.0±24.0^{b}	2431.0±17.6^{c}	157.9±3.9^{a}	73.5±2.3^{c}	1.0±0.03^{cd}	2.5±0.02^{c}	201.9±6.2^{a}	184.4±1.2^{a}	0.3±0.01^{b}	1.0±0.21^{c}
5 min	2675.5±8.5^{b}	2463.5±49.5^{abc}	150.9±2.5^{a}	70.6±0.7^{abc}	1.1±0.06^{d}	3.3±0.02^{f}	208.9±5.4^{bc}	183.4±1.7^{a}	1.0±0.20^{e}	0.5±0.05^{a}
15 min	2664.5±15.9^{a}	2435.3±26.1^{bc}	157.6±1.9^{a}	72.0±3.5^{bc}	1.0±0.04^{cd}	3.2±0.03^{de}	209.9±4.3^{bc}	182.5±0.3^{a}	0.6±0.03^{d}	0.9±0.01^{b}
25 min	2625.0±42.3^{bcd}	2432.0±26.3^{abc}	155.0±2.3^{a}	70.7±3.0^{abc}	0.9±0.02^{cd}	3.4±0.01^{f}	205.8±2.6^{bc}	184.3±2.9^{a}	0.2±0.04^{a}	0.5±0.08^{a}
HHP – 200 MPa										
Control	2703.8±39.8^{bc}	2226.6±22.3^{abc}	155.8±1.4^{b}	64.9±1.4^{abc}	-	-	216.6±0.4^{b}	185.1±2.9^{a}	-	-
1 min	2680.6±41.1^{b}	2232.3±22.4^{abc}	153.9±2.7^{b}	55.8±0.7^{abc}	0.6±0.08^{b}	0.3±0.01^{a}	214.0±0.6^{b}	183.8±1.8^{a}	2.0±0.26^{g}	0.9±0.31^{b}
3 min	2669.3±37.2^{d}	2244.1±28.5^{abc}	153.9±3.2^{b}	55.4±3.0^{abc}	1.0±0.10^{d}	1.8±0.01^{b}	210.2±5.3^{bc}	182.5±3.4^{a}	2.9±0.07^{h}	0.9±0.12^{b}
5 min	2701.8±43.7^{b}	2181.4±3.1^{ab}	155.5±2.7^{b}	49.2±4.2^{ab}	0.2±0.20^{a}	1.8±0.12^{b}	212.9±0.7^{bc}	183.8±2.3^{a}	3.4±0.05^{i}	0.9±0.18^{b}
15 min	2681.0±43.0^{b}	2215.9±20.3^{ab}	146.8±4.2^{b}	49.8±4.6^{ab}	0.7±0.07^{bc}	3.1±0.02^{d}	210.4±1.7^{bc}	181.8±0.2^{a}	0.5±0.07^{c}	1.2±0.02^{c}
25 min	2686.3±3.6^{bcd}	2225.6±13.0^{a}	148.9±2.2^{b}	47.7±4.9^{a}	0.9±0.01^{bcd}	3.2±0.09^{de}	211.2±4.5^{bc}	181.9±3.6^{a}	0.3±0.13^{b}	1.0±0.13^{c}

*expressed as mean ± standard deviation (N=3). ANOVA to compare data; different superscript letters indicate statistical differences among wines of all treatments at the same time (Tukey's test, $p<0.05$). Abbreviations: ΔE, total colour difference value.

even a decrease of ΔE occurred after 90 days of storage. These samples are characterised with ΔE values below 2, meaning that HHP treatment did not affect colour change.

DISCUSSION

Brettanomyces bruxellensis is often considered the main spoilage microorganism in red wines. It causes deterioration of wine quality through the production of volatile phenols, which are the compounds responsible for off-flavours in red wine (Oelofse *et al.*, 2009; Childs *et al.*, 2015). In the wine industry, contamination by *B. bruxellensis* is very hard to control due to its ability to survive and grow in conditions that are inhibitory for most other microorganisms (low pH, anaerobic conditions, high alcohol level, and very low amounts of fermentable sugars), and its ability to enter a VBNC state in stress conditions (Agnolucci *et al.*, 2010, 2017; Serpaggi *et al.*, 2012; Fabrizio *et al.*, 2015). Several other yeast species are also associated with wine quality degradation. Primarily, these yeasts are related to the refermentation of sweet wines and bottled wines with residual sugars. Yeasts such as *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Schizosaccharomyces pombe* and *Saccharomycodes ludwigi* are reported to cause refermentation in various types of wine, while *S. cerevisiae* and *Z. bailii* are most commonly reported to cause refermentation in sweet white wines (Loureiro *et al.*, 2003; Martorell *et al.*, 2005; Enrique *et al.*, 2007; Rai and Bridge, 2009; Alonso *et al.*, 2015).

Among the different methods employed for microbial stabilisation, SO_2 is most common, but as it is not always effective due to its potential negative effect on human health and the presence of resistant yeast strains or the occurrence of cross contaminations in cellar operations (Fabrizio *et al.*, 2015), new technologies for preventing and controlling undesirable yeasts are being developed. One of these technologies is high hydrostatic pressure (HHP), wherein pressures between 100 and 600 MPa are employed to inactivate spoilage microorganisms in beverages and other foods (van Wyk *et al.*, 2018). Until now, this was successfully applied for growth control of several strains of *B. bruxellensis* (AWRI 1499, AWRI 1608, AWRI 1613 and two strains isolated from Rioja red wines) and several strains of *S. cerevisiae* (CBS 1171, KCCM 1224 and different commercial strains) in must and in white, red and rosé wines (Tonello *et al.*, 1998; Perrier-Cornet *et al.*, 2005; Mok *et al.*, 2006; Marx *et al.*, 2011; González-Arenzana *et al.*, 2016; Van Wyk and Silva, 2017a,b; Van Wyk *et al.*, 2019).

1. Short-term effect of HHP

The trends observed (a higher reduction with higher pressures and longer treatment durations) are in line with previous investigations, which showed that microbial inactivation during HHP processing strongly depends on applied pressures and treatment duration (González-Arenzana *et al*., 2016; van Wyk and Silva, 2017a,b). Additionally, a recent investigation suggested a minimum threshold pressure below which no inactivation occurs (van Wyk and Silva, 2019), with a pressure of 100 MPa considered to be the threshold as it did not result in significant inactivation of *B. bruxellensis* yeast (González-Arenzana *et al*., 2016; Van Wyk and Silva, 2017a). Our study additionally confirms that hypothesis, for reduction of both yeasts, as there were no significant differences observed after application of 100 MPa pressure. Although application of the same pressure resulted in statistically significant reduction of *B. bruxellensis* cell culturability (Figure 1a), these changes are negligible as they are under the same decimal dilution. Hence, the results suggest that a more effective reduction could be achieved by applying higher pressures, such as 200 MPa.

Regarding the yeast species treated, a higher reduction was achieved for *B. bruxellensis* (Figure 1a) than for *S. cerevisiae* (Figure 1b). Treatments at 200 MPa for 15 and 25 min resulted in the highest reduction for both yeasts, but these treatments were more effective for *B. bruxellensis* as its culturability was not confirmed after the treatment (Figure 1a). As previously stated, complete reduction of *S. cerevisiae* was not achieved by applied pressures, and the most intensive treatment (200 MPa during 25 min) resulted in only 1.5 log reduction. According to data in the literature (Tonello *et al*., 1998), higher applied pressures (300 and 500 MPa) resulted in a higher reduction in *S. cerevisiae*. Marx *et al*. (2011) showed that application of 600 MPa for 7 min resulted in complete inactivation of this yeast. The discrepancy between the *B. bruxellensis* and *S. cerevisiae* reduction could be due to several factors. First, different yeast species and strains could behave differently under HHP treatment

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(van Wyk and Silva, 2017a). Tonello *et al.* (1996) showed that HHP inactivation depends on type and size of microorganisms targeted, wherein different efficacy was obtained for *S. cerevisiae* and *S. ludwigii* yeasts. Taken together, we can expect that optimal set of processing parameters for preventing wine spoilage will depend not only on yeast species, but also on the strain. Second, the sugar present in wine could also act protectively, as determined for *B. bruxellensis* yeast treated by thermosonication (Križanović *et al.*, 2020). Third, alcohol concentration also proved an important factor for microbial inactivation, with higher reduction achieved in wines with alcohol levels above 13 % (Tonello *et al*., 1996, 1998; van Wyk and Silva, 2017b; Križanović *et al*., 2020), which were significantly higher than those in treated sweet wine (11 %) (Table 1). Generally, previous investigations also established that suspending medium could significantly affect pressure resistance of microorganisms. Moreover, the resistance could be greatly increased when treated in nutritionally rich media containing substances that can act protectively against damage or contain nutrients essential for microorganism repair (van Opstal *et al.*, 2003; Molina-Höppner *et al.*, 2004; Chauvin *et al.*, 2006; Goh *et al.*, 2007; Sokołowska *et al.*, 2013). In other words, the results obtained in this study indicate the baroprotective effect of sugar in sweet wine on *S. cerevisiae* cells during pressurisation.

2. Long-term effect of HHP

Our results (Figure 2) suggest that HHP treatment resulted in *B. bruxellensis* entering an VBNC state; the culturability was not confirmed immediately after 15-min and 25-min treatments (Figure 1a), but after the stress was removed the cells were able to grow on culture media again. Unlike the given results, van Wyk *et al.* (2018) showed that HHP processing at 400 MPa for 5 s resulted in complete inactivation of *B. bruxellensis* yeast. The authors showed that culturability was not recovered during 12 months of storage, meaning that the VBNC state after HHP exposure was not observed, as is the case in our research. The obtained differences could be primarily due to the variation of applied pressures. Hence, the lower pressures used in this research potentially cause *B. bruxellensis* to enter a VBNC state, but higher pressures (400 MPa) used in van Wyk *et al*. (2018) cause complete inactivation. Additionally, a potential explanation could be that the strain (AWRI 1499) used in that study is more susceptible to HHP treatment in comparison to the CBS 2499 strain we used. Generally, there is a large diversity of genotypes and phenotypes of *B. bruxellensis* strains isolated from wine, and previous studies showed that metabolic activity, sulphur dioxide tolerance and behaviour in the VBNC state are strain dependent (Capozzi *et al.*, 2016; Crauwels *et al.*, 2016; Avramova *et al.*, 2019; Dimopoulou *et al.*, 2019). In terms of non-thermal technologies, it has already been demonstrated that HHP inactivation efficiency strongly depends on the yeast strain used, wherein AWRI 1499 was more resistant than AWRI 1608 and AWRI 1613 (van Wyk and Silva, 2017a). Although analyses regarding high tolerance to stress showed that genomic characteristics are distributed in the genome of both CBS 2499 and AWRI 1499 strains (Godoy *et al.*, 2017), it is possible that CBS 2499 could be more resistant to HHP treatments. However, before making any general conclusions it is necessary to conduct further, more extensive studies. Nevertheless, van Wyk *et al*. (2018) showed that, among higher sulphur dioxide, pulsed electric field (PEF) and HHP, the latter was the best and the most reliable treatment for *B. bruxellensis* inactivation, meaning that HHP has a great potential for the wine industry.

Besides provoked stress conditions, the main theory regarding the microbial inactivation by HHP is related to changes in the cell membrane associated with macromolecules such as proteins, as well as to compression of the membrane's bilayer due to changes in permeability (Ross *et al.*, 2003). These changes may not necessarily lead to cell death, although surviving cells showed enhanced sensitivity to different inhibitors (Mackey, 2000). In fact, several investigations showed the occurrence of sublethal injury after HHP exposure using different plating techniques (Alpas *et al.*, 2000; Chilton *et al.*, 2001; Jordan *et al.*, 2001; Somolinos *et al.*, 2008). In other words, and contrary to the VBNC state, sub-lethally injured cells still possess the ability to grow on growth media (Li *et al.*, 2014). This effect is the most probable explanation for trend estimated in the case of *S. cerevisiae* yeast: immediately after the longest HHP treatments (15 and 25 min) sublethal injury occurred and, consequently, cells were able to grow on selective medium. However, after a certain period of storage, enhanced sensitivity resulted in cell death,

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potentially due to higher levels of sugar (osmotic pressure), presence of ethanol, sulphur dioxide, or their combination. Nevertheless, these results imply that HHP treatment could be successfully applied in sweet wine production, in terms of avoiding the risk of a secondary fermentation and subsequent degradation of wine.

3. Changes in wine phenolics and chromatic characteristics

In terms of the phenolic content, the results obtained in this research (Table 3) are in accordance with previous investigations where it was demonstrated that HHP treatment did not result in immediate changes of total phenolics in red and white wines, while changes in concentrations were estimated after 9 and 12 months of storage (Santos *et al.*, 2013a,b). As with sweet wine, these differences were observed in both, control and treated wines, and most probably were due to different chemical and biochemical reaction that occur during wine ageing.

Furthermore, the colour difference value (ΔE) is important because it shows whether the changes in the chromatic characteristics were visually relevant. According to the literature, the difference in colour is clearly perceptible when the value of ΔE between two samples is in the range 2 - 10, while for values higher than 10 the colours are more opposite than similar (Brainard, 2003). Moreover, a recent study showed that all the colour differences higher than 6 should be taken as considerable, clearly visible (Ramirez-Navas and Rodriguez de Stouvenel, 2012), while values higher than 3 were visible to the human eye (Puértolas *et al.*, 2010).

These results confirm the potential of the HHP technique for microbial stabilisation of sweet wines, wherein the yeasts are inactivated without detrimental effects on wine quality parameters, such as visual colour perception and phenolic content. A previous investigation also reported that HHP treatment resulted in low values of ΔE, meaning that although changes in chromatic characteristics occurred, they could not be perceived by the human eye (Tao *et al.*, 2012). Santos *et al.* (2013a) showed that immediately after the HHP treatment and during first six months of storage the ΔE values of red wine were below 3, but increased after 9 and especially after 12 months of storage. A similar trend was observed in white wines, but the values above 3 were observed much earlier, after 3 months of storage (Santos *et al*., 2013b). Even though obtained results suggest application of HHP on wine without effect on main quality parameters, it is necessary to conduct further research on longer storage periods and overall wine quality.

CONCLUSIONS

The results here showed that effectiveness of HHP for microbial stabilisation of wine depends on process parameters, such as applied pressure and treatment duration. Immediately after the treatment, HHP was more effective for reducing *B. bruxellensis* in red wine, wherein its culturability was not confirmed after pressurising by 200 MPa for 15 and 25 min. Conversely, the same process parameters only resulted in a decrease not complete reduction of culturability of *S. cerevisiae* yeast in sweet wine. The opposite result was established during 90 days of storage. Primarily, the culturability of *B. bruxellensis* was confirmed in all analysed samples, indicating that cells entered a VBNC state after HHP exposure, but after a certain period they were able to grow on culture media and were completely recovered. However, culturability of *S. cerevisiae* in samples treated by 200 MPa for 15 and 25 min was not confirmed after 30, 60 and 90 days of storage. The results suggest that HHP is a promising technique for microbial stabilisation of sweet wine, in terms of avoiding refermentation, and consequently it can assure lower concentrations of sulphur dioxide in treated wines. In the case of *B. bruxellensis,* HHP could have a positive effect only in the first stages of wine contamination, when the number of yeast cells is considerably lower. In terms of the quality parameters, applied treatments slightly affected the concentration of total anthocyanins in red wine, while changes in total phenolics and total colour difference value were negligible and unrelated to treatment.

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