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Use of high hydrostatic pressure and non-*Saccharomyces* yeasts for improving aging on lees of white wine

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ABSTRACT

This study aimed to assess the impact of different fermentation lees on enhancing the chemical composition, volatile profile, and sensory characteristics of wine during aging, specifically in relation to autolysis induction treatments. Lees processed by high hydrostatic pressure, especially those obtained from sequential fermentation (*Hanseniaspora* spp. followed by *Saccharomyces* spp.) determined the best color evolution, the highest protection against oxidation together with an overall improvement of volatile profile; these aged wines also obtained the highest scores in floral and fruity notes, resulting the most preferred by the panel. An improvement of the overall wine quality might be reached, by modulating lees composition (*i.e.*, non-*Saccharomyces* spp. as fermentation co-starters), then by applying high hydrostatic pressure as alternative to enzyme addition for managing the subsequent aging on lees. This might possibly implement and improve the current winemaking process with an innovative, sustainable approach.

1. Introduction

Aging on lees (AOL) is a traditional winemaking practice that aims at enriching wine of several compounds, including nitrogen compounds, polysaccharides, lipids and antioxidant molecules, released by yeast cells as a result of their autolysis (Comuzzo et al., 2021). During AOL, the release of mannoproteins may positively contribute to protein (Dupin et al., 2000) and tartaric stabilization (Rodriguez-Nogales et al., 2012). At the same time, the adsorption of phenolic fraction or the antioxidant properties exhibited by lees surface (Comuzzo et al., 2015a; Gallardo-Chacón et al., 2010; Jaehrig et al., 2007) may reduce wine predisposition to browning, improving the protection against oxidation. Furthermore, the interaction between polysaccharides and phenolic compounds may also affect wine color stability and astringency (Del Barrio-Galán et al., 2019; Escot et al., 2001; Rodrigues et al., 2012). On the other hand, the release of volatile compounds from lees (Del Barrio-Galán et al., 2012) or the potential adsorption of aroma molecules by yeast mannoproteins (Juega et al., 2012) may modulate and improve the overall aroma complexity and the sensory perception of wines (Del Barrio-Galán et al., 2011; Loira et al., 2013).

The occurrence of yeast autolysis during AOL is a natural and generally slow process that may last from several months to years, depending on wine typology and aging conditions (Comuzzo et al., 2021). During wine aging in barriques, the periodic agitation of wines and the suspension of lees, namely *bâtonnage*, is generally performed to favor the release of glucidic colloids from yeasts, up to 150–200 mg/L (Ribereau-Gayon et al., 2006). Nevertheless, the most common method used in winemaking for accelerating AOL consists in the addition of enzymes with β-glucanase activity, that allows a faster occurrence of autolysis, reducing the conventional aging period (Palomero et al., 2007).

Over the last decades, emerging technologies have been successfully applied in food industry as an alternative to more traditional thermal treatments (Paniagua-Martínez et al., 2018). Among these, pulsed electric fields (PEF), ultrasounds (US) and high-pressure treatments have been also used for winemaking purposes. The International Organization of Vine and Wine (OIV) has recently authorized the use of US and PEF as tools for enhancing the extraction of compounds from grapes and to reduce the maceration time, whereas high-pressure treatments may be applied for inactivating spoilage microorganisms in grapes and must, to reduce sulfur dioxide addition (International Organization of Vine and Wine, 2022). However, these emerging technologies may play a role also in accelerating AOL, thanks to the induction of autolysis.

The application of PEF seems to be a useful tool for inducing autolysis in *Saccharomyces cerevisiae* and accelerating the release of mannoproteins during AOL (Martínez et al., 2019), whereas the use of US for processing fermentation lees allowed to obtain a high release of soluble

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Abbreviations:

HHP	high hydrostatic pressure
AOL	aging on lees
OIV	International Organization of Vine and Wine;
PEF	pulsed electric fields
US	ultrasounds
HPH	high pressure homogenization
NSY	non-*Saccharomyces* yeasts
GSH	glutathione
RP-HPLC	reversed-phase high performance liquid chromatography
SE-HPLC	size exclusion high performance liquid chromatography
PS	polysaccharides
SPME-GC-MS	solid phase microextraction gas chromatography-mass spectrometry
PCA	principal component analysis

colloids and proteins during wine aging, with results similar to those obtained by enzyme-assisted lysis (Cacciola et al., 2013). Moreover, the possibility to release compounds of enological interest (*e.g.,* polysaccharides) in a shorter time, might allow to reduce the conventional period of contact of wine with lees (Del Fresno et al., 2018), thus also lowering the risk of microbial contamination (González-Marco & Ancín-Azpilicueta, 2006).

Concerning high-pressure processing, interesting results have been reported about the application of high pressure in dynamic conditions, *i.e.,* using high pressure homogenization (HPH), to produce yeast autolysates for winemaking (Comuzzo et al., 2015b), or to obtain yeast extracts and to recover β-glucans for food applications (Dimopoulos et al., 2020). On the other hand, no evidence has been reported yet about the potential role of high-pressure technologies in accelerating yeast autolysis, when pressure is applied in static conditions, *i.e.,* operating with high hydrostatic pressure (HHP) plants.

Regarding the yeast strains, most of the studies carried out on autolysis and AOL mainly focus on *S. cerevisiae*. However, non-*Saccharomyces* yeasts (NSY) represent a consistent part of the natural microflora of grapes and must (Pallmann et al., 2001) and a non-negligible amount of cells are still present and viable in lees after alcoholic fermentation (Comuzzo et al., 2021). Besides their natural presence, the use of these strains as fermentation co-starters is becoming widespread in winemaking, thanks to the high content of polysaccharides released during both fermentation (Domizio et al., 2014; Giovani et al., 2012) and aging on lees (Kulkarni et al., 2015; Loira et al., 2013; Palomero et al., 2009). Interesting results have been also obtained using different strains belonging to *Hanseniaspora* spp., that show a good aptitude to produce polysaccharides (Romani et al., 2010). However, during AOL, the content of polysaccharides released by these strains in model solution seemed to be comparable to those obtained from *Saccharomyces* spp. (Del Fresno et al., 2020).

NSY may also have a role in stabilizing wine color, due to an increased production of pyruvic acid and acetaldehyde that leads to the formation of more stable pigments in red wines (Escribano-Viana et al., 2019; Medina et al., 2016; Mylona et al., 2016). The increasing interest in NSY strains has recently led researchers to investigate their glutathione (GSH) metabolism, too (Torrellas et al., 2020); Binati et al. (2021) also observed a high content of GSH produced during both single and sequential fermentation, highlighting once again the great potential of the use of NSY in winemaking. Despite these results, to the authors' knowledge, no data were reported regarding the role of NSY strains (*e.g.*, *Hanseniaspora* spp.) in the protection of wine against oxidation during aging on lees, particularly in relation to the methods eventually used to accelerate autolysis.

The aim of the present work was to evaluate the impact of lees from single (*S. cerevisiae*) and sequential fermentation (*Hanseniaspora uvarum* and *S. cerevisiae*) on the chemical composition, volatile and sensory profile of a white wine during aging on lees, also in relation to the treatment performed for accelerating autolysis.

2. Material and methods

2.1. Reagents and materials

Sodium chloride for microbiology, bacteriological peptone, Malt Extract broth and technical agar were purchased from Oxoid (Basingstoke, U.K.). Glucose, yeast extract, magnesium sulfate, tetracycline, ethanol (96% v/v), mannan from *S. cerevisiae*, trifluoroacetic acid, riboflavin, L-glutathione reduced (GSH), *p*-benzoquinone, 3-mercaptopropanoic acid, acetaldehyde (ACS reagent, ≥99.5%), (+)-catechin hydrate (≥98%, HPLC grade) and ethyl heptanoate were from Sigma Aldrich Italy (Milan, Italy). HPLC grade methanol was purchased from VWR Chemicals (Milan, Italy). MilliQ water was produced by a MilliQ Advantage A10 apparatus (Merck Millipore, Billerica, MA, USA) and microfiltered at 0.22 μm before use. Commercial β-glucanase enzyme preparation used for autolysis experiments was from Enologica Vason S.p.A. (San Pietro in Cariano, VR, Italy), whereas the white wine used for AOL trials was a blend of Sauvignon Blanc and Pinot Gris (harvest 2021), supplied by a local winery.

2.2. Yeast strains and inoculum preparation

Commercial active dry yeast preparation (*S. cerevisiae,* strain VP5, Enologica Vason S.p.A.) and strain H3 were used in the present experimental trials; concerning the latter, the yeast was previously isolated from red grape must (cv. Merlot) and selected for its good ability to produce and release polysaccharides and antioxidant compounds (Voce et al., 2022). The identification of the strain H3 was performed by molecular methods by 26S rRNA sequencing, as reported in Voce et al. (2022); the strain was then identified as *H. uvarum.*

Regarding inoculum preparation of *H. uvarum*, the biomass was produced at laboratory scale, using a culture medium consisting of glucose (20 g/L), yeast extract (10 g/L), magnesium sulfate (2.5 g/L) and bacteriological peptone (10 g/L) at pH 5. Single pure colonies isolated on Malt Extract agar plates were grown overnight in 10 mL-sterile tubes containing the culture medium; yeast suspensions were then inoculated at 10% (v/v), approximately corresponding to a concentration of 5.5×10^6 CFU/mL in 100 mL-sterile flasks containing 50 mL of the selected culture medium. After the first 24 h of growth, the biomass was collected and fed with fresh culture medium until a final volume of 500 mL, then incubated for further 24 h. The biomass production was carried out in an orbital shaker (SKI4, ArgoLab, Carpi, Modena, Italy) under constant agitation (150 rpm) at 30 °C; then, the biomass was harvested by centrifugation (13,000 rpm for 10 min at 4 °C), resuspended in the same volume of the synthetic must (500 mL) and used for the following fermentation trials. Concerning *S. cerevisiae*, the commercial active dry yeast preparation was rehydrated in ten volumes of warm water (35 °C) for 20 min, according to the supplier's recommendation.

2.3. Fermentation trials and lees production

To obtain sufficient yeast lees biomass to be used in the subsequent experiment of AOL, alcoholic fermentation was performed in synthetic medium in 10 L stainless steel kegs, one keg for each fermentation trial (single and sequential). Synthetic grape must MS300 was prepared as reported by Rossignol et al. (2003); after preparing the inoculum as reported above, both the strains were inoculated at approximately 1×10^7 cells/mL. The single fermentation trial was performed by

inoculating only *S. cerevisiae* (hereafter referred to as S), whereas the sequential fermentation trial was set up by firstly inoculating *H. uvarum*, followed by *S. cerevisiae* after 48 h (hereafter referred to as HS).

Fermentations were carried out at 20 ± 1 °C until the complete consumption of sugars and the recovery of fermentation lees was obtained by decanting and racking.

2.4. Lees treatments and wine aging on lees

After racking, lees from both single and sequential fermentation were recovered and subjected to two different treatments aimed at accelerating autolysis process and cell breakdown.

Enzyme treatment (hereafter referred to as ENZ) was carried out at lab scale, by adding a commercial preparation with β-glucanase activity, at the maximum dosage recommended by the supplier (40 mg/L); lees were added to the wine immediately after enzyme addition.

High hydrostatic pressure processing (hereafter referred to as HHP) was performed by an external facility (HHP Italia S.r.l., Traversetolo, PR, Italy); the equipment used was an Avure HHP machine, model AV-10 (AVURE Technologies, Erlanger, KY, USA), whereas the processing parameters were 400 MPa for 8 min at 30 °C. Untreated lees (hereafter referred to as CON) were used as control.

As for aging on lees, the white wine used in the trials was a blend of Sauvignon Blanc and Pinot Gris (harvest 2021), supplied by a local producer. The main enological parameters determined on the white wine after alcoholic fermentation were reported in Table A1 (Supplementary material).

Treated lees (ENZ and HPP) and CON were added to the white wine (5% v/v) in 750 mL green glass bottles. The wine without lees addition was used as reference (hereafter referred to as RW). Since flavanols were not detectable in the white wine used in the AOL trials, the wine blend was spiked with 30 mg/L of (+)-catechin before bottling and lees addition, in order to increase the potential oxidizability of wine and, consequently, to allow a better evaluation of the protecting ability of the lees.

After preparation, nitrogen was blown in the headspace of the samples and bottles were manually sealed with crown-cap closures. AOL was carried out at 20 ± 1 °C for three months. Bottles were kept in horizontal position throughout the aging period, to maximize the contact surface between lees and wine, and lees were resuspended twice a week to simulate *bâtonnage*. All samples were prepared in triplicate. Analytical determinations were carried out at the end of the aging period as described below.

2.5. Wine color and browning assay (POM-test)

Wine color (Abs 420 nm) and predisposition to browning (POM-test) were analyzed as reported by Comuzzo et al. (2015a). Briefly, wine color was assessed measuring the absorbance at 420 nm in 10 mm optical path length cuvettes and the readings were performed against distilled water. Concerning POM-test, 5 mL of wine were heated at 60 °C for 1 h, after the addition of 25 μL of a 3% (v/v) hydrogen peroxide solution. The predisposition to browning was then estimated based on the percent increase of the absorbance at 420 nm. Moreover, CIELAB parameters (L*, a*, b*) were also evaluated in 10 mm path length quartz cuvettes. Transmittance spectra were acquired in the wavelength range from 380 to 780 nm (0.5 nm intervals), reading against Milli Q water. CIELAB parameters were calculated for the CIE illuminant D65 and 10° standard observer conditions (Pérez-Magariño & González-San José, 2006) and the software used was Spectra Manager for Windows 95/NT (Spectra Analysis, version 1.53.04, Jasco Corporation). Delta E was also calculated (CIE 1976). All the spectrophotometric analyses were performed using a UV/Vis spectrophotometer model V-530 (Jasco Inc, Mary's Court Easton, Maryland, USA).

2.6. Evaluation of glutathione by RP-HPLC

The content of reduced glutathione was determined following the method described by Fracassetti et al. (2011). Analysis was performed using a LC-2010 AHT liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an integrated autosampler and a UV–visible detector, set at 303 nm. The separation column was a 5 μm, 150 × 4.6 mm Zorbax Eclipse Plus C18 (Agilent Technologies, Santa Clara, CA, USA), conditioned at 25 °C. The eluents were 0.05% v/v trifluoroacetic acid in Milli Q water (solvent A) and methanol (solvent B); gradient was set as follows: 10% of solvent B (initial conditions), increased from 10% to 35% in 18 min. Flow rate was 1 mL/min, and the injection volume was 20 μL. Concentrations were calculated in relation to a calibration curve prepared with standard solutions of reduced glutathione (0–400 μmol/L).

2.7. Evaluation of riboflavin by RP-HPLC

Riboflavin content was determined following the method described by Fracassetti et al. (2017) with some modifications. Wine samples were filtered on 0.22 μm cellulose acetate membranes and directly injected. Separation column was a Zorbax Eclipse Plus C18, 5 μm, 150 × 4.6 mm (Agilent Technologies, Santa Clara, CA, USA), conditioned at 25 °C. The mobile phase consisted of 0.05% v/v trifluoroacetic acid in Milli Q water (eluent A) and methanol (eluent B). Elution gradient used was set as follows: 30% solvent B in the first 2 min, then up to 60% B in 8 min; solvent B was increased at 100% at min 11 and held for the following 3 min; equilibration time before the following injection was 3 min at the initial conditions. The flow rate was 0.6 mL/min, and injection volume was 20 μL. Detection was spectrophotometric, at a wavelength of 440 nm and the concentrations were calculated in relation to a calibration curve prepared with standard solutions of riboflavin (0–200 μg/L).

2.8. Evaluation of polysaccharides determination by SE-HPLC

Polysaccharides (PS) were determined by SE-HPLC after ethanol precipitation, as reported by Palomero et al. (2007), with some modifications. Two milliliters of wine were added with 5 vol of ethanol (96% v/v) and stored at 0–4 °C for 24 h. The precipitated pellet was separated by centrifugation, washed twice with ethanol (96% v/v), resuspended in 2 mL of Milli Q water and filtered on 0.22 μm cellulose acetate membrane before injection. SE-HPLC separation was achieved by using a binary pump Model LC 250 (Perkin-Elmer, Waltham, MA, USA), equipped with a Rheodyne 7125 NS manual injection valve (Rheodyne, Rohnert Park, CA, USA) and a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). Separation columns were an 8 μm, 300 × 7.5 mm PL Aquagel-OH MIXED-H (Agilent Technologies, Santa Clara, CA, USA) and a 6 μm, 300 × 7.8 mm, Ultrahydrogel 250 (Waters, Milford, MA, USA). Mobile phase was MilliQ water, and the separation was carried out in isocratic conditions, with a flow rate of 0.7 mL/min and an injection volume of 20 μL. The concentrations of PS were calculated in relation to a calibration curve prepared with standard solutions of mannan (0–1000 mg/L).

2.9. Particle size distribution and filtration assay

Particles size distribution was evaluated by dynamic light scattering as reported by Natolino and Celotti (2022), whereas filtration assay was carried out by measuring the time necessary to filter 20 mL of centrifuged wines on 0.45 μm pore size cellulose acetate membranes (47 mm diameter); the filtration flow rate was calculated and expressed in mL/min/cm^2.

2.10. Wine volatile profile

For analyzing volatile compounds, 10 mL of wine were spiked with 50 μL of ethyl heptanoate (0.0984 g/L in ethanol 96% v/v – internal

standard) in 20 mL glass vials; sodium chloride (3 g) were added, and vials were sealed with PTFE/silicone septa. Wine volatile profile was then analyzed by SPME-GCMS, using a GC2030 Nexis gas chromatograph, coupled with a QP2020NX mass spectrometer (Shimadzu, Kyoto, Japan) and equipped with a GC autosampler (HTA, Brescia, Italy).The operating conditions adopted were reported by Voce et al. (2021). Briefly, the samples were pre-conditioned at 40 °C for 15 min and the microextraction was carried out for 15 min at the same temperature by using a 2 cm 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA). A J&W DB-Wax capillary column, 30 m × 0.25 mm, 0.25 μm film thickness (Agilent Technologies Inc., Santa Clara, CA, USA) was used for the GC separation, with the following operating conditions: 40 °C for 1 min, then 4 °C/min, up to 240 °C, with a final holding of 15 min. Injection was performed in splitless mode with 60 s of splitless time; injection port and transfer line were set at 250 °C and 240 °C respectively. Carrier gas was helium, at a linear flow rate of 35 cm/s. Electron impact mass spectra were recorded at 70 eV and the identification of volatile compounds was tentatively carried out by comparing their mass spectrum with those reported in spectrum library NIST 20. For each detected compound, linear retention index was also calculated based on the retention times of n-alkanes and compared with those reported in literature.

2.11. Wine sensory evaluation

Ethical approval for the involvement of human subjects was granted by the Institutional Review Board of University of Udine (IRB-DI4A, reference number 0006195, date of approval 11/28/2023) and all the tasters were completely voluntary; informed consent was also obtained from each taster.

An attribute difference test was carried out on the wines. All wine samples were labeled with three-digit numerical codes, poured in 20 mL aliquots into ISO wine glasses and presented to each taster, according to a balanced randomized service order; the tasting panel consisted of 12 experts in wine analysis (4 females and 8 males, age 25–60 years). Each panelist was asked to singularly evaluate the wines based on a series of pre-established attributes, in relation to the intensity; the scale used during the test was a line scale. Attributes were referred to wine color (yellow hue and browning), odor attributes (floral, fruity, vegetable/herbaceous, yeast-like notes and bread crust, reduction, oxidation, other defects, *i.e.*, leather and polish), taste (acidity, astringency, bitterness, body), aftertaste (floral, fruity, reduction, oxidation) and global impression (qualitative parameter). The test was carried out at the laboratory of sensory analysis of the University of Udine (LABAS); the software Smart Sensory Box (Smart Sensory Solutions S.r.l, Sassari, Italy) was used for determining the randomized service order and for collecting the data.

2.12. Statistical analysis

One-way ANOVA and Tukey HSD test were carried out for all the analytical parameters evaluated and differences were considered significant at $p < 0.05$. Concerning sensory analysis, data from each panelist and attribute were previously standardized, then subjected to one-way ANOVA and least significant difference (LSD Fisher test, $p < 0.05$). Factor analysis and principal component analysis (PCA) were also used for processing data referred to aroma compounds. All elaborations were carried out by the software Statistica for Windows Version 8.0 (StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Color evolution and protection against oxidation

As reported above, wines were analyzed after three months of AOL. The results obtained for wine color (Abs 420 nm and CIELAB parameters) and predisposition to browning (POM-test) were reported in Table 1, whereas the content of reduced glutathione was reported in Fig. 1.

A better color evolution was observed in all the treated wines compared to the reference sample (RW, no lees addition). In general, wines aged on lees treated by HHP showed the lowest values in terms of color (abs 420 nm) and yellow hue (b*), thus resulting statistically different from the others; furthermore, sample HS_HHP also showed the highest mean value of lightness (L* = 98.4) and the highest color difference compared to the reference wine, with a mean value of 1.8; only for the latter parameter, statistically significant differences were observed among wines aged on lees.

The potential oxidizability of the wines was also strongly affected by the addition of lees; POM-test showed values that tended to be higher in treated wines than in RW, demonstrating a better preservation of the phenolic fraction during the aging period. Sequential fermentation and HHP treatment generally led to better results in terms of protection against oxidation: the highest POM-test value was found in HS_HHP (67), which resulted significantly different from all the other samples, followed by HS_CON (52); furthermore, the addition of S_HHP lees also had a good effect on potential oxidizability (43). On the contrary, RW showed the lowest POM-test, with a mean value of 18. POM-test represents the potential oxidizability of wine, related to the phenolic fractions that are potentially oxidizable, but not yet oxidized. In RW, no lees were added. The absence of lees and free sulfur dioxide, known for their protective effect towards wine oxidation, have possibly determined a more intense and faster oxidation of the phenolic fractions, explaining the lower potential oxidizability and POM-test value observed in such wine compared to wines added with lees. In addition, the presence of *H. uvarum* in the lees biomass from sequential fermentation may have enhanced the release of glutathione in the respective aged wines, thus explaining the higher concentration of this compound detected in such wine samples compared to that released by the lees from single fermentation. Concerning the total GSH content in wines (Fig. 1), it is worth noting that, regardless of the treatment performed, all wines added with lees from sequential fermentation showed a greater amount of the tripeptide (up to 7.2 mg/L in HS_HHP), resulting statistically different from both RW and wines aged on lees from single fermentation. The higher release of GSH observed in HS_HHP wines might have positively contributed to better preserve wine phenolic fractions, resulting in a lower browning and higher POM-test value, as previously observed for this sample (Table 1). Nevertheless, a non-negligible effect of the treatment performed for processing lees was also observed, with the best results in terms of color evolution and protection against oxidation obtained with HHP treatment.

The protective effect of lees against wine oxidation is well known and this activity seems to be related to several factors. Comuzzo et al. (2015a) observed that the addition of yeast lees in white wine determined a faster oxygen consumption and intense radical scavenging activity, together with wine color evolution and mean values of POM-test at the end of aging period similar to those obtained by adding ascorbic acid. Furthermore, antioxidant activity exhibited by yeast lees may be related to the release of glutathione, but also to the presence of other small peptides and compounds containing thiol groups linked to the cell wall (Gallardo-Chacón et al., 2010; Jaehrig et al., 2007; Tirelli et al., 2010).

The possible interaction between polyphenols and yeast cell walls (Del Barrio-Galán et al., 2012; Gallardo-Chacón et al., 2010) has been proposed as another mechanism involved in the protective effect of lees against oxidations, consequentially impacting on wine color and predisposition to browning. Under white winemaking conditions, a reduction of wine color and total polyphenols has been previously observed in wines aged on lees and on yeast derivatives (Del Barrio-Galán et al., 2011). Similarly, the addition of different types of yeast derivatives (both cell walls, autolysates and mannoproteins) determined a reduction of wine color during aging, even if without determining significant

Table 1
Wine color evolution (abs 420 nm and CIELAB parameters) and predisposition to browning (POM-test) after three months of aging. Different letters within the same row marked significant differences among the samples, according to ANOVA and Tukey HSD test ($p < 0.05$). RW: reference wine, without lees addition; S: lees from single fermentation; HS: lees from sequential fermentation; CON: wines added with untreated lees; ENZ: wines added with lees treated by enzyme addition; HHP: wines added with lees treated by high hydrostatic pressure.

	RW			CON						ENZ						HHP					
				S			HS			S			HS			S			HS		
	Mean	SD[a]		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
Color (abs 420 nm)	0.106	± 0.000	d	0.091	± 0.002	bc	0.090	± 0.000	bc	0.098	± 0.005	cd	0.100	+ 0.004	cd	0.085	+ 0.001	ab	0.074	+ 0.003	a
L*	97.7	± 0.8		98.1	± 0.1		98.3	± 0.0		97.5	± 0.4		97.7	+ 0.2		97.8	+ 0.1		98.4	+ 0.3	
a*	−0.6	± 0.0	c	−0.6	± 0.0	abc	−0.7	± 0.0	a	−0.6	± 0.0	abc	−0.7	+ 0.0	ab	−0.6	+ 0.0	bc	−0.6	+ 0.0	abc
b*	6.0	± 0.2	bc	5.9	± 0.1	b	6.0	± 0.0	b	5.9	± 0.0	b	6.3	+ 0.1	c	5.1	+ 0.1	a	4.8	+ 0.0	a
ΔE	–	–		1.3	± 0.1	ab	1.4	± 0.0	ab	1.2	± 0.0	a	1.3	+ 0.0	ab	1.5	+ 0.0	b	1.8	+ 0.1	c
POM-test (%)	18	± 2	a	41	± 4	b	52	± 2	bc	34	± 11	ab	34	+ 8	ab	43	+ 1	b	67	+ 5	c

[a] SD: standard deviation.

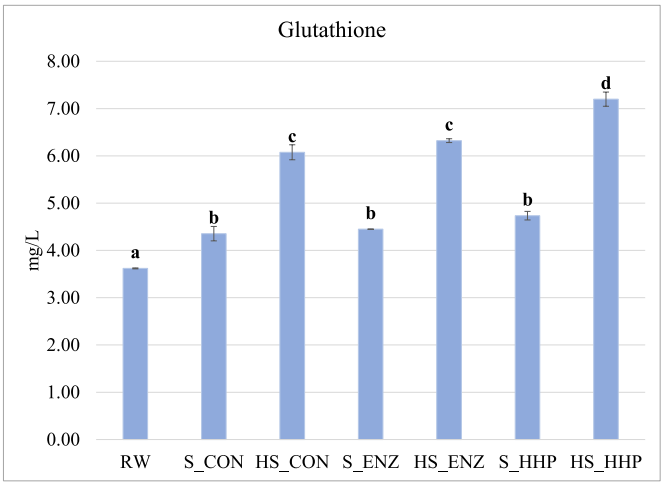

Fig. 1. Content of glutathione (mg/L) detected in wines after three months of aging. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test ($p < 0.05$). RW: reference wine; S: lees from single fermentation; HS: lees from sequential fermentation; CON: untreated lees; ENZ: lees treated by enzyme addition; HHP: lees treated by high hydrostatic pressure.

differences in the amount of polyphenols (Rodriguez-Nogales et al., 2012). Based on this evidence, it might be hypothesized that the adsorption of phenolic compounds on the lees surface could be one of the possible factors that might explain the less intense color observed in wines aged on lees compared to RW.

Furthermore, the release of intracellular components naturally occurs during AOL, also including GSH (Comuzzo et al., 2021), that is well-known for its antioxidant property. Similarly, the release of this tripeptide was observed in all the samples aged on lees compared to the reference wine, presumably improving the protective effect related to lees addition. This was particularly evident for the samples added with lees obtained from sequential fermentation (HS) and treated by HHP that in fact, as discussed above, resulted the richest in terms of GSH content. The highest release of such compound may have positively contributed to better color evolution and protection against oxidation, also confirmed by the lower browning and greater POM-test values observed in such samples.

All these antioxidant effects were well studied in lees, focusing on the autolysis of *Saccharomyces* spp., but few data are available in literature about the potential contribution of non-*Saccharomyces* yeasts on improving the antioxidant activity of lees during AOL. The ability of NSY to produce and release glutathione during single and sequential fermentation was previously observed, possibly reaching concentrations up to 10 mg/L at the end of the alcoholic fermentation (Binati et al., 2021). In previous experiments, Voce et al. (2022) investigated the ability of wild non-*Saccharomyces* strains to produce and release glutathione both after growth and enzyme-assisted lysis, finding interesting results for *Hanseniaspora* spp., as confirmed in the present experiment.

Concerning the technologies investigated in the present paper, to our knowledge, no evidence is reported in literature about the effect of HHP on preserving yeast antioxidant compounds (*e.g.*, glutathione). The low temperature reached during the treatment (about 30 °C) might have reduced the risk of oxidation phenomena or the loss of antioxidant molecules, thus possibly explaining the higher GSH content and the better behavior towards oxidation in wines aged on lees treated with this technology, especially if combined with lees from sequential fermentation. Lastly, to the authors' knowledge, no further literature data are reported about the effect of HHP on the extraction of antioxidant compounds from wine yeast, either for producing derivatives or for processing yeast lees.

3.2. Release of riboflavin

The amount of riboflavin slightly increased in presence of lees (Fig. 2). The highest mean concentration was detected in wines aged on lees from sequential fermentation, up to 93 and 100 μg/L in HS_ENZ and HS_CON, respectively, thus resulting statistically different from RW, where the vitamin was detected at 67 μg/L. It is interesting to note that HHP treatment determined a declining trend in riboflavin concentration, if compared to CON and ENZ samples (76 and 86 μg/L in S_HHP and HS_HHP, respectively); nevertheless, no further statistical differences were observed among treated wines.

A certain amount of riboflavin is naturally present in wines, originating from grapes (Ribereau-Gayon et al., 2006) or produced by yeasts during alcoholic fermentation (Fracassetti et al., 2017). The mean concentration in white wines may vary from 8 to 200 μg/L, depending on grape variety (Mattivi et al., 2000) and the yeast strain used (Fracassetti et al., 2017). It is known that, in bottled white wines and under specific conditions that include the presence of such vitamin (at least 50 μg/L) and methionine (at least 1.5 mg/L), riboflavin may be involved in the appearance of the so-called light-struck defect (Fracassetti et al., 2019). The content of riboflavin observed in the present study was comparable to those detected by Mattivi et al. (2000) in Cava sparkling wines (85.34 μg/L), which are generally subjected to a period of contact with lees of at least nine months (Francioli et al., 2003). Considering these results, the possible occurrence of light-induced reactions and the appearance of light-struck defect might be enhanced by AOL, since the concentration of riboflavin tended to increase with lees contact. However, it is worth noting that the contribution of lees can be considered negligible, since most of the riboflavin content was already present into the wine without lees addition (RW). It is also interesting to highlight that, in terms of mean values, HHP determined a reduced release of riboflavin in the respective aged wines, if compared to the same lees typology subjected to enzyme addition (ENZ) or without treatment (CON).

3.3. Release of polysaccharides

As expected, AOL increased wine polysaccharides content (Table 2). PS were also detected in RW (about 93 mg/L), probably derived from grapes (Guadalupe & Ayestarán, 2007) or released by yeasts during alcoholic fermentation (Escot et al., 2001). In general, the addition of lees from single fermentation, in particular untreated lees (S_CON) and

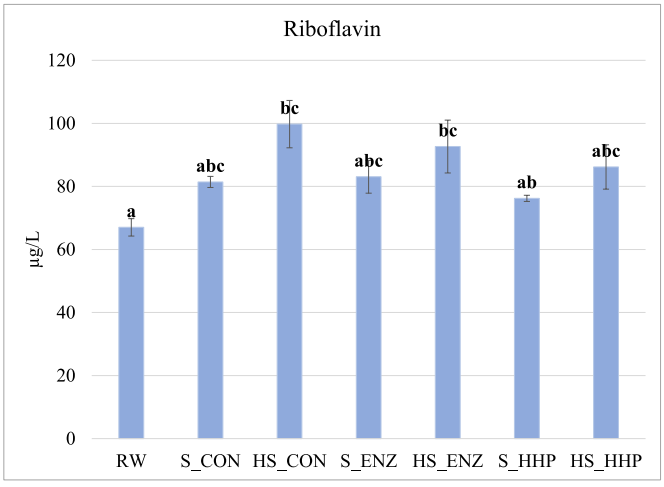

Fig. 2. Content of riboflavin (μg/L) detected in wines after three months of aging. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test ($p < 0.05$). RW: reference wine; S: lees from single fermentation; HS: lees from sequential fermentation; CON: untreated lees; ENZ: lees treated by enzyme addition; HHP: lees treated by high hydrostatic pressure.

Table 2
Polysaccharides content, mean particle size and filtration flow after three months of aging. Different letters within the same row marked significant differences among the samples, according to ANOVA and Tukey HSD test ($p < 0.05$). RW: reference wine, without lees addition; S: lees from single fermentation; HS: lees from sequential fermentation; CON: wines added with untreated lees; ENZ: wines added with lees treated by enzyme addition; HHP: wines added with lees treated by high hydrostatic pressure.

	RW			CON						ENZ						HHP					
				S			HS			S			HS			S			HS		
	Mean		SD[a]	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD	Mean		SD
Polysaccharides (mg/L)	9	±	11	123	±	9	99	±	8	138	±	8	119	±	48	143	±	7	169	±	6
Mean particle size (nm)	514	±	7 a	1096	±	136 b	1203	±	2 b	1464	±	161 b	2069	±	107 c	1047	±	156 b	993	±	213 b
Filtration flow (mL/min/cm^2)	0.8	±	0.0	0.7	±	0.0	0.7	±	0.0	0.7	±	0.0	0.7	±	0.0	0.7	±	0.0	0.7	±	0.0

[a] SD: standard deviation.

lees subjected to enzyme addition (S_ENZ), increased polysaccharides content to about 123 mg/L and 138 mg/L, slightly higher than the mean values detected in wines aged on lees from sequential fermentation (99 mg/L and 119 mg/L detected in HS_CON and HS_ENZ, respectively). However, lees processed by HHP promoted the highest release of PS into the wines, with mean values of 143 mg/L in S_HHP and 169 mg/L in HS_HHP, the latter determining the highest concentration of PS.

The ability of AOL to increase wine polysaccharides is known and it is reported to occur during the first two months of aging (Del Barrio-Galán et al., 2011), thus confirming the trend here observed. In previous studies, different authors highlighted a good ability of *Hanseniaspora* spp. to produce high contents of polysaccharides during fermentation (Domizio et al., 2014; Giovani et al., 2012), with differences also dependent on the strain (Romani et al., 2010). Furthermore, under real winemaking condition, Del Fresno et al. (2021) observed how the release of polysaccharides after sequential fermentation (*Hanseniaspora* spp. followed by *Saccharomyces* spp.) was higher compared to single fermentation, whereas after one year of aging the concentration of PS detected in wines aged on lees from *Hanseniaspora* spp. was lower than those found using only *Saccharomyces* spp. These results might explain the reason why HS wines aged on lees from sequential fermentation – both untreated (CON) and subjected to enzyme-induced lysis (ENZ) – showed a lower PS content compared to those subjected to the same treatment but aged on lees from single fermentation (S).

However, the treatment applied for processing lees also had a pivotal role in promoting PS release. Cacciola et al. (2013) observed that the addition of β-glucanase enzymes to fermentation lees enhanced the release of soluble colloids, thus confirming the greater mean concentration of polysaccharides detected in S_ENZ and HS_ENZ wines compared to CON samples (untreated lees).

On the other hand, HHP also showed a very interesting effect, because of its ability to maximize PS concentration, especially when combined with lees from sequential fermentation (HS_HHP).

High pressure treatments have been reported to be suitable for extracting yeast glucans (Dimopoulos et al., 2020) and for producing yeast derivatives (Comuzzo et al., 2015b; Voce et al., 2021), thus confirming the highest tendential release of polysaccharides in wines added with lees obtained by HHP. Currently, high hydrostatic pressure is recommended by OIV for eliminating wild, spoilage microorganisms in grape must (Resolution Oeno 594A/2019) but, considering the results obtained in the present study, it might be also applied in winemaking as an alternative method to accelerate autolysis, potentially reducing the time needed for AOL.

3.4. Particle size distribution and filtration assay

Both mean particle size and wine filterability were affected by lees addition after the aging period (Table 2); concerning the former, all the aged wines significantly differed from RW samples, except for HS_HHP, the latter showing a mean value of about 993 nm.

The highest mean particles size was observed in the wines added with lees subjected to enzyme treatment; S_ENZ and HS_ENZ showed mean values of 1464 nm and 2069 nm, respectively, with the latter being statistically different from all the other samples. During aging on lees, the release of soluble molecules, *e.g.*, polysaccharides and mannoproteins, and the potential formation of aggregates with wine components (Dupin et al., 2000; Guadalupe & Ayestarán, 2007; Guadalupe et al., 2010) may impact on wine colloidal state, potentially explaining the increase in the mean size of soluble particles here observed. Furthermore, soluble colloidal particles may be subjected to several modifications in terms of molecular weight (Guadalupe & Ayestarán, 2007), particle size and charge also in relation to the treatment performed for inducing autolysis (Cacciola et al., 2013).

In a study carried out by Vaquero et al. (2022), the authors observed that the application of Ultra High-Pressure Homogenization (UHPH) for processing must determined a reduction in the average size of colloidal particles, compared to untreated samples. This finding might in part support the trend here observed, with wines aged on lees treated by HHP with mean values lower than CON (lees without treatment).

As expected, the presence of lees also determined a reduction in filtration flow. The lowest mean values were observed in wines aged on lees, even if no significant differences were observed among the samples, neither from a statistical nor from a practical point of view. In a previous work, the addition of mannoproteins for tartaric stabilization slightly reduced white wine filterability compared to untreated wine, also depending on the dosage used (Puškaš et al., 2021). The results obtained in the present study might suggest that the treatment performed for processing lees have a limited effect on wine filterability, with most of the reduction in filtration flow linked to AOL itself.

3.5. Wine aroma profile

One hundred-fourteen volatile compounds were tentatively identified in the headspace of wines and the results of qualitative and semi-quantitative analysis were reported in Tables A2 and A3, respectively (Supplementary material); in addition, the results of PCA carried out on the concentrations (μg/L) of the most significant variables (volatile compounds), resulting from Factorial Analysis for marked factor loadings >0.7, were reported in Fig. 3 (a and b). The addition of lees determined an enrichment in aroma compounds, with treated samples showing the most intense and complex volatile profile. Reference sample (RW) showed the less characterized volatile profile, with higher average concentrations of aldehydes (ALD02, 2-furfural) and some norisoprenoids, *e.g.*, β-damascenone (N08); the wines aged on untreated lees (CON) and on lees subjected to enzyme addition (ENZ) showed the higher concentration of esters (both ethyl esters and other esters, EE and AE in the PCA plot), 1-decanol (AL15), citronellol (T09) and vitispirane-like compound (N03). It is interesting to note that wines added with both lees treated by HHP, especially those obtained from sequential fermentation, were mainly characterized by a higher concentration of acetic esters, *i.e.*, ethyl acetate, 2- and 3-methyl-1-butanol acetate and hexyl acetate (EA01, EA04, EA03 and EA05, respectively) and β-myrcene (T01), together with some volatile phenols, *i.e.*, 4-ethylguaiacol and 4-ethylphenol (PH01 and PH02 in the PCA plot).

During wine aging on lees, the modification and the subsequent modulation of wine volatile profile occurs, due to the release of aroma compounds from lees as products of yeast metabolism, mainly including esters and higher alcohols (Del Barrio-Galán et al., 2012); their concentration may further increase with an improvement of wine aroma complexity and sensory perception (Loira et al., 2013), thus confirming the trend here observed. By considering the concentrations of volatile compounds reported in Table A3 (Supplementary material), 2- and 3-methyl-1-butanol acetate were found in the highest concentrations in wines aged on lees treated by HHP, especially with those obtained from sequential fermentation, resulting significantly different from the reference sample (RW); furthermore, the same samples showed the higher mean concentration of hexyl acetate. The tendential increase in acetic esters observed in such aged wines might positively impact on wine volatile profile, since these aroma compounds generally confer fruity notes (Swiegers et al., 2005); furthermore, the ability of *Hanseniaspora* spp. to produce high amounts of acetic esters during single (Del Fresno et al., 2020; Lleixà et al., 2016) and sequential fermentation (Medina et al., 2013) has been previously reported, also depending on the strains used (Moreira et al., 2008) and grapevine variety (Martin et al., 2019). This might possibly explain the highest average concentration of acetic esters detected in HS_HHP wines.

Similarly, the total concentration of higher alcohols tended to increase during AOL (Table A3). Generally, it would be said that wines aged on lees obtained from single fermentation showed tendential higher content of total alcohols, regardless of the treatment performed for processing lees. In a study carried out by Lleixà et al. (2016), the authors observed that wines fermented by *S. cerevisiae* were

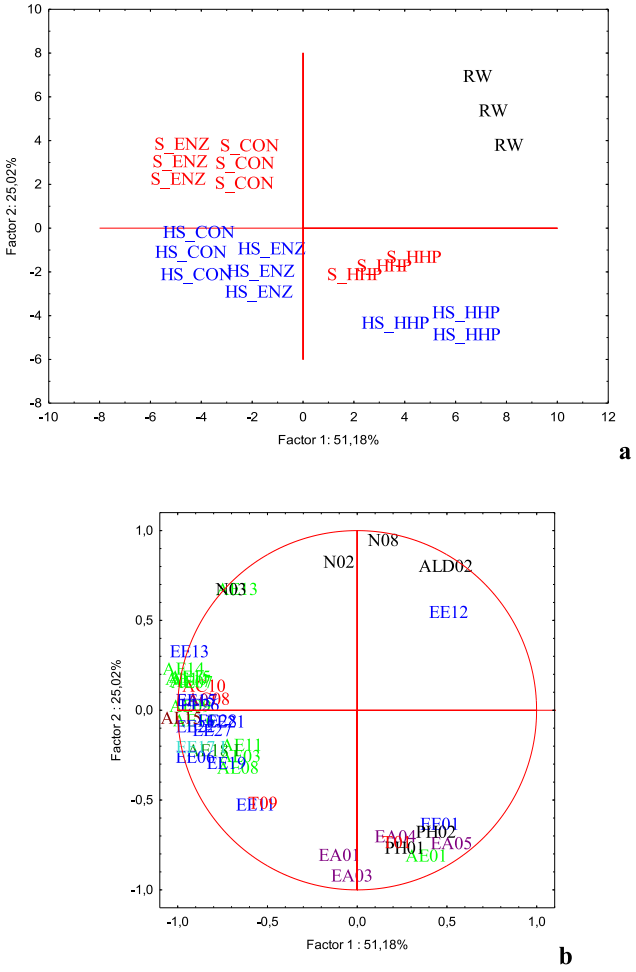

Fig. 3. Results of the PCA carried out on the concentration (μg/L) of volatile compounds detected in the headspace of aged wines. Projection of cases (samples) (**a**) and variables (volatile compounds) (**b**) on the factor-plan were reported. Factor Loadings (FL) were calculated by Factor Analysis, and the most relevant variables were selected for marked FL > 0.7. RW: reference wine; S: lees from single fermentation; HS: lees from sequential fermentation; CON: untreated lees; ENZ: lees treated by enzyme addition; HHP: lees treated by high hydrostatic pressure; AC: acids (red); AL alcohols (brown); ALD: aldehydes (black); AE: other esters (light green); EA: acetate esters (violet); EE: ethyl esters (blue); N: norisoprenoids (black); PH: phenols (black); T: terpenes (red);. The numbers reported after letters indicated the specific aroma compound (for details, see Table A3).

characterized by higher concentration of alcohols compared to those obtained by *Hansensiaspora vinae*, thus confirming the trend observed in the present study. However, the increase of such compounds in wines after AOL might further contribute to improve wine aroma and sensory profile since some of them, *e.g.*, 2-phenylethanol, generally confer floral and rose notes (Swiegers et al., 2005).

Concerning fatty acids, an increase in the total amount of such compounds was observed in all the wines aged on lees compared to the reference sample, with acetic, octanoic and decanoic acids the most representative. Acetic acid was detected in similar concentration among the samples - ranging from 10 to 13 μg/L - whereas significant differences were observed concerning octanoic and decanoic acids (Table A3). The lowest concentrations were generally detected in wines added with lees obtained from sequential fermentation; furthermore, by comparing the same lees typology subjected to different treatments, it is interesting to note that the lowest average content of fatty acids was detected in wines added with lees treated by HHP. This might positively impact on wine aroma profile and sensory perception, since such volatile compounds are described with sour, pungent, and cheese-like notes (Comuzzo et al., 2006).

Aldehydes mostly characterized reference wine (1.18 μg/L), whereas the lowest mean concentration of total aldehydes was detected in wines aged on lees obtained by sequential fermentation. However, HHP treatment gave interesting results, especially in the case of HS lees (0.47 μg/L), determining the lowest concentration of such compounds in the respective added wines. Aldehydes may be produced as intermediate of yeast metabolism (Swiegers et al., 2005) or by the occurrence of oxidation phenomena (Bueno et al., 2016); as previously discussed about wine color evolution, the reference sample resulted the most oxidized sample, thus possibly explaining the tendential higher concentration of aldehydes in such sample due to oxidation phenomena. Furthermore, it has been reported that aroma compounds may be adsorbed on the lees surface, also including aldehydes (Gallardo-Chacón et al., 2009), thus possibly explaining the lowest concentration found in wines aged on lees, compared to the reference wine (RW).

During AOL, an increase in the total content of volatile phenols was observed, except for S_CON that in fact showed the lowest total average concentration (2.93 μg/L, Table A3). The two compounds detected, 4-ethylguaiacol and 4-ethylphenol, were generally found in higher concentration in wines aged on lees from sequential fermentation; furthermore, S_HHP and HS_HHP were characterized by the highest content of such compounds, thus resulting statistically different from all the other samples. Even if such compounds are produced by *Brettanomyces* spp., their presence in wines fermented by *H. vinae* was previously reported (Lleixà et al., 2016), thus possibly explaining the higher average concentration of such compounds in wines aged on lees from sequential fermentation.

3.6. Wine sensory evaluation

The results of sensory analysis carried out on wines after aging were reported in Fig. 4 (a, color and odor attributes; b, taste attributes; c, aftertaste attributes and global impression), whereas the mean scores of the attributes that resulted statistically different are reported in Table A4 (Supplementary material).

After aging on lees, an overall improvement in terms of aromatic intensity and complexity was observed in almost all the treated wines compared to the reference sample, except for HS_CON and HS_ENZ, that in fact obtained the lowest scores in terms of global impression (2.78 and 2.99, respectively), probably due to the presence of reduction notes. This improved wine sensory perception after aging was in line with what previously reported by other authors (Loira et al., 2013) and it might be dependent on several factors, *i.e.*, the release of some volatile compounds from lees (Del Barrio-Galán et al., 2011, 2019), the enhanced volatility of some aroma compounds due to their interaction with yeast mannoproteins (Juega et al., 2012) or the adsorption phenomena of some odor-active compounds onto lees surface (Gallardo-Chacón et al., 2009).

Concerning color attributes, the highest scores in terms of yellow hue and browning were attributed to the reference wine, and the wines added with untread lees and lees subjected to enzyme addition (in particular, S_CON, S_ENZ and HS_ENZ), whereas wines aged on both the lees (S and HS) treated by HHP obtained the lowest ones. This was in line with what previously observed concerning wine color evolution, with the lowest browning intensity attributed to wines aged on lees subjected to HHP treatment. Concerning the odor attributes, S_HHP, S_ENZ and HS_HHP obtained the highest scores in floral and fruity notes, and the lowest values in reduction notes (Fig. 4a; for values, see Table A4).

By considering taste and aftertaste (Fig. 4b and c, respectively), the highest scores in terms of floral and fruity notes together with lower perception of reduction and oxidation notes were attributed to wines aged on lees treated by HHP; as described above, these samples generally showed higher concentrations of acetic esters and some terpenes,

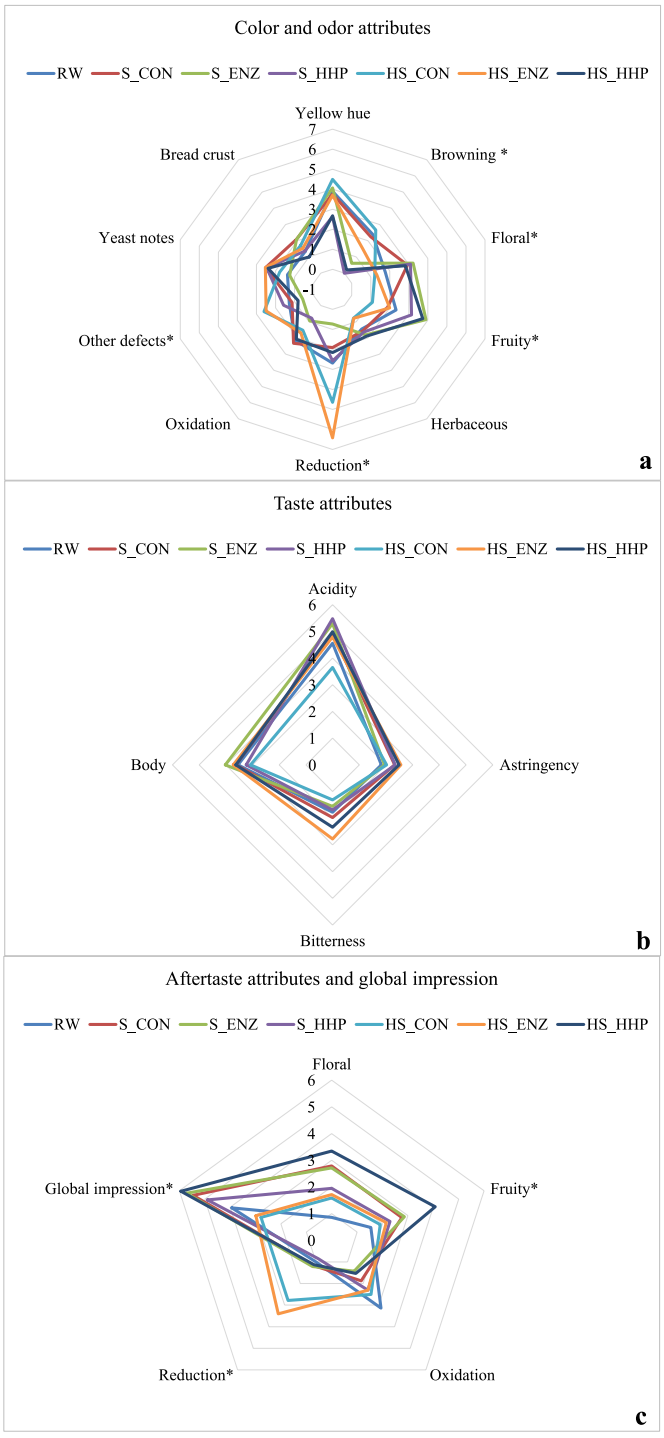

Fig. 4. Mean scores of sensory profiles of wines after three months of aging. Color and odor attributes (**a**), taste attributes (**b**), aftertaste attributes and global impression (**c**). RW: reference wine; S: lees from single fermentation; HS: lees from sequential fermentation; CON: untreated lees; ENZ: lees treated by enzyme addition; HHP: lees treated by high hydrostatic pressure. *Significant different according to ANOVA and LSD Fisher test at $p < 0.05$.

and a lower mean content of aldehydes. Lastly, wines added with lees from sequential fermentation and treated by HHP also resulted the most preferred by the panelist group, with a final score in terms of global impression of 5.9. In general, it might be concluded that sensory analysis confirmed what previously determined from the chemical point of view.

4. Conclusion

The outcomes of this study offer insights to improve the winemaking process, both modulating the composition of lees, adapting them to specific wine targets, and applying suitable processing methods for accelerating yeast autolysis, potentially reducing aging time.

High hydrostatic pressure seems to be a suitable strategy for managing AOL, also leading to a better color evolution and protecting wine against oxidation (both in terms of browning potential and glutathione content), especially when HHP is associated with aging on lees obtained from sequential fermentation, carried out with specific yeast strains.

The approach investigated in the present paper suggests that the antioxidant ability of yeast lees can be improved by integrating biotechnologies and emerging physical treatments, potentially allowing the reduction of sulfur dioxide and improving wine quality.

Further investigations on the performance of different yeast strains (*e.g.*, *Torulaspora* spp., *Hanseniaspora* spp., *Lachancea* spp., *Metschnikowia* spp., *Schizosaccharomyces* spp.) and applied technologies (*e.g.*, high pressure treatments, ultrasounds, pulsed electric fields) will give other opportunities for exploiting the potential of yeast lees as winemaking aid, creating the basis for an innovative and integrated approach, well suited to a context of sustainable enology.

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CRediT authorship contribution statement

Sabrina Voce: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lara Tat:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Anna Bortolini:** Investigation. **Andrea Colautti:** Writing – review & editing, Investigation. **Piergiorgio Comuzzo:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest regarding what discussed in the present paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.104335.

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