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Titolo della tesi

Investigation about new concepts of yeast derivatives for winemaking with enhanced antioxidant properties and polysaccharides content

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Riassunto

I derivati di lievito vengono utilizzati nell'industria enologica allo scopo di arricchire il vino in sostanze colloidali, in particolare polisaccaridi e molecole antiossidanti, e di migliorarne la stabilità nel corso dell'affinamento. Questi prodotti sono ottenuti a partire da biomasse di lievito sottoposte a trattamenti termici o enzimatici, e spesso caratterizzati dalla presenza di sostanze (per es., pirazine, acidi grassi e composti aromatici) che, rilasciate nel vino, possono causare la comparsa di *off-flavor*. Inoltre, i ceppi attualmente autorizzati per la produzione di derivati appartengono al genere *Saccharomyces* spp.

Nella presente tesi di dottorato, l'obiettivo è stato quello di produrre derivati di lievito innovativi per scopi enologici, caratterizzati da una migliore composizione chimica, maggiore attività antiossidante e minore impatto sensoriale, a partire da ceppi non-*Saccharomyces*, mediante l'impiego di tecnologie emergenti, non-termiche.

Tra diversi ceppi di lievito non-*Saccharomyces*, isolati da mosto e vinacce, *Hanseniaspora* spp. ha mostrato una buona attitudine alla produzione di composti di interesse enologico - colloidi solubili e antiossidanti - con buone potenzialità di impiego come co-starter di fermentazione, per il miglioramento delle caratteristiche del vino durante l'affinamento o per la produzione di derivati.

Tra i parametri di processo valutati nel presente studio, diverse condizioni di crescita hanno determinato una diversa produzione di biomassa e un differente rilascio di molecole antiossidanti e colloidi solubili sia in fase di crescita che dopo lisi indotta, con risultati interessanti ottenuti ancora una volta da *Hanseniaspora* spp.

Sia il lievito di partenza che i trattamenti applicati per indurre l'autolisi hanno influenzato la composizione chimica dei derivati di lievito con interessanti risultati ottenuti utilizzando *Pichia* spp. e *Torulaspora* spp.; le tecnologie emergenti come l'alta pressione idrostatica e gli ultrasuoni sono apparse delle valide alternative ai metodi convenzionali per la produzione di derivati (trattamento termico e lisi enzimatica). In fase di affinamento di un vino bianco, l’aggiunta di derivati di lievito ha consentito una buona protezione nei confronti dell’ossidazione e una buona evoluzione del colore dopo sei mesi di contatto, in confronto ai controlli solfitati; vini addizionati con derivati ottenuti per via enzimatica e mediante ultrasuoni hanno mostrato una maggiore caratterizzazione aromatica, mentre derivati ottenuti mediante trattamento termico e alta pressione idrostatica hanno determinato un profilo aromatico più povero nei rispettivi vini.

L’impatto dei lieviti non-*Saccharomyces* è stato valutato anche in condizioni di vinificazione. Un ceppo selezionato di *Hanseniaspora* spp. è stato utilizzato come co-starter di fermentazione, al fine di valutarne l’impatto al termine del processo fermentativo e, soprattutto, in fase di affinamento. L’impiego di fecce ottenute da fermentazione sequenziale ha consentito un miglioramento della composizione chimica - con un maggiore rilascio di polisaccaridi (mannani) e molecole antiossidanti - una buona protezione nei confronti dell’ossidazione, un miglioramento del profilo aromatico e sensoriale del vino, in condizioni reali di vinificazione.

Infine, è stata valutato il potenziale uso dei derivati di lievito per l’alimentazione umana; è stata osservata una certa bioattività, soprattutto in termini di proprietà antiossidanti ed effetto ipolipidemico, rendendo tali prodotti potenzialmente utilizzabili come ingredienti nella preparazione di cibi funzionali o come integratori alimentari.

Parole chiave: non-*Saccharomyces*; antiossidanti; colloidi solubili; derivati di lievito; tecnologie non-termiche; affinamento su feccia

Abstract

Yeast derivatives are used in winemaking for enriching wine in colloidal substances, polysaccharides and antioxidant molecules, and for improving wine stability during aging. Currently, these products are produced starting from yeast biomass subjected to thermal or enzymatic treatments and often characterized by the presence of compounds (i.e., pyrazines, fatty acids, odor active compounds) that may lead to the appearance of *off-flavors* when released into the wine. In addition, the only strains currently authorized for yeast derivatives production belong to *Saccharomyces* spp.

The aim of the present research project was to produce innovative yeast derivatives for winemaking use, characterized by better chemical composition, high antioxidant properties and low odor impact, starting from non-*Saccharomyces* strains, by non-thermal approach.

Among different spontaneous non-*Saccharomyces* strains, isolated from grape must and pomace, *Hanseniaspora* spp. has showed a good aptitude to produce compounds of enological interest – soluble colloids and antioxidant compounds – with potential use as fermentation co-starter, for improving wine aging on lees or for yeast derivatives production.

Among the processing parameters evaluated in the present study, different growth conditions have differently impacted on biomass production and release of antioxidant molecules and soluble colloids, both after growth and induced lysis, with interesting results obtained once again by *Hanseniaspora* spp.

Both strain and treatment for inducing yeast autolysis have also affected the chemical composition of derivatives, with interesting results obtained by *Pichia* spp. and *Torulaspora* spp.; emerging, non-thermal technologies – high hydrostatic pressure and ultrasounds – have seemed to be good alternatives to traditional methods for producing yeast derivatives (thermal inactivation and enzyme addition). During wine aging, the addition of yeast derivatives has allowed a good protection against wine oxidation and good wine color evolution after six months, compared to sulfite samples; wines added with derivatives obtained by enzyme addition and ultrasounds have showed the most characterized volatile profile, whereas the addition of derivatives obtained by thermal inactivation and high hydrostatic pressure have determined the poorest volatile profile in the respective added wines.

The impact of non-*Saccharomyces* yeasts has also been evaluated under winemaking conditions. Selected strain of *Hanseniaspora* spp. has been used as fermentation co-starter in order to assess its impact during alcoholic fermentation and, above all, during aging on lees. The use of lees from sequential fermentation has allowed to improve the chemical composition of wine - with highest release of soluble colloids (mannan) and antioxidant compounds - together with good protection against oxidation and an improved volatile and sensory profile under real winemaking conditions.

Lastly, the potential use of yeast derivatives for human nutrition has also evaluated; a certain bioactivity was observed, especially concerning antioxidant properties and hypolipidemic effect, potentially making them suitable as ingredients for functional foods preparation or dietary supplements.

Keywords: non-*Saccharomyces*; antioxidants, soluble colloids, yeast derivatives, non-thermal technologies, aging on lees

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Chapter 1

Evaluation of the aptitude of non-*Saccharomyces* yeast strains as fermentation co-starters and for yeast derivatives production

1. Introduction

Non-*Saccharomyces* yeasts (NSY) have long been considered as undesirable or spoiling microorganisms in food and wine industry but in the last years, there was an increasing interest on their use for the production of alcoholic beverages, especially wine [1]. This is mainly due to their ability to enhance varietal characteristics and to improve the aroma profile, especially when used in sequential or mixed fermentation with *S. cerevisiae* [2,3].

Generally, NSY show slower growth rate and lower fermentation capacity compared to *S. cerevisiae* [4], with minor yield in ethanol and glycerol and higher production of volatile acidity [2], but an improvement of the aromatic complexity and color stability of wines were observed [5], depending on yeast strain [2,3], nitrogen source [6], metabolism [7,8] and fermentation conditions [9]. Positive effects were also observed during sparkling wine production: Medina-Trujillo and colleagues [10] observed that the sequential inoculation of *T. delbrueckii* and *S. cerevisiae* during the fermentation of base wine improved the foaming properties of the sparkling wine obtained, mainly due to the higher release of proteins with low molecular weight.

An interesting feature that characterizes many NSY is the higher release of soluble colloids during alcoholic fermentation, in comparison with *S. cerevisiae*, maybe related to the greater production capacity or faster cell turnover during the growth phase [4]. The polysaccharide fractions mainly consist in mannoproteins with high mannose/glucose ratio [4] or galactomannoproteins [11,12]; the release may vary during the different stages of alcoholic fermentation, in terms of composition (*e.g.*, monosaccharide moieties) and protein fraction, mainly depending on the yeast strain [4,13]. Furthermore, higher release of soluble colloids was also observed during aging on lees, due to the different structure and chemical composition of cell walls as well as the different autolytic capacity of the strains [12,14]; their use might allow to reduce the conventional period of aging on lees, by releasing greatest amounts of soluble colloids and mannoproteins in a shorter period compared to *S. cerevisiae*.

Concerning the potential of NSY to protect wine against oxidation, most of the studies available in literature mainly focused on their use during alcoholic fermentation, with positive effects on the stability of the phenolic fraction and an improved formation of stable pigments in red wines (*e.g.*, vitisins or other anthocyanin-derived compounds), mainly due to the higher production of some compounds, like pyruvic acid and acetaldehyde [3,15]. Moreover, glutathione (GSH) and other yeast compounds containing cysteine residues (as well as yeast lees themselves) are well known for their antioxidant properties; this feature has mainly been studied in *S. cerevisiae* [16,17], but the increasing interest towards NSY has recently led to investigate their GSH metabolism too. A higher GSH/GSSG ratio in dry cells during active dry yeast preparation [18] and a higher amount of reduced GSH produced during single and sequential fermentation were observed [19], highlighting once again the great potential of the use of NSY in winemaking.

Regarding to nitrogen sources, these compounds are considered an important factor for yeasts metabolism and growth, thus conditioning biomass production, fermentation kinetics and volatile compounds production, with different consumption and utilization that are strain-dependent [6]. On the other hand, amino acids are precursors of biogenic amines, whose presence into the wine is considered harmful for human health, also determining *off-flavors* if in excess. Although a certain amount of biogenic amines is present in the grapes, the major part is produced by microorganisms during fermentation and aging on lees: in this regard, the use of NSY in spontaneous or sequential fermentation with *S. cerevisiae* seems to be a good strategy to reduce the amount of abovementioned compounds, determining a lower production or preventing the occurrence of malolactic fermentation [20].

2. Aim of the work

The aim of this work was to investigate the ability of different non-*Saccharomyces* wild strains isolated from red grape pomace and must, to produce soluble colloids and antioxidant compounds, with the purpose of evaluating their potential application during fermentation, for improving wine aging on lees, or for yeast derivatives production. Twenty yeast strains were isolated and identified, based on their colony and cell morphology (evaluated on differential growth media and by optical microscopy) as well as by 26S rRNA sequencing. The aptitude of the strains isolated to be used for winemaking applications was evaluated in terms of yeast cell viability and biomass production, release of soluble colloids, antioxidant compounds and amino nitrogen after growth and after autolysis induced by β-glucanase enzymes, in comparison with commercial strains of *S. cerevisiae* and *T. delbrueckii*.

3. Materials and Methods

3.1 Reagents

Sodium chloride for microbiology, bacteriological peptone, Malt Extract Broth, WL Nutrient agar (WLN) and technical agar were purchased from Oxoid (Milan, Italy); Yeast-Extract Peptone Dextrose broth (YPD) and tetracycline, sodium hydroxide, N-acetyl-L-cysteine, *o*-phthaldialdehyde (OPA), isoleucine, ethanol (96 % v/v), mannan from *S. cerevisiae*, 4,4′-dithiodipyridine (DTDP), sodium acetate, ethylenediaminetetraacetic acid (EDTA), L-glutathione reduced, sodium phosphate, yeast glutathione reductase, NADPH, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were from Sigma Aldrich Italy (Milan, Italy). Boric acid and hydrochloric acid (37 % v/v) were purchased from Carlo Erba Reagents (Milan, Italy). MilliQ water was produced by MilliQ Advantage A10 apparatus (Merck Millipore, Billerica, MA, USA) and microfiltered at 0.22 μm before use. Commercial β-glucanase preparation and commercial active dry yeasts (ADY) *S. cerevisiae* and *T. delbrueckii* were from Enologica Vason S.p.A. (San Pietro in Cariano, VR, Italy).

3.2 Isolation and identification of wild yeast strains

Wild yeast strains were isolated from pomace and must of red grapes cv. Merlot, supplied by a local producer (Friuli Venezia Giulia – North-East Italy). Ten (10) g of pomace were transferred into a sterile stomacher bag, 90 mL of saline-peptone water (8 g/L NaCl, 1 g/L bacteriological peptone) were added and mixed for 1.5 min in a Stomacher machine (PBI, Milan, Italy); in the case of must, 1 mL was directly sampled after homogenization. Decimal dilutions were made in the same physiological solution and yeasts were counted on WL Nutrient Agar, incubated at 30 °C for 48 hours under aerobic conditions. Twenty colonies were isolated from the agar plates in relation to their morphology, color and size. Isolates were streaked and purified on Malt

Extract agar plates and then stored at -80 °C in the Malt extract broth, supplemented with glycerol (30% final concentration). The isolates were then identified.

A first identification step was performed by observing the morphology of the colonies on WL Nutrient agar, whereas cells morphology was observed by phase-contrast optical microscopy (magnification 100x). Based on their morphological features, the strains were then grouped and at least one strain per group was selected to confirm the preliminary identification via 26S rRNA gene sequencing as described below.

3.3 DNA extraction from pure cultures

One milliliter of an overnight culture was centrifuged at 14,000 x g for 10 min at 4°C to pellet the cells and the pellet was subjected to DNA extraction, according to Andrighetto et al. [21].

3.4 Identification of yeast isolates

Yeasts were identified by molecular methods by 26S rRNA sequencing. DNA of the isolated strains was used as target for PCR reaction using primers NL1, 5'-GCC ATA TCA ATA AGC GGA GGA AAA G-3' and NL4, 5'-GGT CCG TGT TTC AAG ACG G-3' according to the reaction mixture and amplification protocol described by Iacumin et al. [22]. After purification using the QIAquick PCR Purification Kit (Qiagen, Milan, Italy), following the Handbook instruction, products were sent to a commercial facility for sequencing (Eurofins MWG GmbH, Martinsried, Germany). Lastly, sequences were aligned with those in GeneBank with the Blast program [23] to determine the closest known relatives, based on the partial 26S rRNA gene homology.

3.5 Cell viability, biomass production and lysis treatment

For each strain identified, a single pure colony was transferred into sterile test tubes containing 3 mL of YPD broth and incubated at 30°C for 48 hours; three aliquots of 100 μL of the suspensions were then transferred in sterile test tubes, each containing 10 mL of YPD broth and further incubated at 30°C for 48 hours.

Viable cells were counted after growth, by plating 100 μL of proper serial dilutions of each sample onto Malt Extract Agar added with tetracycline and incubated for 48 hours at 30°C, with results expressed as the logarithm of colony forming units (log CFU/mL). Furthermore, the production of biomass was assessed by filtering under vacuum 10 mL of yeast suspensions on pre-weighted 0.45 μm cellulose acetate membranes; after filtration, membranes were washed with sterile water and placed in oven at 60 °C for 2 hours up to constant weight. The difference between the initial and final weight was used to calculate the biomass and the results were expressed in g/L.

After growth, additional aliquots of yeast suspensions were centrifuged (5 min, 3000 rpm), the biomass was washed twice with sterile water, resuspended in 10 mL of MilliQ water and subjected to enzymatic lysis. A commercial β-glucanase preparation was added (5 % w/v) and samples were incubated at 45 °C for 24 hours.

Both suspensions (at the end of the growth phase) and autolyzed cultures (after enzyme treatment) were subjected to the analytical determinations reported below and the data were standardized to 10^9 cells. All trials were performed in triplicate and the performances of the strains isolated were compared with those of two commercial active dry yeast preparations of *S. cerevisiae* and *T. delbrueckii* (hereafter referred as S-COMM and T-COMM, respectively). Concerning the samples analyzed at the end of the growth phase, because of the presence of compounds deriving from the yeast extract present in the YPD broth (*e.g*, amino acids, soluble colloids, glutathione and thiol molecules), the growth medium was also subjected to the same analytical determinations and the results obtained were used to correct the data.

3.6 Evaluation of free amino acids

The amino acids released during growth and after enzymatic lysis were quantified spectrophotometrically by *o*-phthaldialdehyde (OPA) derivatization as reported by Dukes & Butzke [24].

Before analysis, yeast suspensions collected after the growth phase and the autolysates obtained by enzymatic treatment were centrifuged (10 min, 3000 rpm) and 50 µL of the supernatant were subjected to derivatization. A calibration curve with isoleucine (Ile) was prepared (0-10 mM) and the results were expressed in mg per 10^9 cells.

3.7 Evaluation of total soluble colloids

Soluble colloids, including soluble cell wall polysaccharides and glycoproteins, were determined by SE-HPLC after ethanol precipitation. Yeast suspensions (after growth) and autolysates (after enzyme treatment) were centrifuged (10 min, 3000 rpm) and 5 mL of supernatant was added to 5 volumes of ethanol (96 % v/v). Samples were stored at 0-4 °C for 24 hours. The precipitated pellet was separated by centrifugation, washed twice with ethanol (96 % v/v), resuspended in 5 mL of MilliQ water and filtered on 0.22 µm cellulose acetate membrane before injection. SE-HPLC separation was achieved using a binary pump Model LC 250 (Perkin-Elmer, Waltham, MA, USA), equipped with a manual injection valve (type 7125 NS Rheo-dyne, Rohnert Park, CA, USA) and a refractive index detector RID-10A (Shimadzu, Kyoto, Japan). The columns were a PL Aquagel-OH MIXED-H, (8 µm, 300 x 7.5 mm, Agilent Technologies, Santa Clara, CA, USA) and a Ultrahydrogel 250 (6 µm, 300 x 7.8 mm, Waters, Milford, MA, USA). The mobile phase was MilliQ water; the separation was performed in isocratic conditions, with a flow rate of 0.7 mL/min and the injection volume was 20 µL. Total soluble colloids were quantified by a calibration curve prepared with mannan (0-1000 mg/L) and the results were expressed in mg per 10^9 cells.

3.8 Evaluation of antioxidant compounds

Antioxidant compounds, mainly consisting of thiol molecules and glutathione, were determined spectrophotometrically on suspensions collected after growth and enzymatic lysis after centrifugation (10 min at 3000 rpm), to eliminate insoluble residues and cell debris, and the supernatant was subjected to the following analyses.

Thiol compounds were determined by derivatization with 4,4′-dithiodipyridine (DTDP), according to Gallardo-Chacón et al. [17]. The content of soluble thiols was expressed in µmol of GSH per 10^9 cells, using a calibration curve prepared with glutathione (0-650 µmol/L).

Total glutathione was analyzed by the enzymatic assay described by Adams & Liyanage [25] and the results were expressed in µmol of glutathione per 10^9 cells.

3.9 Statistical analysis

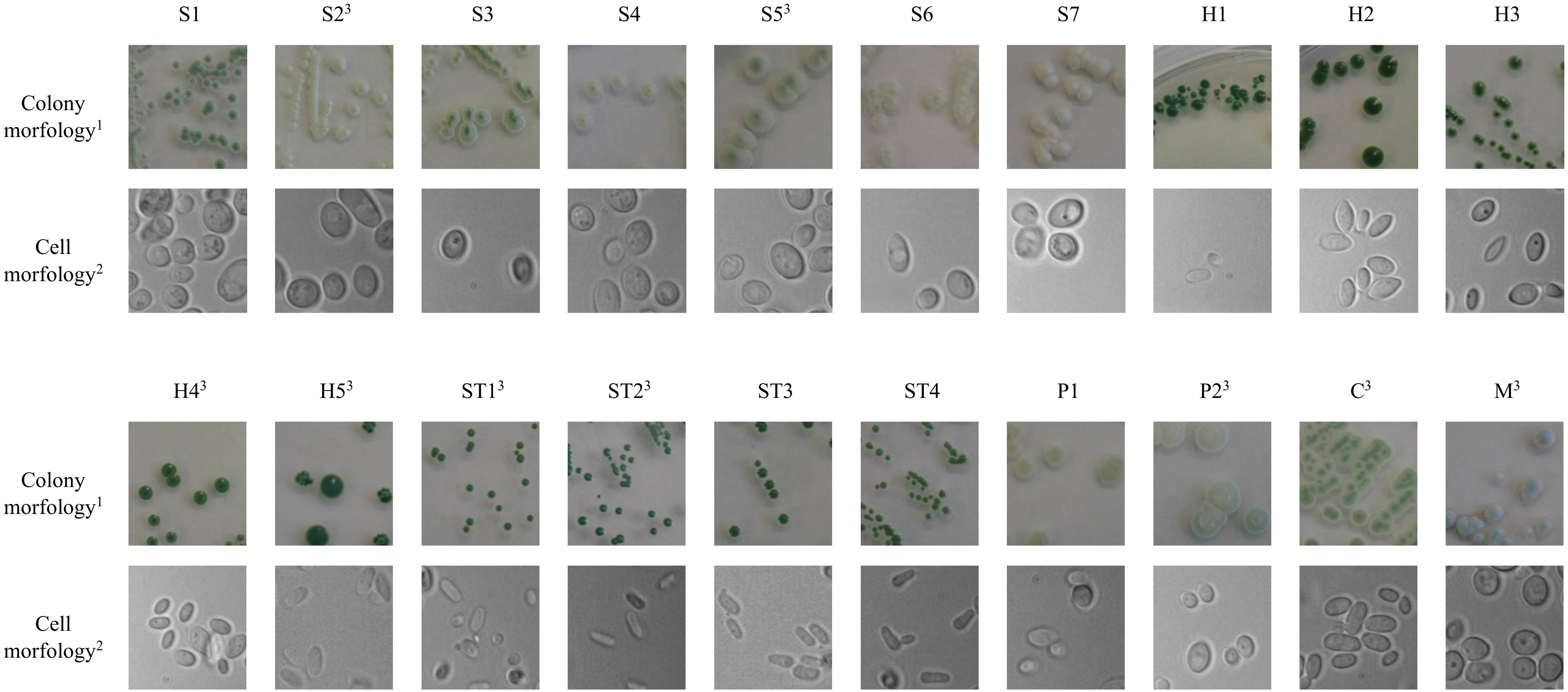
The results obtained were means and standard deviations of three repeated trials. One-way ANOVA and Tukey HSD test were carried out for all the parameters evaluated and differences were considered significant at $p < 0.05$. All elaborations were carried out by the software Statistica for Windows Version 8.0 (StatSoft, Tulsa, OK, USA).

4. Results and discussion

4.1 Isolation and identification of yeast strains

The different morphotypes isolated on the differential culture medium (WL Nutrient agar) were reported in table 1, allowing a preliminary tentative identification of the strains.

Table 1. Yeast strains isolated from red grape pomace and must and their morphological features.



[1] Colonies grown on WL Nutrient agar after incubation at 30°C for 48 hours
[2] Phase-contrast optical microscopy (magnification 100x)
[3] Identification by PCR amplification and 26S rRNA sequencing
S: *Saccharomyces* spp.; H: *Hanseniaspora* spp.; ST: *Starmerella* spp.; P: *Pichia* spp.; C: *Candida* spp.; M: *Metschnikowia* spp.

Knoblike colonies with cream to light green color, smooth opaque surface and creamy consistency characterized *Saccharomyces* spp., whereas dark green, flat colonies with smooth surface and butter consistency were typical features of *Hanseniaspora* spp. Such morphotypes were comparable to those observed by other authors after isolation and identification of native grape microflora on the same differential culture medium [26,27]. For each strain, single pure colony was also analyzed by phase-contrast optical microscopy (magnification 100x), thus confirming the belonging to the previously hypothesized genera (table 1).

Based on the morphological similarities, strains were then divided in six groups tentatively corresponding to the genera; at least one strain per group was subjected to PCR amplification and 26S rRNA sequencing. The molecular analysis further confirmed the previous identification (Annex 1).

Based on this approach, twenty native yeast strains were tentatively identified in red must and pomace (table 2), of whom seven belonged to the genus *Saccharomyces*, whereas the remaining thirteen were recognized as NSY, *Candida* spp. (n=1), *Hanseniaspora* spp. (n=5), *Metschnikowia* spp. (n=1), *Pichia* spp. (n=2) and *Starmerella* spp. (n=4). All strains were representative of the spontaneous microflora of grapes and musts.

Table 2. Yeast strains isolated from red grape pomace and must.

Yeast strain code	Species	Identification method	Accession Number, NCBI (Score, Identities, Gaps)
C	*Candida californica*	g[1]	MH681756.1 1014 bits (549), 554/556 (99%), 2/556 (0%)
H1	*Hanseniaspora* spp.	m[2]	
H2	*Hanseniaspora* spp.	m	
H3	*Hanseniaspora* spp.	m	
H4	*Hanseniaspora uvarum*	g	KT922411.1 1033 bits (559), 564/566 (99%), 2/566 (0%)
H5	*Hanseniaspora opuntiae*	g	HQ149320.1 1057 bits (572), 581/585 (99%), 2/585 (0%)
M	*Metschnikowia fructicola*	g	MW648802.1 880 bits (476), 476/476 (100%), 0/476 (0%)
P1	*Pichia* spp.	m	
P2	*Pichia kluyveri*	g	KY108823.1 1053 bits (570), 575/577 (99%), 2/577 (0%)
S1	*Saccharomyces* spp.	m	
S2	*Saccharomyces cerevisiae*	g	JQ771733.1 1061 bits (574), 574/574 (100%), 0/574 (0%)
S3	*Saccharomyces* spp.	m	
S4	*Saccharomyces* spp.	m	
S5	*Saccharomyces cerevisiae*	g	JX141382.1 1061 bits (574), 579/581 (99%), 1/581 (0%)
S6	*Saccharomyces* spp.	m	
S7	*Saccharomyces* spp.	m	
ST1	*Starmerella bacillaris*	g	MW171262.1 913 bits (494), 499/501 (99%), 2/501 (0%)
ST2	*Starmerella bacillaris*	g	MN337262.1 900 bits (487), 497/501 (99%), 4/501 (0%)
ST3	*Starmerella* spp.	m	
ST4	*Starmerella* spp.	m	

[1] g: identification by PCR amplification and 26S rRNA sequencing

[2] m: identification by colony morphology on WL Nutrient agar plates and phase-contrast optical microscopy (100x).

4.2 Cell viability and biomass production

Yeast cell viability (as log CFU/mL) and biomass recovery (as g/L) were evaluated after growth (in YPD broth, at 30°C for 48 h) and the results were reported in table 3.

Table 3. Yeast cells viability and biomass recovery determined after growth in YPD broth at 30°C for 48 h. Mean and standard deviation (SD) of three replicates were reported. Different letters within the same column marked significant differences among yeast strains, according to ANOVA and Tukey HSD test at $p < 0.05$.

Yeast code	Yeast genus	log (CFU/mL)	Biomass (g/L)
		Mean ± SD	Mean ± SD
S1	*Saccharomyces* spp.	7.51 ± 0.10 cdef	4.80 ± 2.50 cd
S2	*Saccharomyces* spp.	7.59 ± 0.03 ef	1.37 ± 0.07 abc
S3	*Saccharomyces* spp.	7.60 ± 0.15 ef	1.68 ± 0.09 abcd
S4	*Saccharomyces* spp.	7.64 ± 0.09 ef	1.45 ± 0.04 abc
S5	*Saccharomyces* spp.	7.13 ± 0.10 abc	1.55 ± 0.09 abc
S6	*Saccharomyces* spp.	7.60 ± 0.10 ef	1.77 ± 0.13 abcd
S7	*Saccharomyces* spp.	7.51 ± 0.03 def	4.42 ± 2.61 cd
H1	*Hanseniaspora* spp.	7.13 ± 0.12 abc	1.85 ± 0.21 abcd
H2	*Hanseniaspora* spp.	7.10 ± 0.29 ab	2.84 ± 0.78 abcd
H3	*Hanseniaspora* spp.	6.99 ± 0.18 a	3.41 ± 2.55 abcd
H4	*Hanseniaspora* spp.	7.15 ± 0.10 abcd	3.32 ± 0.52 abcd
H5	*Hanseniaspora* spp.	7.41 ± 0.14 bcde	0.50 ± 0.06 ab
ST1	*Starmerella* spp.	7.15 ± 0.04 abcd	0.34 ± 0.23 a
ST2	*Starmerella* spp.	7.56 ± 0.09 ef	2.75 ± 0.53 abcd
ST3	*Starmerella* spp.	7.62 ± 0.03 ef	1.96 ± 0.61 abcd
ST4	*Starmerella* spp.	7.48 ± 0.06 bcdef	0.39 ± 0.06 a
P1	*Pichia* spp.	7.86 ± 0.04 f	1.50 ± 0.07 abc
P2	*Pichia* spp.	7.83 ± 0.01 f	5.34 ± 1.24 d
C	*Candida* spp.	7.17 ± 0.07 abcd	0.34 ± 0.15 a
M	*Metschnikowia* spp.	7.60 ± 0.08 ef	1.47 ± 0.69 abc
S-COMM	*Saccharomyces* spp.	7.32 ± 0.11 abcde	4.08 ± 1.37 bcd
T-COMM	*Torulaspora* spp.	7.56 ± 0.26 ef	3.34 ± 2.33 abcd

Concerning viable cells, statistical differences resulted both inter- and intra-species. The two *Pichia* strains (P1 and P2) showed significantly higher values than those observed for *Candida* spp., *Hanseniaspora* spp. and commercial *S. cerevisiae* (S-COMM), as well as, within *Hanseniaspora* spp., the strain H5 significantly differed from strain H3, the latter with the lowest cell viability (6.99 log CFU/mL). Despite these variations appeared statistically relevant, from the practical point of view the number of viable cells detected after growth were comparable for all the strains, highlighting a good growth capacity in the tested conditions.

Concerning the production of biomass, the highest amount was observed for strain P2 with 5.34 g/L; this value significantly differed from those found for P1 (*Pichia* spp.), C (*Candida* spp.) and M (*Metschnikowia* spp.), H5 (*Hanseniaspora* spp.), ST1 and ST4 (*Starmerella* spp.), and for some *Saccharomyces* strains (S2, S4, S5). *Saccharomyces* (both commercial and strains S1 and S7), commercial *T. delbruecki* and strains H2, H3 and H4 (*Hanseniaspora* spp.) also showed interesting biomass recoveries, with values ranging from 2.8 to 4.4 g/L. Even if no other significant differences were observed, the amount of biomass produced by some *Saccharomyces* strains, as well as by certain strains of *Hanseniaspora* spp. was not negligible from a practical point of view. The results obtained in terms of microbial counts (about 10^7 CFU/mL for all the strains) and biomass production were in agreement with those observed by other authors under winemaking conditions [4,7], with differences that seemed to be strain-dependent, probably due to the different utilization of growth factors, sugars and amino acids [6,7,28].

4.3 Release of free amino acids after growth and lysis treatment

The content of amino acids released by yeast strains after growth and lysis treatment was reported in table 4.

Table 4. Free amino acids content determined after growth and lysis treatments. Mean and standard deviation (SD) of three replicates were reported. Different letters within the same column marked significant differences among yeast strains, according to ANOVA and Tukey HSD test at $p < 0.05$. Concerning data “after growth”, positive and negative values (green and red bars) indicated a release or a consumption respectively, in relation to the initial composition of YPD broth.

		Free amino acids (mg/10^9 cells)							
		after growth				after lysis			
Yeast code	Yeast genus	Mean	±	SD		Mean	±	SD	
S1	*Saccharomyces* spp.	-1.8	±	2.2	bcd	14.7	±	3.1	ab
S2	*Saccharomyces* spp.	1.9	±	2.6	bcd	16.3	±	2.5	abc
S3	*Saccharomyces* spp.	6.1	±	3.2	cde	17.0	±	5.6	abc
S4	*Saccharomyces* spp.	-2.3	±	0.7	bcd	11.0	±	3.6	ab
S5	*Saccharomyces* spp.	-7.9	±	6.1	bc	25.0	±	4.6	abc
S6	*Saccharomyces* spp.	0.2	±	1.7	bcd	10.3	±	5.7	a
S7	*Saccharomyces* spp.	-2.1	±	0.7	bcd	14.3	±	0.6	ab
H1	*Hanseniaspora* spp.	-24.1	±	15.4	a	14.3	±	5.0	ab
H2	*Hanseniaspora* spp.	-1.2	±	7.8	bcd	22.0	±	3.6	abc
H3	*Hanseniaspora* spp.	19.6	±	0.6	ef	31.3	±	22.5	bc
H4	*Hanseniaspora* spp.	11.6	±	9.5	def	20.7	±	15.3	abc
H5	*Hanseniaspora* spp.	-4.5	±	2.1	bc	14.0	±	3.6	ab
ST1	*Starmerella* spp.	-7.5	±	2.0	bc	24.3	±	1.2	abc
ST2	*Starmerella* spp.	2.8	±	1.0	cd	13.7	±	2.5	ab
ST3	*Starmerella* spp.	0.3	±	0.9	bcd	12.3	±	2.1	ab
ST4	*Starmerella* spp.	1.2	±	0.5	bcd	16.7	±	3.8	abc
P1	*Pichia* spp.	1.9	±	0.8	bcd	7.3	±	0.6	a
P2	*Pichia* spp.	0.0	±	0.6	bcd	7.0	±	0.0	a
C	*Candida* spp.	22.9	±	3.4	f	36.3	±	4.2	c
M	*Metschnikowia* spp.	1.3	±	0.9	bcd	12.7	±	2.9	ab
S-COMM	*Saccharomyces* spp.	-13.1	±	6.8	ab	8.0	±	2.6	a
T-COMM	*Torulaspora* spp.	-4.7	±	4.2	bc	5.0	±	2.6	a

Concerning the growth phase, different trends were observed, both among yeast genera and within the same genus. Generally, *Saccharomyces* spp. showed a consumption of amino nitrogen during growth, except for strains S2 and S3 for which a release of amino acids was observed (1.9 and 6.1 mg/10^9 cells, respectively). The highest producers of amino nitrogen were *Candida californica* (strain C) with an average concentration after growth of approximately 23 mg/10^9 cells, followed by the two *Hanseniaspora* H3 and H4 (with 19.6 and 11.6 mg/10^9 cells, respectively), resulting significantly different from all the other strains. Both the commercial ADY, *S. cerevisiae* and *T. delbrueckii*, on the contrary, showed the tendency to consume amino nitrogen (-13.1 and -4.7 mg/10^9 cells, respectively). By considering the same genus, the most evident differences were observed in the case of *Hanseniaspora* spp.: as reported above, a release in amino acids was observed for H3 and H4, whereas strains H1, H2 and H5 showed a variable ability to use amino acids, with a consumption ranging from -1.2 to -24.1 mg/10^9 cells. Lastly, *Pichia* spp., *Metschnikowia* spp. and almost all *Starmerella* spp. showed a weak release of amino nitrogen, with no significant differences compared to the commercial yeasts used as reference.

As expected, after lysis induced by enzyme addition, the amounts of amino acids released was higher, ranging from the lowest value observed for commercial *T. delbrueckii* (5 mg /10^9 cells) to the highest amount released by *Candida* spp. (36.3 mg/10^9 cells). The latter, together with *Hanseniaspora* H3 (with a mean concentration of 31.3 mg/10^9 cells), was the strain characterized by the greatest production of amino acids, both after growth and lysis. It is interesting to observe that certain strains, which during growth released low amounts of amino acids (*e.g.*, ST3, ST4 and S2) or even tended to slightly consume them (*e.g.*, S5 and ST1), showed a not negligible ability to release amino nitrogen after lysis, even twice or three times higher than commercial yeast strains used as reference.

The different utilization and preference of nitrogen sources observed by some authors under winemaking conditions [6,28] might explain the different behavior towards nitrogen metabolism of the strains evaluated in the present study. Similarly, higher release of amino acids after enzymatic lysis observed for some strains, particularly for *Hanseniaspora* spp., could be due to the faster, early occurrence of the autolytic process during wine aging on lees [29] or to a possible, higher cell susceptibility to the enzymatic attack.

From the practical point of view, the amino acids content is an important, not negligible aspect to be considered, mainly depending on the final utilization: strains with higher production/release of amino acids could be useful in mixed fermentation or to produce yeast derivatives to be used as alcoholic and malolactic fermentation enhancers. On the other hand, if the improvement of wine organoleptic and sensory attributes is the main purpose, high level of such compounds might lead to spontaneous, undesirable fermentations during aging on lees, thus compromising the wine quality.

4.4 Release of soluble colloids after growth and lysis treatment

The content of soluble colloids released after growth and lysis treatment was reported in table 5.

Table 5. Soluble colloids content determined after growth and lysis treatments. Mean and standard deviation (SD) of three replicates were reported. Different letters within the same column marked significant differences among yeast strains, according to ANOVA and Tukey HSD test at $p < 0.05$. Concerning data "after growth", positive and negative values (green and red bars) indicated a release or a consumption respectively, in relation to the initial composition of YPD broth.

Yeast code	Yeast genus	Soluble colloids (mg/10^9 cells)							
		after growth				after lysis			
		Mean	±	SD		Mean	±	SD	
S1	*Saccharomyces* spp.	-7.7	±	11.1	ab	0.9	±	0.2	a
S2	*Saccharomyces* spp.	-12.3	±	2.2	ab	0.0	±	0.0	a
S3	*Saccharomyces* spp.	-11.4	±	4.0	ab	0.1	±	0.2	a
S4	*Saccharomyces* spp.	-3.3	±	3.8	ab	0.3	±	0.1	a
S5	*Saccharomyces* spp.	17.6	±	5.7	abcd	0.6	±	0.3	a
S6	*Saccharomyces* spp.	2.0	±	3.8	abc	0.4	±	0.2	a
S7	*Saccharomyces* spp.	4.9	±	7.0	abcd	0.6	±	0.1	a
H1	*Hanseniaspora* spp.	121.0	±	23.8	f	1.3	±	1.0	ab
H2	*Hanseniaspora* spp.	-17.0	±	44.2	ab	2.9	±	0.4	bc
H3	*Hanseniaspora* spp.	66.9	±	25.2	e	3.0	±	1.7	bc
H4	*Hanseniaspora* spp.	68.4	±	17.8	e	3.2	±	1.7	c
H5	*Hanseniaspora* spp.	21.9	±	5.6	bcd	0.6	±	0.3	a
ST1	*Starmerella* spp.	-9.4	±	4.5	ab	0.0	±	0.0	a
ST2	*Starmerella* spp.	10.9	±	0.9	abcd	0.1	±	0.1	a
ST3	*Starmerella* spp.	-24.0	±	2.2	a	0.0	±	0.0	a
ST4	*Starmerella* spp.	8.1	±	5.6	abcd	0.1	±	0.1	a
P1	*Pichia* spp.	5.7	±	2.0	abcd	0.4	±	0.1	a
P2	*Pichia* spp.	-11.0	±	0.5	ab	0.3	±	0.1	a
C	*Candida* spp.	15.6	±	3.3	abcd	0.0	±	0.0	a
M	*Metschnikowia* spp.	-10.1	±	1.2	ab	0.4	±	0.0	a
S-COMM	*Saccharomyces* spp.	47.3	±	9.6	de	1.7	±	0.4	abc
T-COMM	*Torulaspora* spp.	42.9	±	19.5	cde	0.8	±	0.7	a

After the growth, a different trend in the soluble colloids content was observed. Strains belonging to *Hanseniaspora* spp. (except for H2) showed the greater release of soluble colloids with the highest concentration observed for strain H1 (121 mg/10^9 cells), thus resulting the only significantly different from all the other strains. H3 and H4 (66.9 and 68.4 mg/10^9 cells, respectively) and the two commercial yeasts used as reference (T-COMM and S-COMM) also showed a good ability to release soluble colloids. This trend might highlight a higher production rate of cell wall soluble colloids (*e.g.*, mannoproteins and glucans), or a faster cell turnover during the growth phase. Although the amount of biomass produced by *Hanseniaspora* spp. were slightly lower than that observed for some *Saccharomyces* spp. (table 3), the ability of the former to produce a higher ratio of soluble colloids per gram of cell dry weight during alcoholic fermentation has already been reported [4].

The aptitude of *Hanseniaspora* spp. to release soluble colloids was also confirmed after β-glucanase treatment (table 5). The best results were observed for strains H2, H3 and H4 (about 3 mg/10^9 cells), followed by the commercial *S. cerevisiae*, whereas for the remaining yeasts, the amounts of soluble colloids released was less than 1 mg/10^9 cells; this might be probably due to a lower biomass produced in the tested conditions or it could be dependent on a different cell wall composition and autolytic behavior. However, even if the amounts of soluble colloids detected after lysis were less consistent that those released after growth, these results agreed with what previously observed by Dupin et al. [30].

However, concerning the ability of *Hanseniaspora* spp. to release greater amounts of soluble colloids compared to *Saccharomyces* spp., it seemed to be related to an earlier occurrence of the autolytic process. In a previous study carried out by Giovani et al. [13], lower cell viability and higher ratio of polysaccharides content to cell dry weight were observed in *Hanseniaspora* spp.; after seven days of fermentation, the content of polysaccharides was greater (almost twice) than that detected for *Saccharomyces* spp. This interesting feature of *Hanseniaspora* spp. recently led to investigate its contribution to wine quality during fermentation and aging on lees, with interesting positive effects on astringency, body and structure as well as on the aroma profile of the resulting wines [29,31].

4.5 Release of antioxidant molecules after growth and lysis treatment

Antioxidant molecules, thiol compounds and total glutathione (GSH) were determined on yeast suspensions after growth and after lysis treatment; the results were both expressed in µmol/10^9cells of glutathione and reported in table 6.

Table 6. Thiol compounds and total glutathione (GSH) determined after growth and lysis treatments. Mean and standard deviation (SD) of three replicates were reported. Different letters within the same column marked significant differences among yeast strains, according to ANOVA and Tukey HSD test at $p < 0.05$. Concerning data "after growth", positive and negative values (green and red bars) indicated a release or a consumption respectively, in relation to the initial composition of YPD broth.

		Thiol compounds (µmol/10^9 cells)								GSH (µmol/10^9 cells)							
Yeast code	Yeast genus	after growth				after lysis				after growth				after lysis			
		Mean	±	SD		Mean	±	SD		Mean	±	SD		Mean	±	SD	
S1	*Saccharomyces* spp.	0.17	±	0.25	cdef	0.00	±	0.00	a	-0.17	±	0.06	a	0.30	±	0.26	a
S2	*Saccharomyces* spp.	-0.87	±	0.06	abcd	0.00	±	0.00	a	-0.23	±	0.15	a	0.30	±	0.36	a
S3	*Saccharomyces* spp.	-0.93	±	0.35	abcd	0.00	±	0.00	a	-0.17	±	0.06	a	0.23	±	0.25	a
S4	*Saccharomyces* spp.	-0.53	±	0.60	abcde	0.00	±	0.00	a	-0.07	±	0.25	a	0.23	±	0.21	a
S5	*Saccharomyces* spp.	-1.70	±	0.46	abc	0.00	±	0.00	a	-0.83	±	0.06	a	0.90	±	0.78	a
S6	*Saccharomyces* spp.	-0.37	±	0.67	bcde	0.00	±	0.00	a	-0.30	±	0.10	a	0.23	±	0.25	a
S7	*Saccharomyces* spp.	-0.03	±	0.12	cde	0.00	±	0.00	a	-0.47	±	0.40	a	0.37	±	0.35	a
H1	*Hanseniaspora* spp.	1.27	±	0.38	ef	1.07	±	0.23	cd	0.43	±	0.93	a	0.73	±	0.64	a
H2	*Hanseniaspora* spp.	1.27	±	1.07	ef	1.70	±	1.04	d	0.33	±	0.65	a	0.53	±	0.55	a
H3	*Hanseniaspora* spp.	3.30	±	1.15	g	0.70	±	0.36	abc	0.20	±	1.04	a	2.27	±	0.21	b
H4	*Hanseniaspora* spp.	2.03	±	1.14	fg	0.53	±	0.38	abc	-0.17	±	0.15	a	0.80	±	0.56	a
H5	*Hanseniaspora* spp.	-0.40	±	1.21	bcde	0.00	±	0.00	a	-0.23	±	0.23	a	0.27	±	0.25	a
ST1	*Starmerella* spp.	-2.43	±	1.10	a	0.00	±	0.00	a	-0.27	±	0.31	a	0.43	±	0.38	a
ST2	*Starmerella* spp.	0.57	±	0.45	def	0.00	±	0.00	a	-0.20	±	0.35	a	0.23	±	0.21	a
ST3	*Starmerella* spp.	0.07	±	0.06	cde	0.03	±	0.06	ab	-0.13	±	0.15	a	0.17	±	0.15	a
ST4	*Starmerella* spp.	-1.17	±	0.15	abcd	0.00	±	0.00	a	-0.27	±	0.15	a	0.47	±	0.12	a
P1	*Pichia* spp.	-0.10	±	0.10	cde	0.00	±	0.00	a	-0.07	±	0.12	a	0.13	±	0.12	a
P2	*Pichia* spp.	0.17	±	0.06	cdef	0.43	±	0.21	abc	-0.07	±	0.12	a	0.23	±	0.15	a
C	*Candida* spp.	-2.07	±	0.67	ab	0.00	±	0.00	a	0.27	±	1.15	a	0.63	±	0.15	a
M	*Metschnikowia* spp.	0.33	±	0.12	def	0.00	±	0.00	a	-0.03	±	0.06	a	0.57	±	0.06	a
S-COMM	*Saccharomyces* spp.	0.53	±	0.25	def	0.87	±	0.29	bcd	-1.00	±	0.98	a	0.97	±	0.47	a
T-COMM	*Torulaspora* spp.	0.30	±	0.20	def	0.50	±	0.26	abc	-0.50	±	0.78	a	0.37	±	0.47	a

The highest producers of thiol compounds were *Hanseniaspora* spp. strains: in particular, H3 resulted the only statistically different from all the other yeasts with a production of 3.30 µmol/10^9 cells, followed by strain H4 (2.03 µmol/10^9cells), the second in terms of mean value. For the other strains that showed a production of thiol compounds during growth, the concentration ranged from about 0.1 to 0.6 µmol/10^9 cells, whereas the remaining ones, mainly

Saccharomyces spp. and two strains of *Starmerella* spp. (ST1 and ST4) showed a consumption of thiol molecules (*e.g.*, glutathione or cysteine). Similarly, after lysis treatment, *Hanseniaspora* spp. showed the highest value in terms of release: in this case, the strain H2 showed the highest concentration of thiol compounds with an amount of 1.70 µmol/10^9cells, resulting significantly different from all the other yeasts, except from H1 (1.07 µmol/10^9cells).

By considering the amount of total glutathione detected after growth (table 6), even in this case, the strains belonging to *Hanseniaspora* spp. showed the highest content: strains H1, H2 and H3, together with *Candida* spp., were the only strains able to produce glutathione with a concentration ranging from about 0.20 to 0.43 µmol/10^9cells, whereas all the other yeasts seemed to consume glutathione; nevertheless, no significant differences were observed by the statistical analysis.

A release of intracellular glutathione was observed after lysis treatment by all the strains tested, with the highest amount detected for the strain H3 (2.27 µmol/10^9cells), thus resulting the only significantly different from all the others. In terms of mean values, *Hanseniaspora* spp. was confirmed the genus characterized by the highest release of total glutathione, with an average of 0.53-0.80 µmol/10^9cells, together with some strains of *Saccharomyces* spp. (S-COMM and strain S5, with 0.97 and 0.90 µmol/10^9cells, respectively).

It is interesting to underline that, by comparing the values detected for thiol compounds with those obtained for glutathione, the ability of some strains to release GSH did not always correspond to a comparable production of thiols (*e.g.*, strain C or H4). This might relate to different factors. First, the enzymatic method used for evaluating GSH [25] detects total GSH, both reduced (GSH) and oxidized (GSSG), but only the former is detectable as a thiol by reaction with DTDP; moreover, the sulfur dioxide probably produced by the strains during growth phase might react with DTDP. Furthermore, the derivatizing agent used (DTDP) might react with other thiol molecules produced by yeasts, such as cysteine and certain cysteine-containing proteins [32].

Up to now the production of antioxidant compounds as well as the antioxidant properties of yeast lees or walls [16,17,32] was mainly focused on *S. cerevisiae* - even if, recently, some evidence has been reported in literature about the aptitude of NSY to produce GSH in higher amount compared to *S. cerevisiae*. Lemos Junior et al. [19] observed that some NSY strains (*L. thermotolerans, S. bacillaris* and *Metschnikowia* spp.) produced relevant amounts of glutathione during growth under aerobic conditions and the release was higher at the end of the exponential phase. Furthermore, they also observed how yeast strains, medium composition (synthetic or grape must) and fermentation conditions (single or sequential fermentation with *S. cerevisiae*) might also influence the amount of intracellular glutathione. In another study carried out by the same research group, it was observed that the sequential fermentation with NSY led to an increase of glutathione released into the wine, up to 10 mg/L compared to the control wine, obtained by single fermentation with *S. cerevisiae* [33]. In light of these considerations, the mixed fermentation as well as the highest content of intracellular glutathione could be a good strategy to improve and enhance the protection against wine oxidation not only at the end of fermentation process but also during wine aging, when the occurrence of autolysis might allow the release of glutathione accumulated into the cells.

5. Conclusions

Within the non-*Saccharomyces* strains evaluated in this study, *Hanseniaspora* spp. showed a good aptitude to produce and release soluble colloids, amino acids and antioxidant compounds during growth and after induced lysis, with a production of biomass and cell viability comparable with those of the commercial ADYs used as reference. By considering the preliminary results obtained in the present study, strain H3 (*Hanseniaspora* spp.) was chosen for further investigations aimed to assess *i)* the most suitable growth conditions to enhance the production of biomass and compounds of enological interest, especially soluble colloids and antioxidant compounds (Chapter 2) and *ii)* the role this strain may have in sequential fermentation (Chapter 5) and subsequent aging on lees (both in model and real wine, Chapter 6), in comparison with commercial *S. cerevisiae* alone. Furthermore, strains H4 (*Hanseniaspora* spp.) and P2 (*Pichia* spp.) were selected for assessing the effect of the yeast strain and the production process on the composition of the resulting yeast derivatives, in comparison with commercial *S. cerevisiae* (Chapter 3, Part I).

Chapter 2

Effect of growth conditions on biomass production and release of soluble colloids and antioxidant molecules after growth and enzyme-assisted lysis in *Hanseniaspora* spp. and *Saccharomyces* spp.

1. Introduction

At large-scale level, the production of yeast biomass is currently based on the use of baker's yeast as starting microorganism; beet or cane molasses supplemented with several macro and micro-nutrients are generally used as substrates [34], even if at laboratory scale, agri-food waste has been proposed as alternatives for obtaining yeast biomass [35,36]. The production is generally carried out in bioreactor in different batch fermentation steps during which several parameters are managed to achieve the maximum growth rate and, consequentially, higher biomass production; among these, sugar concentration, temperature and pH, and presence of oxygen are the most important [37]. During the first step of cultivation, the high sugar concentration determines fermentative metabolism even in presence of oxygen, due to the Crabtree effect [38]. This metabolic pathway is considered necessary since the accumulation of reserve metabolites occurs [39], also depending on the strain and carbon source used [40]. However, the type of carbon and nitrogen sources are important factors to be considered since they influence the specific growth rate of the strains [41], as well as different preferences in terms of nutrient sources and different behavior towards Crabtree effect have been reported [42,43]. Temperature of 30°C and pH value of 5 are generally applied, suitable for promoting the growth of different yeast strains [42,44]. Nevertheless, the most important parameter to be controlled and managed to ensure the maximum biomass production is oxygen [45]; it was observed that the production of biomass was enhanced when yeasts were grown under constant oxygenation together with limited sugar concentration [34], probably due to the fact that ethanol production was limited or avoided, thus maximizing biomass yield [46].

During the growth phase, several compounds of enological interest are synthetized by yeast metabolism - including glutathione and polysaccharides – whose production may be controlled by managing growth conditions. Glucose and peptone (at 4%) and magnesium sulfate (at 0.5%)

seemed to be the best nutrient sources and the best combination for allowing both biomass and glutathione production by *S. cerevisiae* in batch fermentation conditions [47]. Combined strategy of batch and fed-batch cultivations, together with the addition of cysteine and control of dissolved oxygen level allowed highest biomass (102 g/L) and total glutathione production (981 mg/L) in *C. utilis* [48]; furthermore, the constant feeding of glucose (6g/L) during the final step of cultivation process (between 60 and 72 hours) seemed to enhance the production of intracellular glutathione, up to 2177 mg/L [49]. Wei et al. [50] demonstrated that, during batch fermentation, the addition of cysteine at the stationary phase combined with a temperature shift (from 30°C to 26°C) allowed to increase the production of both biomass (16 g/L) and total glutathione (527 mg/L); furthermore, the authors also observed that fed-batch fermentation was more suitable for obtaining greater yield of biomass and glutathione (even twice higher) than simple batch fermentation.

Polysaccharides - mainly consisting in mannoproteins and β-glucans - are produced by yeasts as the main components of cell walls [51,52]; such compounds are produced during the growth phase [53] and released into the wine during alcoholic fermentation [54], in a way that is strain-dependent [4,11,13]. Mannan production by *S. cerevisiae* seemed to be highly affected by the type of sugar and its concentration - with sucrose as the most preferred carbon source at a concentration of 5% - together with organic nitrogen (*e.g.*, peptone and yeast extracts) and glycerol [55]; furthermore, the volume of culture medium, followed by temperature, pH and starting inoculum [55], as well as the concentration of dissolved oxygen [56] seemed to be the growth parameters mostly affecting the production of such compounds. However, differences in terms of polysaccharides production under agitation conditions were also reported, in relation to the type of yeast strains [57] and to the carbon source preferences [58].

2. Aim of the work

The aim of the work was to investigate the influence of growth conditions on biomass production and on release of compounds of enological interest, with the purpose of obtaining both fermentation starters - potentially able to improve wine aging on lees - and yeast derivatives specifically tailored for winemaking use. In this regard, strain H3 (*Hanseniaspora* spp.) was selected for its non-negligible ability to produce biomass and for the high release of antioxidant compounds and soluble colloids, as resulting from its characterization in Chapter 1. After optimizing the culture medium, two parameters influencing growth - mainly temperature and aeration - were studied; the chemical evaluations were focused on cell viability and biomass production, as well as on the release of compounds of enological interest, *i.e.*, nitrogen compounds, soluble colloids, and antioxidant molecules, both after growth and enzyme-assisted lysis, in comparison with commercial *S. cerevisiae*.

3. Materials and methods

3.1 Reagents

Sodium chloride for microbiology, bacteriological peptone, Malt Extract Broth, and technical agar were purchased from Oxoid (Milan, Italy); glucose, fructose, sucrose, yeast extract, magnesium sulfate, tetracycline, sodium hydroxide, N-acetyl-L-cysteine, *o*-phthaldialdehyde (OPA), isoleucine, ethanol (96 % v/v), mannan from *S. cerevisiae*, sodium citrate, trifluoroacetic acid, riboflavin, L-glutathione reduced, L-cysteine, *p*-benzoquinone, 3-mercaptopropionic acid, trichloroacetic acid (TCA) and acetaldehyde (ACS reagent, ≥99.5%) were from Sigma Aldrich Italy (Milan, Italy). Methanol (HPLC grade) were purchased by VWR Chemicals (Milan, Italy). MilliQ water was produced by MilliQ Advantage A10 apparatus (Merck Millipore, Billerica, MA, USA) and microfiltered at 0.22 µm before use. Commercial β-glucanase preparation and commercial active dry yeast (ADY) *S. cerevisiae* were supplied by Enologica Vason S.p.A. (San Pietro in Cariano, VR, Italy).

3.2 Yeast strains and growth kinetics

Commercial active dry yeast preparation of *S. cerevisiae* and strain H3 (*Hanseniaspora* spp., Chapter 1) were purified on Malt Extract Agar plates and one single pure colony was grown overnight in sterile tubes each containing 3 mL of Malt Extract broth; then, 100 µL of yeast suspensions were transferred in sterile tube containing 10 mL of culture media. For the preliminary tests, in order to evaluate the growth kinetics and to assess the most suitable growth factors for enhancing biomass production - in particular, type of sugars and yeast extract concentration - different combinations were used, as reported in table 7. For each medium the pH was adjusted at 5.

Table 7. Composition of the different culture media used for testing growth kinetics.

Sugar	Code	Sugar (g/L)	Yeast extract (g/L)	Magnesium sulfate (g/L)	Peptone (g/L)
Glucose	G1	20	5	2.5	10
	G2	20	10	2.5	10
Fructose	F1	20	5	2.5	10
	F2	20	10	2.5	10
Sucrose	S1	20	5	2.5	10
	S2	20	10	2.5	10

The growth kinetics were evaluated by an absorbance microplate reader (Sunrise, Tecan Italia S.r.l., Cernusco sul Naviglio, MI, Italy), equipped with shaker and temperature control unit, interfaced with a computer for processing the absorbance data (OD 600 nm); the inoculum was 6.3 x 10^4 cells/mL for *Saccharomyces* spp. and 2.7 x 10^5 cells/mL for *Hanseniaspora* spp. and the kinetics was followed up to 72 hours of incubation; all the experimental trials were performed in duplicate.

3.3 Growth conditions and enzyme-induced lysis

Culture medium containing glucose (20g/L), yeast extract (10g/L), magnesium sulfate (2.5g/L) and bacteriological peptone (10g/L) was chosen for the following step of biomass production. For each experimental trial, the inoculum was prepared as reported above, starting from single pure culture and by incubating yeast suspensions overnight in the selected medium; yeast suspensions were then inoculated at 10 % (v/v) - corresponding to 4.7 x 10^6 cells/mL for *Hanseniaspora* spp. and 6.4 x 10^6 cells/mL for *Saccharomyces* spp. - in 200 mL-sterile flasks containing 90 mL of the selected culture medium. For biomass production, the conditions tested were temperature (30°C and 22°C) and aeration. Regarding the latter, yeast suspensions were incubated in an orbital shaker (SKI 4 shaking incubator, Argolab, MO, Italy) set at 150 rpm also equipped with temperature controller, to mimic aerobic condition (hereafter referred to as

+), whereas static cultivation without shaking (unshaken flasks) was performed to mimic anaerobic condition (hereafter referred to as -). For all the experimental trials, the biomass was recovered after 24 hours of incubation that corresponded approximately to the end of the exponential phase; all the tests were performed in triplicates. The suspensions were transferred in 50 mL-sterile Falcon tubes, centrifuged at 13000*g* for 10 min at 4°C and the supernatants were used for the analytical determinations referred to the growth phase, as reported below; furthermore, because of the presence of peptone and yeast extract in the culture broth, the medium was also subjected to the same analyses and the results were used for correcting data referred to the growth phase.

Yeast biomasses were washed twice with saline-peptone water (8 g/L of sodium chloride, 1 g/L of bacteriological peptone) and resuspended in 20 mL of citrate buffer at pH 5 for the following enzyme-assisted lysis. For inducing autolysis, a commercial preparation of enzyme with β-glucanase activity was added at a concentration of 3 % (w/w); after incubation at 45°C for 24 hours, the autolyzed suspensions were centrifuged (3000 rpm for 15 min) and both the supernatants and pellets were subjected to the chemical evaluations as better described below. The supernatant was used for determining the amount of soluble molecules (cell wall polysaccharides and intracellular components, *i.e.,* amino acids and peptides, also including glutathione) that are released as result of autolysis; the pellet was instead analyzed because of the possible presence of antioxidant molecules, *i.e.*, thiol-groups containing compounds, linked to the insoluble cell wall residues.

3.4 Cells viability and biomass recovery

Yeast viable cells, expressed as logarithm of colony forming units (log CFU/mL) and biomass recovery (g/L) was determined on yeast suspensions after 24 h of growth, as reported in Chapter 1, section 3.5.

3.5 Free amino acids and total soluble colloids

These parameters were evaluated after the growth phase and lysis treatment; concerning free amino acids, the suspensions were first centrifuged at 3000 rpm for 15 min and the supernatants were subjected to the analysis, as reported in Chapter 1, section 3.6.

Total soluble colloids were determined on the supernatant after the growth phase; in the case of autolyzed suspensions (after lysis treatment), in order to remove the excess of proteins previously detected, the samples were first subjected to a purification step with trichloroacetic acid (TCA), as reported by Pintado et al. [59] with slight modification: TCA was added at a concentration of 4% w/v; the mixture was incubated at 20°C for 1 h, then centrifuged (3000 rpm for 15 min) and the supernatant was subjected to alcoholic precipitation and SE-HPLC analysis, as reported in Chapter 1, section 3.7. The results were expressed in mg/L and mg/g in relation to the calibration curve made with a standard of mannan (0-1000 mg/L).

3.6 Glutathione and cysteine content by RP-HPLC

The total content of glutathione and cysteine was evaluated on the supernatants, whereas the pellets were used for determining the amount of reducing proteins containing cysteine-residues (RPC), possibly linked to insoluble cell wall residues. For the analysis, 2 mL of suspension was added with acetaldehyde as described by Fracassetti et al [60], then subjected to the derivatization procedure as reported by Tirelli et al. [32]. The RP-HPLC was performed with a LC-2010 AHT liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an integrated autosampler and UV–visible detector set at 303 nm; the separation column was a Zorbax Eclipse Plus C18, 5 µm, 150 x 4.6 mm (Agilent Technologies, Santa Clara, CA, USA), conditioned at 25°C. The eluting solvents were water/trifluoroacetic acid (0.05% v/v) (eluent A) and methanol (eluent B); the concentration of the latter increased from 10% to 35% in 18 min, with a flow rate of 1 mL/min and the injection volume was of 20 µL. The results were expressed as µmol/L and µmol/g in relation to calibration curves made with glutathione (0-400 µmol/L) and cysteine (0-400 µmol/L), whereas the concentration of RPC was calculated as reported in the reference method [32].

3.7 Riboflavin content by RP-HPLC

The content of riboflavin was determined after filtration of 1 mL of supernatant on 0.22 µm MCE syringe-filter, then subjected to RP-HPLC analysis. The separation column was a Zorbax Eclipse Plus C18, 5 µm, 150 x 4.6 mm (Agilent Technologies, Santa Clara, CA, USA), conditioned at 25°C. The mobile phase consisted of water/trifluoroacetic acid (0.05% v/v) (eluent A) and methanol (eluent B); the elution gradient used was the following: 30% B in the first 2 min, 60% B at min 10, 100% B at min 11, maintained for 3 min and equilibration time of 3 min; the flow rate was 0.6 mL/min, and the injection volume was 20 µL. Detection was carried out at wavelength of 440 nm and the content of riboflavin was determined by a calibration curve obtained with a standard of riboflavin (0-200 µg/L); the results were expressed in µg/L and µg/g.

3.8 Statistical analysis

The results obtained were means and standard deviations of three repeated trials. Homogeneity of variance was evaluated by Brown-Forsythe and Cochran, Hartley, Bartlett test; ANOVA and Tukey HSD test were carried out for all the parameters analyzed and differences were considered significant at $p < 0.05$. All elaborations were carried out by the software Statistica for Windows Version 8.0 (StatSoft, Tulsa, OK, USA).

4. Results and discussion

For all the parameters evaluated, means and standard deviations were calculated after growth (Annex 2) and enzyme-assisted lysis (Annex 3); furthermore, the effect of the two factors – temperature and aeration - and their interaction (temperature x aeration) on the parameters evaluated after growth and after lysis were reported in tables 8 and 9, respectively. All the parameters evaluated were discussed in depth in their respective, following sections.

Table 8. Cell viability, biomass production and release of compounds of enological interest after growth phase. Positive and negative values indicated a release or a consumption respectively, in relation to the initial composition of culture medium. The concentrations were expressed per liter and standardized to gram of biomass. Different letters within the same row marked significant differences, according to two-ways ANOVA and Tukey HSD test at $p < 0.05$; the analysis was carried out separately for the two different strains. H: *Hanseniaspora* spp.; S: *Saccharomyces* spp.; - : growth without shaking to mimic anaerobic condition; + : growth with constant shaking (150 rpm) to mimic aerobic conditions.

	Temperature				Aeration				Temperature x aeration							
	H		S		H		S		H				S			
Parameter	22°C	30°C	22°C	30°C	-	+	-	+	H_30°C-	H_22°C-	H_30°C+	H_22°C+	S_30°C-	S_22°C-	S_30°C+	S_22°C+
Viable cells (log CFU/mL)	8.1	8.1	7.7	7.8	7.7 a	8.5 b	7.7	7.8	7.8 a	7.6 a	8.4 b	8.6 b	7.9	7.5	7.7	8.0
Biomass (g/L)	2.4	2.5	7.1	7.2	1.6 a	3.2 b	9.7 b	4.6 a	1.6 a	1.6 a	3.3 b	3.2 b	9.2 b	10.3 b	5.2 a	3.9 a
Mannans (mg/L)	4 a	84 b	1 a	102 b	28	60	30 a	73 b	70 ab	-15 a	98 b	22 ab	90 c	-30 a	114 c	32 b
Mannans (mg/g)	-1 a	39 b	3 a	16 b	20	18	3 a	15 b	49	-9	30	7	10 b	-3 a	22 c	8 b
Riboflavin (µg/L)	136 b	84 a	168 b	131 a	116	103	139 a	160 b	83 a	150 c	84 a	121 b	120 a	159 b	142 ab	178 b
Riboflavin (µg/g)	72 b	42 a	30 b	20 a	81 b	32 a	14 a	36 b	58 ab	105 b	26 a	39 a	13 a	15 a	27 b	45 c
Glutathione (µmol/L)	37.0 a	38.3 b	42.4	38.0	37.9 b	37.3 a	36.9	43.5	38.4 b	37.5 ab	38.1 b	36.4 a	37.5 a	36.2 a	38.4 a	48.6 b
Glutathione (µmol/g)	18.8	18.9	8.0	5.8	26.0 b	11.6 a	3.9	9.9	26.2	25.8	11.5	11.7	4.1 a	3.6 a	7.4 b	12.4 c

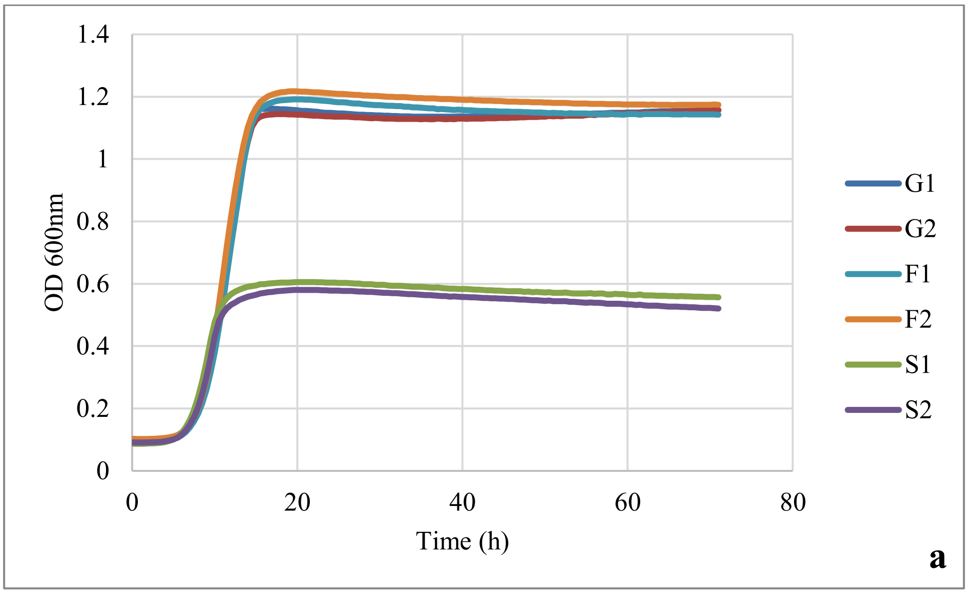
Table 9. Chemical composition of autolyzed suspensions after enzyme addition. The concentrations were expressed per liter and standardized to gram of biomass. Different letters within the same row marked significant differences, according to two-ways ANOVA and Tukey HSD test at $p < 0.05$; the analysis was carried out separately for the two different strains. H: *Hanseniaspora* spp.; S: *Saccharomyces* spp.; - : growth without shaking to mimic anaerobic condition; + : growth with constant shaking (150 rpm) to mimic aerobic conditions.

	Temperature				Aeration				Temperature x aeration							
	H		S		H		S		H				S			
Parameter	22°C	30°C	22°C	30°C	-	+	-	+	H_30°C-	H_22°C-	H_30°C+	H_22°C+	S_30°C-	S_22°C-	S_30°C+	S_22°C+
Amino acids (mg/L)	88	107	309	295	32 a	164 b	70 a	534 b	49 a	14 a	165 b	162 b	95 a	46 a	496 b	573 b
Amino acids (mg/g)	32	42	76	53	22 a	52 b	8 a	121 b	34	10	50	54	11 a	5 a	96 b	146 b
Mannan (mg/L)	689	770	392	277	264 a	1194 b	160 a	510 b	316 a	212 a	1224 b	1165 b	194 a	125 a	360 ab	660 b
Mannan (mg/g)	256	291	91 b	46 a	178 a	369 b	18 a	119 b	211 a	144 a	370 b	367 b	22 a	13 a	69 a	169 b
Riboflavin (µg/L)	103 b	92 a	95	112	54 a	141 b	36 a	171 b	59 a	50 a	125 b	157 c	49 a	24 a	176 b	166 b
Riboflavin (µg/g)	66	61	35	30	58	68	6 a	59 b	62	55	59	78	9 a	4 a	52 b	66 b
GSH (µmol/L)	113.1 b	85.8 a	185.3	216.5	55.6 a	143.3 b	46.7 a	355.1 b	51.8 a	59.3 a	119.8 b	166.9 c	56.2 a	37.2 a	376.8 b	333.4 b
GSH (µmol/g)	47.5	36.0	44.5	39.2	38.8	44.7	5.0 a	78.6 b	35.6	42.0	36.3	53.1	6.3 a	3.8 a	72.1 b	85.2 b
CYS (µmol/L)	50.0	40.2	96.8	95.3	37.7 a	52.5 b	36.2 a	155.8 b	38.2 a	37.2 a	42.1 a	62.9 b	42.4 a	30.0 a	148.1 b	163.5 b
CYS (µmol/g)	22.8	19.5	22.4 b	16.6 a	26.1 b	16.2 a	3.9 a	35.1 b	26.2	26.0	12.7	19.7	4.7 a	3.0 a	28.4 b	41.8 c
RPC (µmol/L)	628.9	818.4	nd[*]	nd	nd a	1447.2 b	nd	nd	nd a	nd a	1636.8 b	1257.7 b	nd	nd	nd	nd
RPC (µmol/g)	199.0	244.1	nd	nd	nd a	443.1 b	nd	nd	nd a	nd a	488.2 b	398.0 b	nd	nd	nd	nd

[*] nd: not detected

4.1 Growth kinetics of Hanseniaspora spp. and Saccharomyces spp.

The effect of sugar and yeast extract concentration on growth kinetics of *Hanseniaspora* spp. and *Saccharomyces* spp. were reported in figure 1 (a and b), respectively.



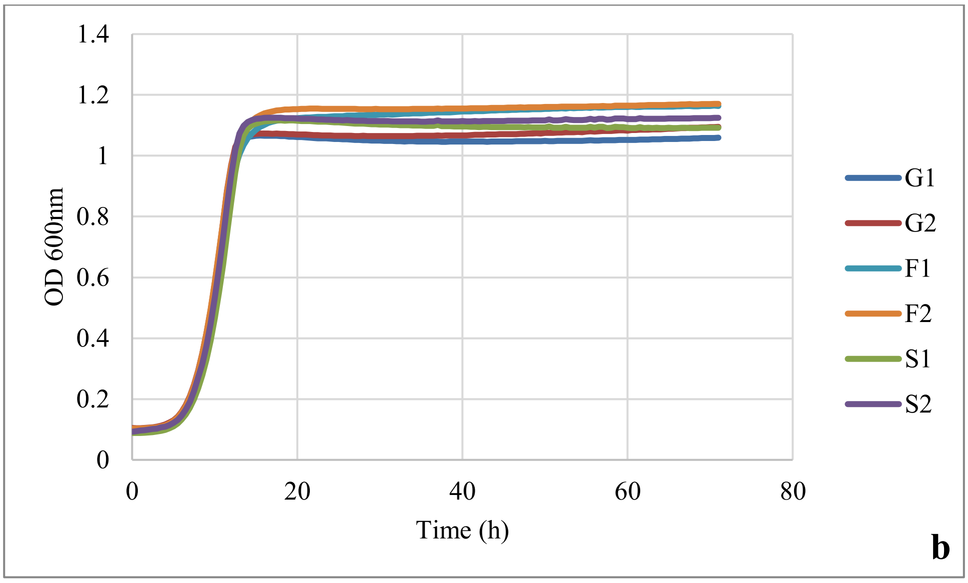


Figure 1. Growth kinetics of *Hanseniaspora* spp. (**a**) and *Saccharomyces* spp. (**b**) at 30°C in different culture medium. G1 (glucose 20 g/L, yeast extracts 5 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L); G2 (glucose 20 g/L, yeast extracts 10 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L); F1 (fructose 20 g/L, yeast extracts 5 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L); F2 (fructose 20 g/L, yeast extracts 10 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L); S1 (sucrose 20 g/L, yeast extracts 5 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L); S2 (sucrose 20 g/L, yeast extracts 10 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L). Data were means of two repeated treatments.

Hanseniaspora spp. showed a reduced growth rate when sucrose was used as the main carbon source, whereas in presence of glucose and fructose the strain reached the maximum value of cell density (OD 600 nm = 1.2), similarly to *Saccharomyces* spp., without differences in relation to the content of yeast extract. On the other hand, *Saccharomyces* spp. well grew under the different growth conditions tested, without differences in cell density neither in relation to the type of carbon source nor to yeast extract concentration. Contrasting results were reported in literature about the sugar utilization by *Hanseniaspora* spp.: similar consumption of the two monosaccharides was previously observed by Ciani and Fatichenti [61], with a more pronounced utilization and preference towards glucose or fructose that seemed to be strain-dependent; conversely, Mateus et al. [62] observed a slight consumption rate of these monosaccharides, reduced growth rate and lower biomass production during fermentation of synthetic must by *H. uvarum*. However, a possible lack of invertase enzyme and the inability to ferment sucrose, as exhibited by some non-*Saccharomyces* strains [8,63], might explain the weak growth and the lower cell density observed for *Hanseniaspora* spp. in presence of this disaccharide.

4.2 Cell viability and biomass production under different growth conditions

The effect of temperature and aeration on cell viability of *Hanseniaspora* spp. and *Saccharomyces* spp. was reported in figure 2.

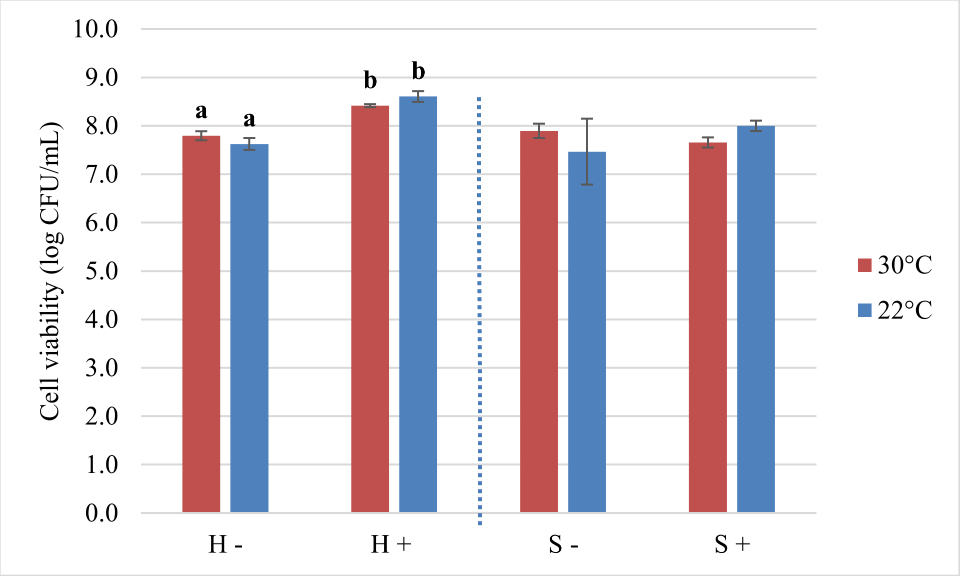


Figure 2. Cell viability (expressed as log CFU/mL) after 24 hours of growth under different conditions. Data were means and standard deviations of three replicates. For each yeast strain, different letters marked significant differences among treatments, according to ANOVA e Tukey HSD test at $p < 0.05$. H: *Hanseniaspora* spp., S: *Saccharomyces* spp.; - : growth without shaking to mimic anaerobic conditions; + : growth under constant shaking (150 rpm) to mimic aerobic conditions.

The starting inoculum of *Hanseniaspora* spp. was 6.4 log CFU/mL that corresponded to 4.7 x 10^6 CFU/mL; after 24 hours of growth, the viable cells reached a value of 7.8 and 7.6 log CFU/mL in anaerobic conditions (H -) at 30°C and 22°C respectively, whereas under aerobic conditions (H +) the cell viability was slightly higher, reaching a value of 8.4 and 8.6 log CFU/mL at 30°C and 22°C respectively, thus resulting significantly different from the former samples. On the other hand, *Saccharomyces* spp. (starting inoculum of 6.5 log CFU/mL that corresponded to 6.4 x 10^6 CFU/mL) showed the highest mean value of cell viability after 24 hours of growth under aerobic condition (S +) at 22°C (8.0 log CFU/mL), even if no significant differences were observed among all the conditions tested. For both the yeasts, no effect of temperature on cell viability was observed, whereas aeration mostly affected this parameter only in case of *Hanseniaspora* spp., with significant difference between aerobic (+) and anaerobic (-) conditions (table 8).

The influence of temperature and aeration on biomass production (expressed in g/L of cell dry weight) was reported in figure 3.

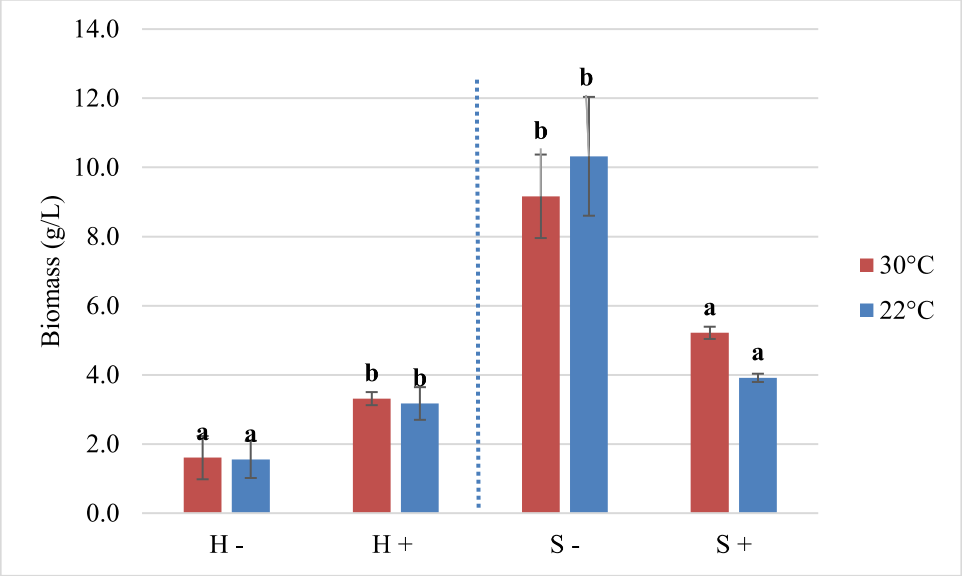


Figure 3. Biomass production (expressed in g/L of cell dry weight) after 24 hours of growth under different conditions. Data were means and standard deviations of three replicates. Different letters marked significant differences among treatments for each yeast strain, according to ANOVA e Tukey HSD test at $p < 0.05$. H: *Hanseniaspora*; S: *Saccharomyces*; - : growth without shaking to mimic anaerobic conditions; + : growth under constant shaking (150 rpm) to mimic aerobic conditions.

Similarly, biomass production was mainly affected by aeration for both the yeasts, whereas temperature did not influenced the production rate (table 8); however, by considering all the treatments, the highest biomass production was reached under aerobic conditions for *Hanseniaspora* spp. (H +) with 3.3 g/L and 3.2 g/L at 30°C and 22°C respectively, and under anaerobic conditions for *Saccharomyces* spp. (S -), with a value of 9.2 g/L (at 30°C) and 10.3 g/L (at 22°C). Higher cell viability of non-*Saccharomyces* yeasts – ranging from 8 and 8.5 log CFU/mL - after 24 hours of growth under aerobic conditions were observed, as well as higher biomass was reached when yeasts were grown in presence of oxygen [33]. Higher biomass production was also obtained when *Hanseniaspora* spp. was grown under aerobic conditions compared to static cultivation [64], thus confirming the trend here observed. Conversely, *Saccharomyces* spp. showed an opposite behavior, with anaerobic state (S-) the most suitable for higher biomass production (around 10 g/L), even two times greater compared to the aerobic condition tested (3.9 g/L and 5.2 g/L at 22°C and 30°C, respectively). These results were in part in line with those observed by Perez-Torrado et al. [39] that obtained a similar final biomass of 10 g/L when cells were grown in non-aerated fermentations, whereas the amount of biomass here detected under aerobic conditions agreed with those reported in literature, in which similar growth conditions were performed, reaching a value of about 4 g/L [33,42]. On the other hand, by comparing the two different conditions of aeration, the results obtained disagree with those reported by Lemos Junior et al. [33] that determined a value of 0.6 g/L of biomass produced by *Saccharomyces* spp. after fermentation, much lower compared to the same yeast grown in aerated condition (about 4 g/L); similarly, Schnierda et al. [42] obtained a biomass production of 3.79 g/L in static cultivation, lower than obtained in bioreactor fermentation under controlled dissolved oxygen level (10.14 g/L).

Above certain glucose concentration, even in presence of oxygen, *Saccharomyces* spp. switches from respiratory to fermentative metabolism [65], thus resulting in lower biomass yield [66]; furthermore, Cowland et Maule [67] observed how *Saccharomyces* spp. showed a good growth rate during aerobic fermentation and the presence of oxygen seemed to increase the production of biomass, at least until certain levels. On the other hand, the faster consumption of glucose observed during anaerobic fermentation [65] together with a possible better adaptation to such condition, might have in part determined higher cell growth rate, thus possibly explaining the highest biomass produced by this strain in the anaerobic condition here performed (S -).

4.3 Release of compounds of enological interest under different growth conditions and after lysis treatment

The amounts of amino acids and mannans, riboflavin and glutathione were determined after 24 hours of growth under different conditions and the results, with means and standard deviations, were reported in Annex 2; the same parameters were also analyzed on the starting culture medium because of the presence of yeast extract in its formulation; the concentrations were used for correcting the data referred to the growth phase. The characterization of autolyzed suspensions after enzyme-assisted lysis, with means and standard deviations, was reported in Annex 3.

All the parameters abovementioned were discussed in depth in their respective, following sections, by comparing the amounts released after growth and after enzyme-assisted lysis; this has allowed to evaluate how starting growth conditions have influenced the synthesis and the subsequent release of such compounds after lysis treatment.

4.3.1 Release of amino acids after growth and lysis treatment

A consumption of amino acids was observed after growth in all the conditions tested (Annex 2), conversely to the results obtained during the initial screening (Chapter 1, section 4.3). This was probably due to the fact that the growth conditions used in the two experimental trials were different; furthermore, the chemical characterization of compounds released after growth were performed approximately at the end of the exponential phase in the present study and during the stationary phase (after 48 hours of growth) in the preliminary screening when cell lysis might be occurred, thus determining a release of such compounds into the culture medium.

The concentration of amino acids (expressed in mg/g of biomass) released after enzyme-assisted lysis by *Hanseniaspora* spp. and *Saccharomyces* spp. was reported in figure 4 (a and b, respectively).

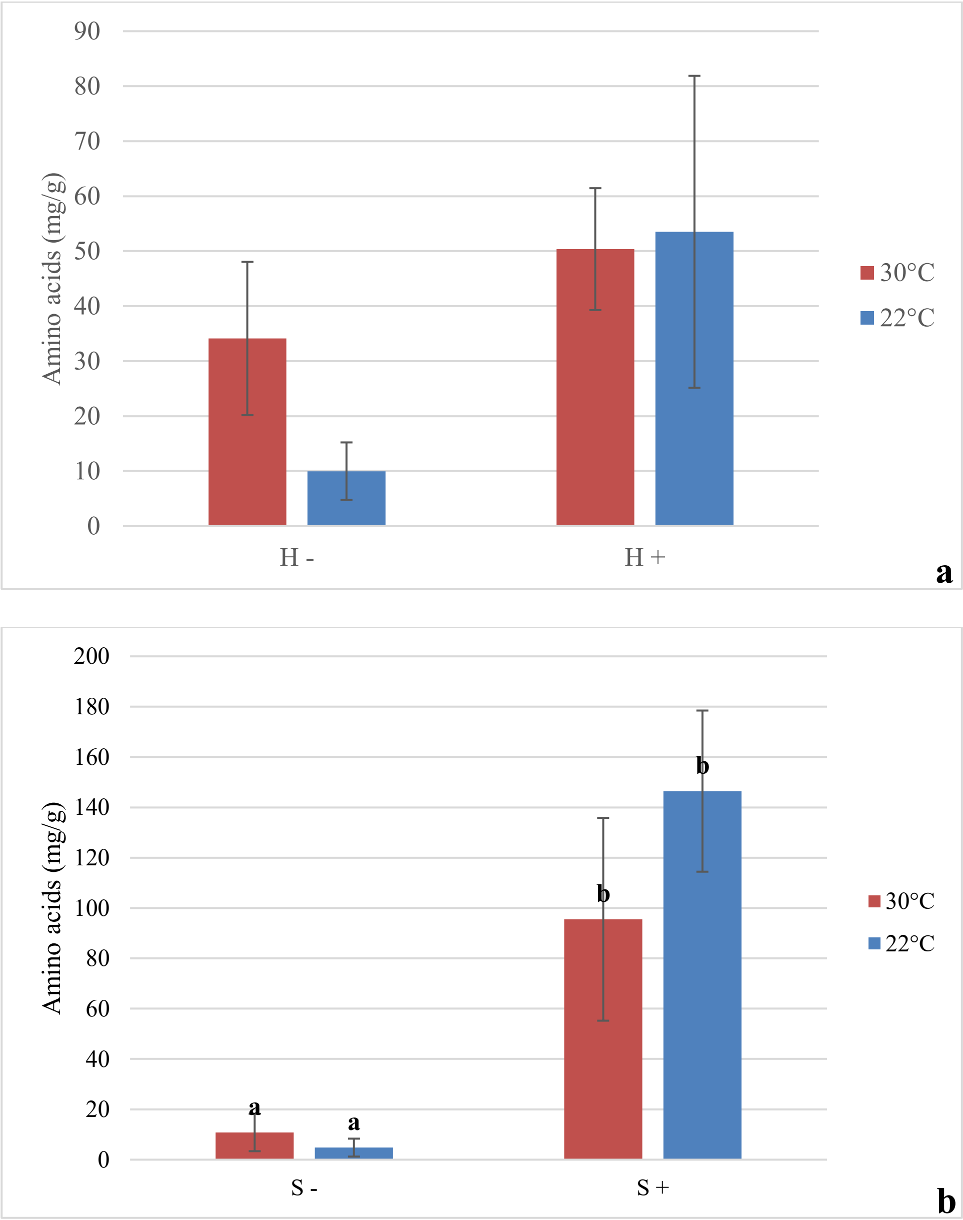


Figure 4. Release of amino acids (standardized to gram of biomass) after enzyme-assisted lysis by *Hanseniaspora* spp. (**a**) and *Saccharomyces* spp. (**b**). Data were means and standard deviations of three replicates. For each strain, different letters marked significant differences among treatments, according to ANOVA e Tukey HSD test at $p < 0.05$. H: *Hanseniaspora* spp.; S: *Saccharomyces* spp.; - : growth without shaking to mimic anaerobic conditions; + : growth under constant shaking (150 rpm) to mimic aerobic conditions.

For both the strains, no effect of the temperature applied during the growth phase was observed (table 9) whereas the growth performed in aerobic condition (+) determined the highest release of such compounds after lysis, with 52 mg/g in H+ and 121 mg/g in S+, significantly different from the respective anaerobic conditions (22 mg/g and 8 mg/g in H- and S-, respectively).

However, the highest mean concentrations were found at 22°C, with 54 mg/g for *Hanseniaspora* spp. and 146 mg/g for *Saccharomyces* spp. (fig. 4 a and b).

Different nitrogen source preference and utilization by several yeast strains was previously observed under winemaking conditions [6,28]; this different behavior might have affected the nitrogen metabolism during the growth phase and, consequently, the amount of intracellular amino acids, thus possibly explaining the different concentration released by the two strains after lysis. Under aerobic conditions, nitrogen sources utilization was improved [68], as well as an increase in intracellular proteins content during growth at lower temperature was also reported [69], possibly justifying the highest mean concentration of amino acids released when both the yeasts were grown under aerobic conditions at 22°C.

4.3.2 Release of mannans after growth and lysis treatment

The amount of mannans (expressed in mg/g of biomass) released after growth and after lysis was reported in table 10.

Table 10. Release of mannans (standardized to gram of biomass) after growth and enzyme-assisted lysis. Data were means and standard deviations (SD) of three replicates. For each yeast strains, different letters within the same column marked significant differences among treatments, according to ANOVA and Tukey HSD test at $p < 0.05$. H: *Hanseniaspora* spp.; S: *Saccharomyces* spp.; - : growth without shaking to mimic anaerobic conditions; + : growth under constant shaking (150 rpm) to mimic aerobic conditions. Concerning data "after growth", positive and negative values (blue and red bars) indicated a release or a reduction respectively, in relation to the initial composition of culture medium.

				Mannans (mg/g)							
Yeast	Temperature	Aeration	Sample code	After growth				After lysis			
				Mean	±	SD		Mean	±	SD	
Hanseniaspora	30°C	-	H_30°C-	49	±	51		211	±	57	a
	22°C	-	H_22°C-	-9	±	22		144	±	63	a
	30°C	+	H_30°C+	30	±	6		370	±	27	b
	22°C	+	H_22°C+	7	±	2		367	±	13	b
Saccharomyces	30°C	-	S_30°C-	10	±	2	b	22	±	10	a
	22°C	-	S_22°C-	-3	±	2	a	13	±	12	a
	30°C	+	S_30°C+	22	±	4	c	69	±	39	a
	22°C	+	S_22°C+	8	±	3	b	169	±	35	b

Starting from data referred to the growth phase (table 8), the release of mannans was significantly affected by temperature for both the yeasts, with the highest amounts of 39 mg/g for *Hanseniaspora* spp. and 16 mg/g for *Saccharomyces* spp. at 30°C. About the effect of aeration, this parameter significantly affected the amount of mannans released after growth in the case of *Saccharomyces* spp., with the highest mean content under aerobic condition. In general, as reported in table 10, the highest mean concentrations were found for *Hanseniaspora* spp. at 30°C in both non-aerated and aerated conditions (49 mg/g and 30 mg/g for H_30°C – and H_30°C +, respectively), even if no statistical differences were observed; for *Saccharomyces* spp. the highest production was observed in S_30°C +, with a content of 22 mg/g, thus resulting significantly different from all the other treatments.

The growth factors that significantly influenced the release of mannans after lysis were aeration for both the strains and temperature for *Saccharomyces* spp. (table 9): autolyzed suspensions obtained from both the yeasts previously grown under aerobic condition were characterized by the highest release of mannans, with values of 369 mg/g and 119 mg/g for *Hanseniaspora* spp. and *Saccharomyces* spp., respectively, as well as the lowest temperature determined the highest release in autolyzed suspensions from *Saccharomyces* spp. (91 mg/g and 46 mg/g in S_22°C and S_30°C, respectively). By considering the results reported in table 10, concerning *Hanseniaspora*, spp., the highest amount was released by yeast suspensions previously grown under aerobic condition at both the temperatures, with a content of 367 mg/g and 370 mg/g for H_22°C+ and H_30°C+, respectively. Even for *Saccharomyces*, the highest amount was found in S_22°C+ with 169 mg/g, resulting significantly different from all the other treatments, followed by S_30°C+ (69 mg/g), in terms of mean values.

It is worth noting the tendential, higher mean content of mannans released by *Hanseniaspora* spp. after lysis treatment, even two times greater compared to *Saccharomyces* spp. Similar trend was previously observed by Domizio et al. [4], reporting the ability of this strain to release higher amounts of polysaccharides per gram of dry cell weight compared to *Saccharomyces* spp. For both the yeasts, the amounts of mannans detected were almost comparable to the content of polysaccharides released during alcoholic fermentation, with a further increase when cells were not yet alive, especially for *Hanseniaspora* spp., probably due to a greater susceptibility and faster autolysis [13].

The effect of temperature on mannan production by *S. cerevisiae* during growth was previously observed, with an enhanced synthesis at lower temperature [55]; in addition, decreased dissolved oxygen level seemed to enhance the production of cell wall mannans and chitin-glucan complexes in *P. pastoris* in bioreactor fermentation at 30°C [56]. It would be hypothesized that the aerobic conditions performed in the present study by constant shaking might allow to dissolve oxygen in suspensions, compared to the unshaken samples (anaerobic condition), thus possibly enhancing the synthesis of cell wall mannans during the growth phase. Consequently, the amount of cell wall polysaccharides might be potentially released after lysis; this might explain the highest concentration of such compounds in autolyzed suspensions obtained from both the yeasts previously grown under aerobic conditions (+), and even at lower temperature in the case of *Saccharomyces* spp.

4.3.3 Release of riboflavin after growth and lysis treatment

The amount of riboflavin (expressed in μg/g of biomass) released after growth and lysis treatment was reported in table 11.

Table 11. Release of riboflavin (standardized to gram of biomass) after growth and enzyme-assisted lysis. Data were means and standard deviations (SD) of three replicates. For each yeast strains, different letters within the same column marked significant differences among treatments, according to ANOVA and Tukey HSD test at $p < 0.05$. H: *Hanseniaspora* spp.; S: *Saccharomyces* spp.; - : growth without shaking to mimic anaerobic conditions; + : growth under constant shaking (150 rpm) to mimic aerobic conditions. Concerning data "after growth", the concentrations were referred to the difference in relation to the starting composition of the culture medium.

Yeast	Temperature	Aeration	Sample code	Riboflavin (μg/g)							
				After growth				After lysis			
				Mean	±	SD		Mean	±	SD	
Hanseniaspora	30°C	-	H_30°C-	58	±	23	ab	62	±	22	
	22°C	-	H_22°C-	105	±	36	b	55	±	19	
	30°C	+	H_30°C+	26	±	5	a	59	±	3	
	22°C	+	H_22°C+	39	±	5	a	78	±	14	
Saccharomyces	30°C	-	S_30°C-	13	±	1	a	9	±	4	a
	22°C	-	S_22°C-	15	±	1	a	4	±	3	a
	30°C	+	S_30°C+	27	±	1	b	52	±	16	b
	22°C	+	S_22°C+	45	±	5	c	66	±	6	b

Starting from the growth phase (table 8), the amount of extracellular riboflavin (standardized to gram of biomass) was significantly affected both by temperature and aeration; regarding the former, the highest production were observed when both the yeasts were grown at lower temperature (72 μg/g and 30 μg/g, in H_22°C and S_22°C, compared to 42 μg/g and 20 μg/g in H_30°C and S_30°C, respectively). Regarding aeration, its effect towards the production of riboflavin during the growth phase was different for the two yeasts: anaerobic condition enhanced the production in *Hanseniaspora* spp., conversely to what observed for *Saccharomyces* spp., for which the highest concentration was found under aerobic condition. In general, the highest amount was found in H_22°C- (105 μg/g), and under aerobic condition at the same temperature for *Saccharomyces* (45 μg/g detected in S_22°C+).

After lysis (table 9), no significant differences were observed in the amount of intracellular riboflavin released by *Hanseniaspora* spp., independently on the growth conditions previously used; however, the sample that showed the highest extracellular release during growth (H_22°C-, with 105 µg/g) conversely showed the lowest amount of intracellular riboflavin released after lysis (55 µg/g), even if only in terms of mean value (table 11). On the contrary, in the case of *Saccharomyces* spp., the condition that enhanced the release of riboflavin during growth phase – aeration at both temperatures - also determined the highest release of such molecule after lysis, with a concentration of 66 µg/g and 52 µg/g for S_22°C+ and S_30°C+, respectively. Riboflavin is produced by yeasts during alcoholic fermentation in different concentration (up to 170 µg/L), mainly dependent on the strains used [70]. The growth conditions here adopted were quite similar to those reported in literature for enhancing microbial production of riboflavin, thus possibly explaining the amounts detected: glucose and yeast extract seemed to be the best nutrient sources [71,72], whereas temperature of 28°C and shaking speed of 170 rpm were the most suitable manufacturing parameters for enhancing the production of this vitamins by *Candida* spp. [73]. Nevertheless, no further studies have been reported in literature about how manufacturing process may affect the production during yeast biomass propagation, like the comparative effect of low and high temperatures, or anaerobic and aerobic conditions, as here assessed. However, the production of riboflavin should be limited since at a concentration higher than 50 µg/L, this vitamin may be involved in light-induced reaction determining the appearance of the so-called light-struck defect [74], particularly relevant for white wines.

4.3.4 *Release of thiol compounds after growth and lysis treatment*

The amounts of antioxidant compounds (expressed in µmol/g) after growth and after lysis were reported in table 12. Concerning the growth phase, only glutathione was quantified, whereas cysteine was also detected on autolyzed suspensions; for these latter, the insoluble fraction was subjected to the same analysis because of the possible presence of other compounds (such as reducing proteins containing-cysteine residues, RPC), linked to insoluble cell wall residues.

Table 12. Release of thiol compounds (expressed in µmol/g) after growth and enzyme-assisted lysis. Data were means and standard deviations (SD) of three replicates. For each yeast strains, different letters within the same column marked significant differences among treatments, according to ANOVA and Tukey HSD test at $p < 0.05$. H: *Hanseniaspora* spp.; S: *Saccharomyces* spp.; - : growth without shaking to mimic anaerobic conditions; + : growth under constant shaking (150 rpm) to mimic aerobic conditions. Concerning data "after growth", the concentrations were referred to the difference in relation to the starting composition of the culture medium. *nd: not detected. GSH: glutathione; CYS: cysteine; RPC reducing proteins containing-cysteine residues)

Yeast	Temperature	Aeration	Sample code	After growth				After lysis											
				GSH				GSH				CYS				RPC			
				Mean	±	SD		Mean	±	SD		Mean	±	SD		Mean	±	SD	
Hanseniaspora	30°C	-	H_30°C-	26.2	±	8.6		35.6	±	12.9		26.2	±	9.1		nd*			a
	22°C	-	H_22°C-	25.8	±	7.4		42.0	±	17.3		26.0	±	8.9		nd			a
	30°C	+	H_30°C+	11.5	±	0.8		36.3	±	3.5		12.7	±	1.2		488.2	±	227.8	b
	22°C	+	H_22°C+	11.7	±	2.0		53.1	±	9.4		19.7	±	1.4		398.0	±	16.2	b
Saccharomyces	30°C	-	S_30°C-	4.1	±	0.6	a	6.3	±	2.4	a	4.7	±	1.5	a	nd			
	22°C	-	S_22°C-	3.6	±	0.6	a	3.8	±	1.7	a	3.0	±	0.6	a	nd			
	30°C	+	S_30°C+	7.4	±	0.4	b	72.1	±	17.2	b	28.4	±	6.3	b	nd			
	22°C	+	S_22°C+	12.4	±	0.2	c	85.2	±	3.2	b	41.8	±	2.6	c	nd			

Starting from data referred to the growth phase (table 8), extracellular glutathione content was only affected by aeration, with a significant release under anaerobic conditions for *Hanseniaspora* spp. (26 µmol/g in H-) and, at least in terms of mean values, under aerobic condition for *Saccharomyces* spp. (9.9 µmol/g in S+). This trend was also confirmed by looking at the results reported in table 12.

After lysis treatment, intracellular glutathione together with intracellular cysteine and RPC linked to the cell wall fractions were also detected in autolyzed suspensions (table 9). Temperature influenced the release of antioxidant compounds, with tendential highest concentration of glutathione and cysteine when both the yeasts were previously grown at low temperature; however, significant difference was only observed for cysteine released by S (22.4 µmol/g in S_22°C and 16.6 µmol/g in S_30°C).

Furthermore, the release of antioxidant compounds was also influenced by aeration: a significant impact on the release of both glutathione and cysteine was observed for *Saccharomyces* spp. when yeast was grown under aerobic condition. For *Hanseniaspora* spp. the aerated conditions seemed to enhance the production of glutathione - even if no significant differences emerged - whereas a significant impact of such condition was observed on the amounts of RPC; conversely, the release of cysteine after lysis seemed to be significantly affected when this strain was previously grown under anaerobic condition.

Looking at the results reported in table 12, it is interesting to note that, independently on the growth conditions, *Hanseniaspora* spp. released a non-negligible amount of all the antioxidant compounds analyzed, with slight differences among the treatments: the content of intracellular glutathione ranged from 35.6 µmol/g to 53.1 µmol/g (H_30°C– and H_22°C+, respectively), whereas the concentration of cysteine ranged from 12.7 µmol/g to 26.2 µmol/g (H_30°C+ and H_30°C-); it is worth noting that this strain showed a good synthesis of reducing proteins under aerobic condition, with a mean content of 488.2 µmol/g and 398 µmol/g detected in H_30°C+ and H_22°C+, respectively. On the other hand, in the case of *Saccharomyces* spp., the conditions that enhanced the production of glutathione during growth phase - S_22°C+ (aeration at 22°C) - also determined the highest release of the intracellular glutathione (85.2 µmol/g) and cysteine (41.8 µmol/g) after lysis; surprisingly, no reducing proteins were detected in all the conditions tested.

A non-negligible concentration of intracellular glutathione produced by non-*Saccharomyces* yeasts was observed [18], with amounts comparable or even higher than *Saccharomyces* spp. after 24 hours of growth, with a further increase during the stationary phase [19]. By simulating biomass propagation in batch fermentation at 30°C in aerated condition (shaking of 180 rpm), different strains of *Hanseniaspora* spp. showed an intracellular glutathione content lower than *Saccharomyces* spp. [18], thus possibly explaining the lower mean concentration detected in H+ (44.7 µmol/g) compared to S+ (78.6 µmol/g) or in H_30°C+ (36.3 µmol/g) compared to S_30°C+ (72.1 µmol/g). On the other hand, the higher activity of enzymes involved in glutathione synthesis showed by *Hanseniaspora* spp. [18,75] might have determined a higher accumulation of intracellular glutathione during growth; this might partially explain the higher concentration detected after lysis, together with a similar release from all the samples, independently on the growth conditions previously adopted. During biomass production of *S. cerevisiae*, Perez-Torrado et al. [39] observed that, after sugars consumption in aerated conditions, a metabolic change - from fermentative to respiratory - occurred, determining a further expression of genes involved in glutathione pathway; in addition, lower temperature seemed to enhance the glutathione production in *C. utilis* during batch fermentation under aerated conditions, determining an increase in the intracellular glutathione content up to 2.5% w/w [76]. Even if no information was about the metabolic changes occurred during the growth conditions evaluated in the present study, it would be hypothesized a similar metabolism for *Saccharomyces* spp., thus possibly explaining the highest mean content of intracellular glutathione produced and then released by this strain when grown at lower temperature in aerated condition.

5. Conclusion

Growth conditions adopted for producing yeast biomass may strongly affect the production of compounds of enological interest both during growth and after lysis treatment. Among these, aeration seemed to be the most impacting parameter, with differences also depending on the strain. In general, aerated conditions have enhanced cell viability, also determining the highest release of amino acids and riboflavin, mannans and antioxidant compounds by both the yeasts after lysis treatment; regarding biomass, aerated condition have enhanced the production in *Hanseniaspora* spp., whereas the most suitable condition for *Saccharomyces* spp. seemed to be anaerobiosis.

It is worth noting the considerable amount of mannans released by *Hanseniaspora* spp., much higher compared to those detected in *Saccharomyces* spp. grown in the same conditions, and above all, the greater ability of this strain to produce and release notable amount of antioxidant compounds compared to the other strain. Independently on the growth conditions adopted, *Hanseniaspora* spp. released an appreciable amount of glutathione and cysteine, comparable or even higher than those detected in *Saccharomyces* spp. grown in the same conditions; furthermore, the former was the only strain able to produce a considerable amount of reducing proteins in the conditions here adopted.

The right management of growth conditions for obtaining yeast biomass may also enhance the production of compounds of enological interest, *i.e.*, soluble colloids and antioxidant molecules; this information might be useful for understanding the contribution of yeast (*e.g.*, used as fermentation starter) during fermentation and wine aging on lees, or how the composition of yeast derivatives might vary in relation to the conditions used to produce the initial biomass.

Considering the interesting results obtained in the present study, the same strain of *Hanseniaspora* spp. was used as co-starter in sequential fermentation with *S. cerevisiae*, in order to evaluate its potential contribution on the chemical composition and volatile profile of wine both during alcoholic fermentation (Chapter 5) and wine aging on lees (Chapter 6).

Chapter 3

Influence of strain and processing technology on yeast derivatives composition

1. Introduction

1.1 Composition of yeast derivatives

Yeasts derivatives are conventionally classified in four groups in relation to the manufacturing process: *i)* inactive yeasts obtained after thermal inactivation; *ii)* yeast hulls or walls consisting of the insoluble fractions of yeast cells without cytoplasmic content; *iii)* yeast extracts, mainly characterized by the soluble fraction after total degradation of intracellular components; *iv)* yeast autolysates obtained after thermal inactivation followed by an incubation period to allow the activity of endogenous enzymes, resulting in partial degradation of intracellular content [77]. Regarding the latter, they consist of cell wall residues that remain after production process, thus representing the insoluble fraction; furthermore, as consequence of the autolytic process and degradation, intracellular components (*i.e.*, amino acids and peptides, also including glutathione) and cell wall polysaccharides may be solubilized into the wine, thus representing the soluble fraction of this type of yeast derivatives.

Yeast derivatives represent a considerable source of proteins, amino acids and polysaccharides as the main components of yeast cells.

As concerns nitrogen compounds, about 39.6% of cell dry weight consists of protein fractions, with increasing content up to 62.4% in autolysates, extracts and protein concentrates [78,79]; this percentage is also in relation to the extraction and precipitation protocols [80] and methods used for inducing cell lysis [81,82]. The amino acids profile varies in terms of composition and amount, consisting of both essential and non-essential amino acids that, in some cases, may be present in excess compared to WHO reference [80,82]; however, their content, together with proteins, increases in yeast derivatives - especially in yeast extracts - thanks to the activities of endogenous enzymes and their subsequent release in the soluble fraction [78,82–84].

Polysaccharides mainly consist of β-(1,3)- and β-(1,6)-glucans and mannoproteins as the main components of yeast cell walls [51,52]. They represent about 30% of cell dry weight [79], with amounts varying in relation to the kind of products, with the highest content in yeast autolysates and cell wall preparations [85,86].

Lastly, yeast derivatives also show an appreciable amount of lipids [87,88], B complex vitamins and minerals [84], nucleic acids and flavoring agents [89,90], making them suitable for different applications.

1.2 Manufacturing process of yeast derivatives

In food industry, the most common methods used for producing yeast derivatives include thermolysis, generally employed for extracting mannoproteins [91,92], enzyme-assisted lysis and autolysis employed for both polysaccharides and intracellular components release [82,93,94]. However, the high temperatures reached during manufacturing process – like induced autolysis or thermolysis – may trigger the well-known Maillard reaction [95], thus determining browning and changes in the volatile profile of the resulting yeast derivatives [94,96]. Furthermore, pH [97] and temperature [98], autolysis promoters [86,97], type of enzyme and dosage [99] and enzyme to substrate ratio [100] may strongly affect the release of both intracellular and cell wall components.

On the other hand, during the last decades some emerging, non-thermal technologies have been successfully applied in food industry as alternative to traditional pasteurization [101]. Among these, pulsed electric fields (PEF), ultrasounds 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 ultrasounds [102] and pulsed electric fields [103] as tools for enhancing the extraction of compounds from grapes to reduce the maceration time, whereas high-pressure treatments may be applied for inactivating spoilage microorganisms in grape and must [104,105], in order to reduce sulfur dioxide addition.

The induction of autolysis in *S. cerevisiae* via PEF was reported, thus being useful both for producing yeast extracts [106] and for accelerating the release of mannoproteins during wine aging on lees [107–109]. Regarding ultrasounds, the efficiency of this technology on extracting polysaccharides was assessed [110], as well as the processing of fermentation lees allowed a higher release of soluble colloids and proteins during wine aging, with results similar to enzyme-assisted lysis [111]; moreover, the release of compounds of enological interest (especially polysaccharides) in a shorter period might possibly reduce the conventional period of aging on lees [112,113]. Interesting results have been also reported about the use of high-pressure homogenization to produce yeast autolysates [114,115], allowing to obtain products with chemical composition and volatile profile similar to those obtained after thermal inactivation or to commercial preparations [114,116]. High-pressure treatments might be also useful to produce yeast extracts via autolysis and to favor the recovery of β-glucans for food applications, mainly depending on treatment intensity and autolysis time [117,118].

2. Aim of the work

In this chapter, different processing parameters were evaluated in order to assess their potential effects on the chemical composition of yeast derivatives, with the purpose to obtain products specifically tailored for winemaking use. In the following chapters, the term “yeast derivatives” is used for indicating autolysates, because the products contain both insoluble and soluble fractions, as the result of yeast inactivation and partial degradation of cellular components.

The present experimental plan consisted of three parts.

In the first part, two non-*Saccharomyces* yeast strains - characterized in Chapter 1, strain H4 (*Hanseniapora* spp.) and strain P2 (*Pichia* spp.) - were chosen for evaluating their aptitude to obtain yeast derivatives. These strains were selected as the best compromise between biomass production and lower release of amino acids, together with high production of soluble colloids and antioxidant compounds. Biomass was produced at laboratory scale and the release of nitrogen compounds, soluble colloids and antioxidant molecules was evaluated after thermal inactivation and high hydrostatic pressure treatment, in comparison with commercial *S. cerevisiae* (the results were reported in Part I “*Potential use of Hanseniaspora spp. and Pichia spp. for yeast derivatives production*”).

In the second part, the application of ultrasounds treatment was optimized with the purpose to obtain yeast derivatives starting from *S. cerevisiae* and *T. delbrueckii* (the results were reported in Part II “*Effect of ultrasounds on yeast inactivation and autolysis for derivatives production*”).

Consequentially, in the third part the experiment was aimed at comparing methods commonly used for producing yeast derivatives (*e.g.*, thermolysis and enzyme treatment) with selected emerging technologies, with the purpose of both evaluating the impact of the production process on the chemical composition, and the possibility to use such emerging techniques as alternatives to the traditional methods. In this regard, thermal inactivation, β-glucanase addition, ultrasounds and high hydrostatic pressure were compared; the chemical characterization of the yeast derivatives obtained was carried out in terms of cell viability and release of compounds of enological interest, *i.e*., nitrogen compounds, antioxidant molecules and soluble colloids, by using commercial *S. cerevisiae* and *T. delbrueckii* yeasts as starting material (the results were reported in Part III "*Effect of yeast strains - S. cerevisiae and T. delbrueckii - and lysis treatments on inactivation and chemical composition of yeast derivative powders"*).

3. Materials and methods

3.1 Reagents

Sodium chloride for microbiology, bacteriological peptone, Sabouraud Dextrose Agar (SDA), Malt Extract Broth, and technical agar were purchased from Oxoid (Milan, Italy); tetracycline, sodium hydroxide, N-acetyl-L-cysteine, *o*-phthaldialdehyde (OPA), isoleucine, ethanol (96 % v/v), mannan from *S. cerevisiae*, sodium citrate, trifluoroacetic acid, riboflavin, L-glutathione reduced, L-cysteine, *p*-benzoquinone, 3-mercaptopropionic acid, bovine serum albumin (BSA) fraction V, Folin Ciocalteu's phenol reagent, 4,4′-dithiodipyridine (DTDP), sodium acetate and ethylenediaminetetraacetic acid (EDTA), 2,2-diphenyil-picrylhydrazyl (DPPH$^{\bullet}$) were from Sigma Aldrich (St. Louis, MO, USA). Absolute ethanol and methanol (HPLC grade) were purchased by VWR Chemicals (Milan, Italy). Tartaric acid, boric acid, hydrochloric acid (37 % v/v), copper sulfate pentahydrate, sodium tartrate and sodium carbonate were purchased from Carlo Erba Reagents (Milan, Italy). MilliQ water was produced by MilliQ Advantage A10 apparatus (Merck Millipore, Billerica, MA, USA) and microfiltered at 0.22 µm before use. Commercial β-glucanase preparation and commercial active dry yeasts (ADY) *S. cerevisiae* and *T. delbrueckii* were from Enologica Vason SpA (San Pietro in Cariano, VR, Italy).

3.2 Yeast biomass

For strain H4 (*Hanseniaspora* spp.) and strain P2 (*Pichia* spp.) the production of biomass was carried out at laboratory scale. Single pure colony were grown on Malt Extract Agar slants, then transferred in sterile tubes containing 100 mL of Malt Extract Broth and incubated at 30°C for 24 hours in an orbital shaker (85 rpm). After the first growth phase in liquid culture medium, the suspensions were homogenized and inoculated on Sabouraud (SDA) and Malt Extract Agar plates, then incubated at the 30°C under constant shaking (85 rpm) for further 48 hours. The biomass was recovered by scraping and washing with saline-peptone water and harvested by centrifugation (8000 rpm x 10 min, at 4°C). The biomasses were then resuspended in distilled water and subjected to lysis treatment as reported below. Commercial active dry yeast preparations of *S. cerevisiae* and *T. delbrueckii* were instead rehydrated in 10 volumes of distilled water, then subjected to lysis treatments.

3.3 *Lysis treatments*

Thermal inactivation (hereafter referred to as THERM) was performed by autoclaving 200 mL of yeast suspensions at 120°C for 2 h, whereas 100 mL of suspensions were transferred in polyamide/polyethylene sterile bags then subjected to high hydrostatic pressure (hereafter referred to as HHP). The treatment was carried out by an external facility (HPP Italia S.r.l., Traversetolo, PR, Italy); the processing system used was an Avure HHP machine, model AV-10 (AVURE Technologies, Erlanger, KY, USA), whereas the processing parameters were 600 MPa for 8 min at 30°C. For enzyme-assisted lysis (hereafter referred to as ENZ), a commercial preparation of enzyme with β-glucanase activity was added at a concentration of 3 % (w/w).

Ultrasounds treatment (hereafter referred to as US) was carried out by using an ultrasonic homogenizer Sonopuls (Bandelin electronic; Germany) equipped with titanium probe (13 mm diameter) (Sonotrode S26d14, Hielscher Ultrasonics Gmbh, Teltow, Germany). The depth of probe was set at 10 mm from the bottom of a 150 mL-Becker containing 100 mL of cell suspension; during the treatment, samples were put in an ice bath to avoid excessive heating. The treatment was performed in continuous pulse mode, at constant frequency of 20 kHz, amplitude of 30% and 90%, sonication time of 10 min, one to five cycles of sonication treatment, and temperature range of 20-45°C.

Expect for thermal inactivation, the pH of the treated suspensions was adjusted at 5 and the suspensions were incubated at 45°C for 24 h to allow yeast autolysis by favoring the activity of endogenous/exogenous enzymes; the suspensions were then heated for 2 min at 70°C to inhibit enzymes activity and to stop autolysis. The treated suspensions were then freeze-dried; the treatments were performed in triplicate. Yeast derivative powders were characterized in model solution (ethanol 12% v/v, 33 mM of tartaric acid, pH 3.2) in order to assess the amounts of soluble molecules potentially releasable into the wine; after resuspension, the samples were centrifuged (3000 rpm for 10 min) and both the supernatants and pellets were collected for the analyses, thus representing the soluble molecules and the insoluble fractions of yeast derivatives, respectively. The latter was also analyzed because of the possible presence of antioxidant molecules, *i.e.*, thiol-groups containing compounds, linked to the insoluble cell wall residues. The chemical evaluations were performed as better described below.

3.4 Cells viability

Yeast viable cells, expressed as logarithm of colony forming units (log CFU/mL) was determined before and after lysis treatments, as reported in Chapter 1, section 3.5.

3.5 Free amino acids and soluble proteins

In the case of US treatments (Part II), treated suspension were centrifuged and the amount of amino acids was determined on the supernatant; in the case of yeast derivatives (Part I and Part III), the powders were first resuspended in model solution at a final concentration of 1% (w/v); after 10 min, the mixtures were centrifuged at 3000 rpm for 15 min and the supernatants were subjected to the analysis.

Free amino acids were determined as reported in Chapter 1, section 3.6, whereas the concentration of soluble proteins was evaluated by Lowry assay as reported by Comuzzo et al. [115]. The concentrations were calculated according to a calibration curve prepared with a standard of bovine serum albumin (0-1000 mg/L) and the results were expressed in mg/g.

3.6 Evaluation of total soluble colloids by SE-HPLC

In the case of US treatments (Part II), treated suspension were centrifuged and the amount of total soluble colloids was determined on the supernatant; in the case of yeast derivatives (Part I and Part III), the powders were previously resuspended in model solution (at final concentration of 10% w/v); after 10 min, the mixtures were centrifuged at 3000 rpm for 15 min. The supernatants thus obtained were subjected to alcoholic precipitation and total soluble colloids were determined by SE-HPLC as described in Chapter 1 section 3.7.

The concentrations were calculated according to a calibration curve prepared with a standard of mannan (0-1000mg/L). and the results were expressed in mg/g.

3.7 Total insoluble solids of yeast derivatives powder by weighing

Yeast derivatives were resuspended in model solution at a concentration of 1% (w/v) and the suspensions were then filtered under-vacuum on pre-weighed 0.45µm pore size cellulose membranes. The membranes were kept in oven at 60 °C for 2 hours up to constant weight; the difference between final and initial weight was used for calculating total insoluble solids content and the results were expressed in mg/g.

3.8 Particle size distribution by dynamic light scattering (DLS)

On yeast derivative powders, mean particles diameter distribution was evaluated by dynamic light scattering (DLS) measurement as reported by Natolino & Celotti (2022). After resuspending powders in model solution at a concentration of 1% (w/v) for 10 min, the mixtures were centrifuged (3000 rpm for 10 min) and the supernatant was subjected to the analysis. Measurements give an estimation of mean particles diameter, together with the distribution of different diameters for particles with mean size below 1 µm, both expressed in nanometers (nm).

3.9 *Evaluation of antioxidant properties*

The antioxidant properties were evaluated by spectrophotometric assays (thiol compounds and DPPH assay) and by RP-HPLC analysis (content of glutathione, cysteine and reducing proteins containing cysteine-residues, RPC). As concerns thiol compounds, yeast derivative powders were first resuspended at 1% (w/v) in acetate buffer (0.3M, pH 3.6); after 10 min, the mixtures were centrifuged (3000 rpm for 15 min). The supernatants were collected, the pellets were washed twice and carefully resuspended in the same buffer, thus represented the soluble and the insoluble fractions of derivatives, respectively. The following analysis was performed as reported in Chapter 1, section 3.8. DPPH assay was carried out following the method described by Da Porto et al. [119] with slight modification. Yeast derivative powders was resuspended in model solution at a concentration of 1 % (w/v); 500 µL of the mixture were then transferred into 10 mL-Pyrex tubes and added with 2.5 mL of DPPH solution (80µM in absolute ethanol), vigorously mixed and left in darkness for 60 min; this fraction was used for evaluating the antioxidant property of the whole yeast derivative. The remaining suspension was centrifuged (3000 rpm for 15 min) and 500 µL of the supernatant was subjected to DPPH assay as previously reported, thus representing the soluble fraction. The absorbance was measured against absolute ethanol - at the beginning and after the reaction, and the difference between the initial and final values was used for calculating the radical scavenging activity. The antioxidant properties were expressed in µmol/g, in relation to a calibration curve made with standard solution of glutathione (0-650 µmol/L).

The content of glutathione and cysteine were evaluated on the supernatants, whereas the pellets were used for determining the amount of reducing proteins containing cysteine-residues possibly linked to insoluble cell wall residues. For yeast derivatives (Part III), the powders were first resuspended in 50 mM of citrate buffer pH 5 as described in Tirelli et al. [32], then subjected to derivatization and RP-HPLC analysis as reported in Chapter 2, section 3.6.

3.10 Riboflavin content by RP-HPLC

The content of riboflavin was determined on yeast derivatives (Part III) by resuspending powders in model solution (final concentration of 1% w/v); after 10 min, the mixtures were centrifuged at 3000 rpm for 15 min; 1 mL of the supernatant was filtered on 0.22 µm MCE syringe-filter, then subjected to RP-HPLC analysis as reported in Chapter 2, section 3.7.

3.11 Aroma profile of yeast derivatives powders by SPME-GC-MS

Two grams of freeze-dried powder were introduced in 20 mL glass vials sealed with PTFE/silicone septa. The volatile profile was 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 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 x 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°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 of external standard and 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.

3.12 Statistical analysis

The results obtained were means and standard deviations of three repeated trials. Homogeneity of variance was evaluated by Brown-Forsythe and Cochran, Hartley, Bartlett test; ANOVA and Tukey HSD test were carried out for all the parameters analyzed and differences were considered significant at $p < 0.05$. Due to the non-homogeneity of the variances, groups were compared with Kruskal-Wallis. Multiple comparisons to detect significant differences among groups were carried out using the Bonferroni adjusted Mann-Whitney U test. Factorial analysis and principal component analysis (PCA) were also performed. All elaborations were carried out by the software Statistica for Windows Version 8.0 (StatSoft, Tulsa, OK, USA).

4. Results and discussion

Part I: ***Potential use of Hanseniaspora spp. and Pichia spp. for yeast derivatives production***

In this section, the results about the potential use of two non-*Saccharomyces* yeast strains, namely strains H4 (*Hanseniapora* spp.) and P2 (*Pichia* spp.) for obtaining yeast derivatives were reported. As already written, these strains were selected based on the first characterization (Chapter 1), as the best compromise between biomass production and non-negligible release of soluble colloids and antioxidant compounds. Biomass was produced at laboratory scale and the release of nitrogen compounds, soluble colloids and antioxidant molecules was evaluated after thermal inactivation and high hydrostatic pressure, in comparison with commercial *S. cerevisiae*.

The chemical characterization of yeast derivative powders was carried out in model solution and the results were discussed in the following paragraphs.

4.1 Release of nitrogen compounds and soluble colloids

The content of nitrogen compounds, free amino acids and soluble proteins (in mg/g of yeast derivative powder) were reported in figure 5.

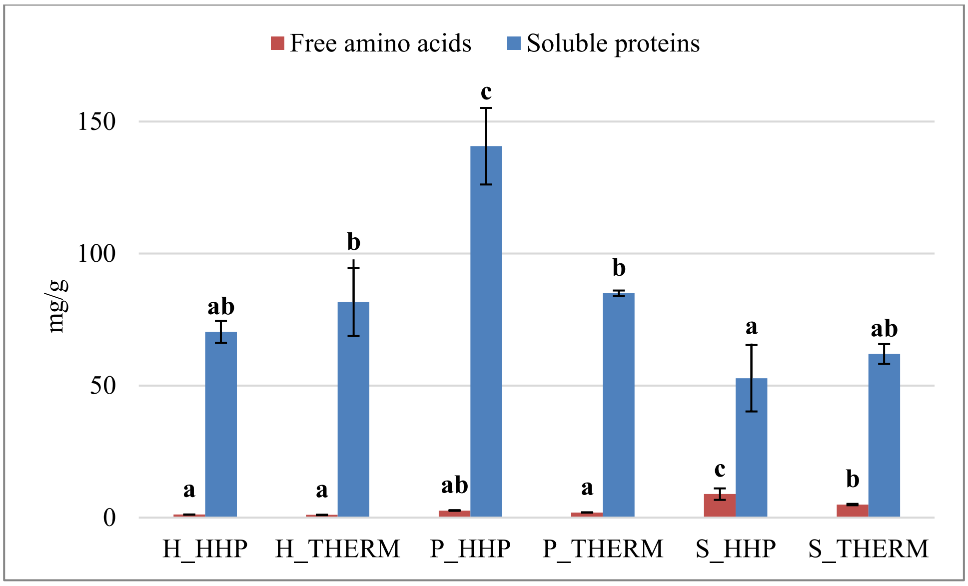


Figure 5. Nitrogen compounds (free amino acids and soluble proteins) expressed in mg/g of powder. Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. H: *Hanseniaspora* spp.; P: *Pichia* spp.; S: *S. cerevisiae*; THER: trmal inactivation; HHP: high hydrostatic pressure.

Concerning amino acids released from each strain, the content was quite similar between the two treatments (ranging from 1 to 3 mg/g), except for S_HHP (9 mg/g) and S_THERM (5 mg/g), that resulted significantly different between them and compared to the other samples. The content of soluble proteins was not affected by the treatment applied in the case of H and S, with no statistical differences between H_HHP and H_THERM (70 and 82 mg/g, respectively), and between S_HHP and S_THERM (53 and 62 mg/g); it is interesting to highlight the content of soluble proteins released by P_HHP, with a value of 141 mg/g, thus resulting significantly different from all the other samples, followed by P_THERM (85 mg/g) in terms of mean values. The different amounts released by the strains might be probably related to a different nitrogen metabolism [6,28] and protein synthesis [120], thus influencing the following release; furthermore, the highest content of soluble proteins detected in P_HHP might be probably due to a higher susceptibility of this strain to high-pressure treatment, resulting in a higher cell disintegration rate.

The concentration of total soluble colloids (in mg/g of yeast derivative powder) was reported in figure 6.

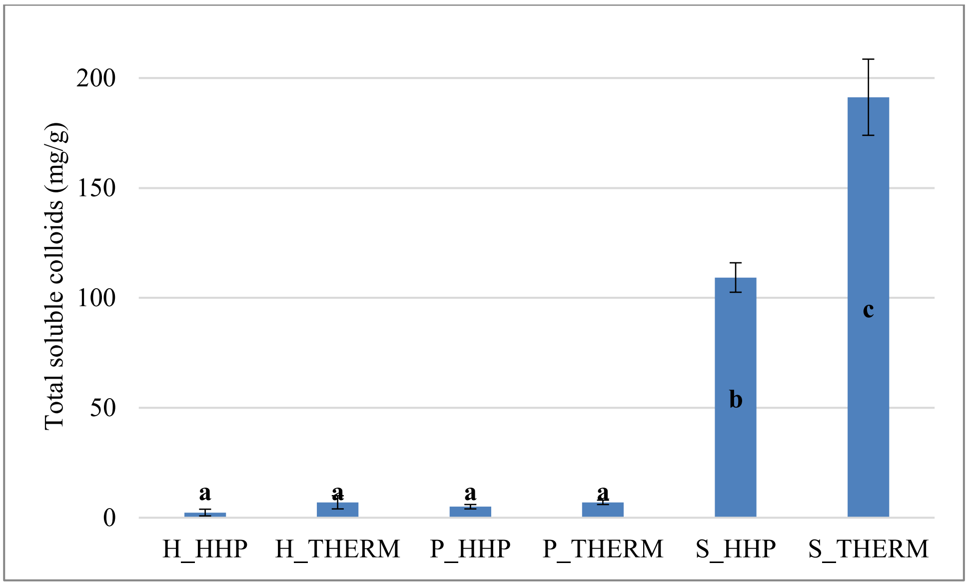


Figure 6. Total soluble colloids content expressed as mg/g of powder. Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. H: *Hanseniaspora* spp.; P: *Pichia* spp.; S: *S. cerevisiae*; THER: thermal inactivation; HHP: high hydrostatic pressure.

Derivatives from S were the most characterized in terms of soluble colloids, with highest amounts detected in T_THERM (191 mg/g), resulting statistically different from the S_HHP, the other derivative with the greatest content (109 mg/g).

On the other hand, the amounts of soluble colloids released by both non-*Saccharomyces* strains used in the present study was very low, with a concentration ranging from 2 mg/g (H_HHP) to 7 mg/g (detected in P_THERM and H_THERM). These results were in contrast with those reported by other authors about the higher release of polysaccharides by *Pichia* spp. and *Hanseniaspora* spp. compared to *S. cerevisiae* during alcoholic fermentation [4,13]. However, in the present study the concentrations detected in derivatives from P was higher compared to the previous characterization (0.3 mg/10^9 cells, Chapter 1, table 5), whereas the amounts of soluble colloids released from H was quite similar to what previously detected (3.2 mg/10^9 cells after lysis treatment, Chapter 1, table 5). This different behavior might be explained by the different lysis treatments performed in the two experimental trials: during the first characterization, enzyme-assisted lysis was carried out, probably resulting more efficient in releasing such compounds compared to THERM and HHP, at least for H. In addition, the growth conditions previously used (YPD broth and incubation at 30°C for 48) were different from the conditions here adopted for producing biomass (Sabouraud and Malt Extract Agar plates, incubation at 30°C for 48 h in orbital shaker), thus probably affecting the growth kinetics and the production of cellular components.

4.2 Antioxidant compounds

The concentration of thiol compounds (in µmol/g of yeast derivative powder) evaluated on both soluble and insoluble fraction was reported in figure 7.

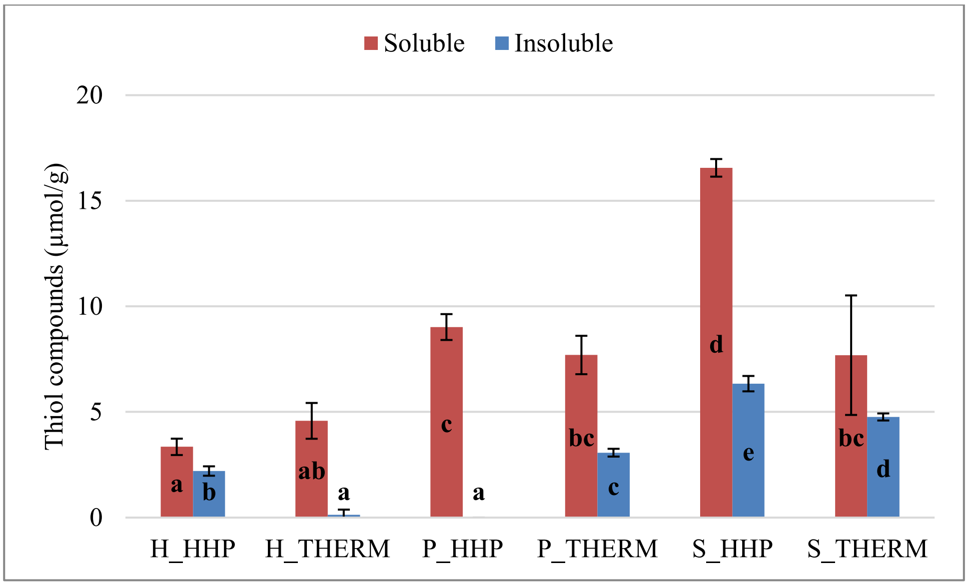


Figure 7. Thiol compounds (soluble and insoluble fraction) expressed as µmol/g of powder. Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. H: *Hanseniaspora* spp.; P: *Pichia* spp.; S: *S. cerevisiae*; THER: thermal inactivation; HHP: high hydrostatic pressure.

For all the strains tested, the highest antioxidant activity seemed to be related to the soluble fraction. The lowest amount was found for strain H processing both by THERM and HHP (3.3 and 4.6 µmol/g, respectively), whereas S_HHP showed the highest content of thiol compounds on both soluble and insoluble fraction (16.6 and 6.3 µmol/g, respectively), thus resulting statistically different from all the other samples. Conversely to what observed during the initial characterization of wild non-*Saccharomyces*, P released a relevant amount of compounds with antioxidant properties, higher than H; in the previous study (Chapter 1, table 6), the amount released after lysis was only 0.23 µmol/10^9 cells for strain P2, whereas strain H4 showed a content of 0.80 µmol/10^9 cells. However, both the strains demonstrated how - in suitable growth conditions and after appropriate lysis treatments – the ability to release antioxidant molecules in model solution was not negligible and, in the case of P, the amount was almost comparable to S treated by THERM. Good antioxidant properties in terms of radical scavenging activity and inhibition of lipids oxidation were observed in intracellular free-cell extracts obtained by *P. fermentans* compared to *S. cerevisiae* [121], depending on the highest content of glutathione and the positive correlation between the antioxidant activity and the content of soluble proteins [122]. These results might in part support the findings obtained in the present study; higher content of intracellular glutathione possibly produced in the growth conditions here tested, together with higher amount of proteins (possibly containing thiol groups) might explain the non-negligible content of antioxidant molecules detected in the soluble fraction, especially as concerns derivatives obtained from *Pichia* spp.

4.3 Conclusion

The aptitude of non-*Saccharomyces* strains to release compounds of enological interest was observed, confirming the possibility to use them for producing yeast derivatives. A significant release of proteins was observed in the case of *Pichia* spp. possibly using this strain for producing protein extracts or derivatives to be employed as fermentation enhancers. However, in more suitable growth conditions and after appropriate lysis treatment, both the strains showed a good ability to release non-negligible amounts of antioxidant molecules, especially in the soluble fractions and, in the case of *Pichia* spp., this feature was comparable to *S. cerevisiae*; this may contribute to enrich wine of antioxidant compounds both during fermentation and wine aging on lees, possibly enhancing the protection against oxidation.

***Part II*: Effect of ultrasounds on yeast inactivation and autolysis for derivatives production**

In this section, the results about the potential application of ultrasound treatment and the optimization of processing parameters for yeast derivatives production were reported.

This section was divided in two parts.

In the first part (section 4.1), the results of preliminary tests performed for yeast inactivation and autolysis induction in *S. cerevisiae* and *T. delbrueckii* were reported; the starting processing parameters were selected among those reported in literature, concerning the use of ultrasounds for accelerating aging on lees.

In the second part (section 4.2), the most suitable operating conditions were then applied with the purpose of obtaining yeast derivatives, and the chemical characterization of treated suspensions was performed in order to evaluate the potential release of compounds of enological interest. The treatments were performed in duplicates, and the chemical evaluation was carried out on yeast suspensions.

4.1. Effect of amplitude and sonication cycles on cell viability and autolysis

The preliminary test about the effect of ultrasounds (hereafter referred to as US) on yeast inactivation and autolysis induction was first performed on *S. cerevisiae*. The US treatments were carried out at an amplitude of 30% and 90% for 10 min, together with an untreated sample as control. In figure 8, the cell viability determined after two and six days of incubation at 45°C was reported.

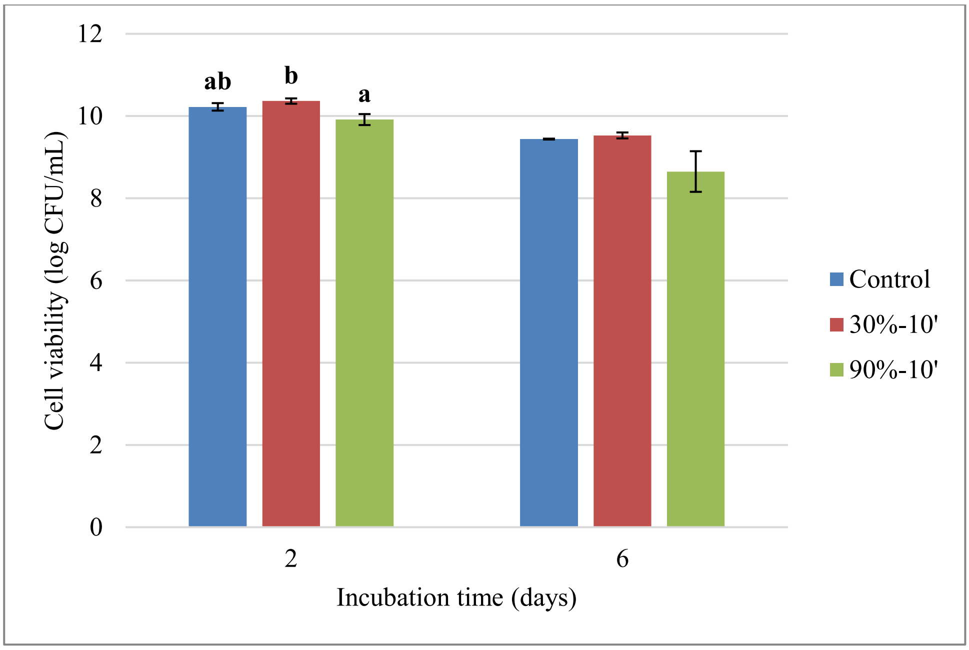


Figure 8. Cell viability (as log CFU/mL) determined on suspensions of *S. cerevisiae* after two and six days of incubation at 45°C. Data were means and standard deviations of two replicates. Control (untreated sample), 30%-10’ (amplitude of 30% for 10 min), 90%-10’ (amplitude of 90% for 10 min). Different letters marked significant differences among treatments at each sampling time, according to ANOVA and Tukey HSD test at $p < 0.05$.

The starting cell concentration was 9.90 log CFU/mL that corresponded to 8.1 x 10^9 CFU/mL. The US treatments performed did not allow a relevant reduction of cell viability neither after two nor after six days of incubation. After two days, the sample treated at 30% showed the highest mean value in the number of viable cells that was comparable to that observed in the untreated sample (10.36 and 10.22 log CFU/mL, respectively), whereas the sample treated at 90% showed the lowest mean value (9.91 log CFU/mL), resulting significantly different from the former. After six days of incubation, for all the samples a reduction in cell viability was observed, with the highest mean value detected in the sample treated at 30% (9.53 log CFU/mL), followed by untreated sample (9.44 log CFU/mL) and sample processed at 90% (8.65 log CFU/mL); however, no significant differences among the treatments were observed.

As concerns the content of free amino acids (figure 9), this parameter was used as index for monitoring the autolytic process.

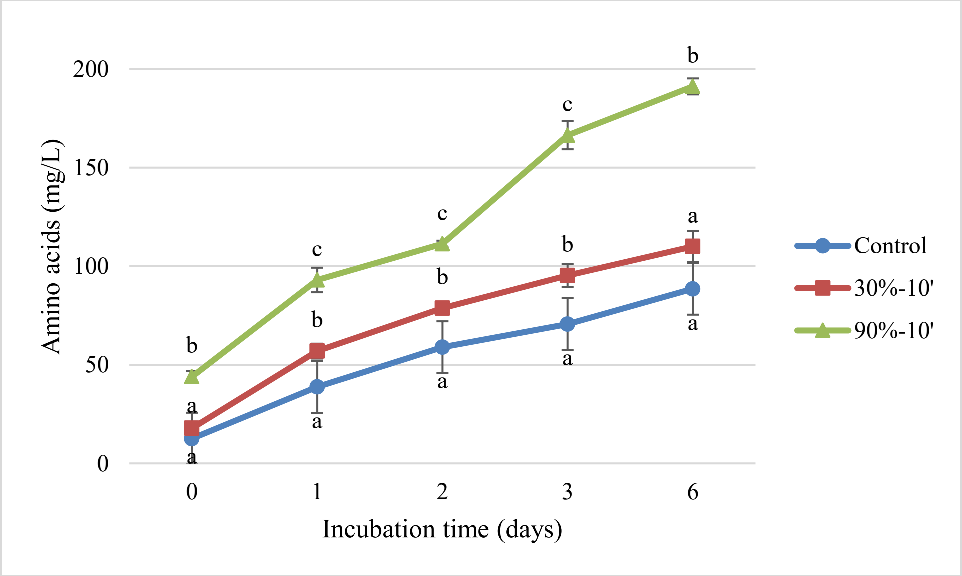


Figure 9. Release of free amino acids (in mg/L) determined on suspensions of *S. cerevisiae* during six days of incubation at 45°C Data were means and standard deviations of two replicates. Control (untreated sample), 30%-10’ (amplitude of 30% for 10 min), 90%-10’ (amplitude of 90% for 10 min). Different letters marked significant differences among treatments at each sampling time, according to ANOVA and Tukey HSD test at $p < 0.05$.

Just after the treatment (time 0), the sample processed at 90% showed the highest release of amino acids (44 mg/L), thus resulting significantly different from the control and the sample treated at 30% (with 13 and 18 mg/L, respectively). During incubation, a continuous release from yeast cells was observed, with significant differences among the treatments at each sampling time. This increase was particularly evident for the sample treated at 90% that showed the highest release of amino acids at the end of incubation, with an amount of 191 mg/L, followed by the sample treated at 30% (110 mg/L) and the control (89 mg/L).

By this preliminary test, the treatment performed at 30% of amplitude seemed to activate yeast cells; furthermore, no difference was observed on cell viability when this sample was compared to the control, whereas a slight, higher release of amino acids was observed. On the other hand, the treatment performed at 90% seemed to allow an induction of autolysis, with a reduction in the number of viable cells of about one logarithmic unit compared to the starting cell concentration (from 9.90 to 8.65 log CFU/mL), together with a considerable release of amino acids after the treatment and, above all, during incubation. Therefore, this treatment was selected for testing the possible induction of autolysis on suspension of *T. delbrueckii*; untreated sample was used as control.

In figure 10, cell viability determined on yeast suspensions of *T. delbrueckii* after two and six days of incubation was reported.

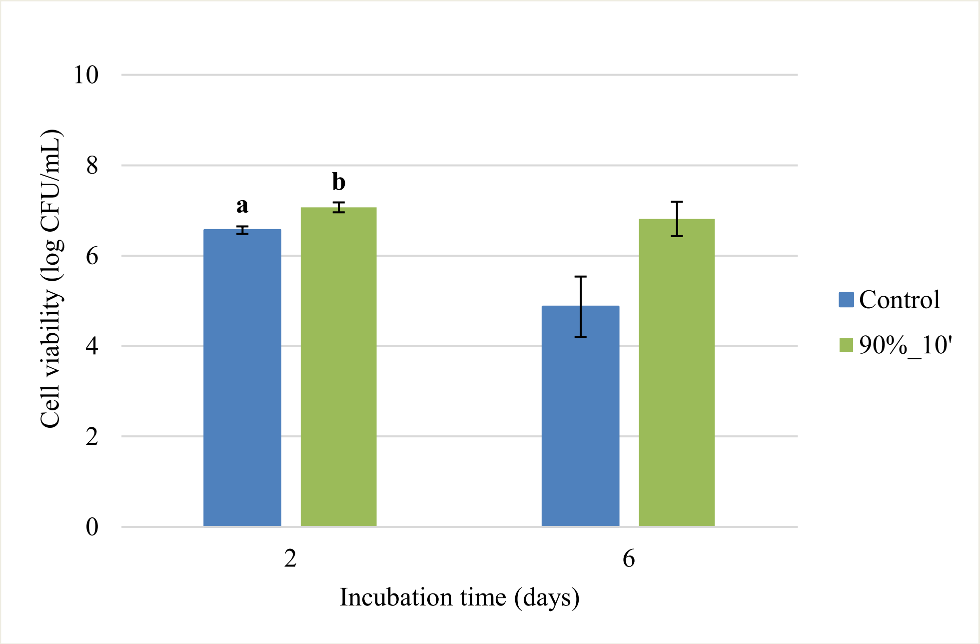


Figure 10. Cell viability (as log CFU/mL) determined on suspensions of *T. delbrueckii* after two and six days of incubation at 45°C. Data were means and standard deviations of two replicates. Control (untreated sample), 90%-10’ (amplitude of 90% for 10 min). Different letters marked significant differences among treatments at each sampling time, according to ANOVA and Tukey HSD test at $p < 0.05$.

The cell concentration determined on suspension before US treatment was 8.30 log CFU/mL, (corresponding to 2 x 10^8 CFU/mL). Contrary to what observed for *S. cerevisiae*, after two days of incubation the microbial counts were lower than the starting cell concentration, with a reduction of 1.74 and 1.23 log CFU/mL observed in the control and the sample treated with US, respectively. However, the number of viable cells was slightly higher in the treated sample (7.07 log CFU/mL) compared to the control (6.56 log CFU/mL), resulting significantly different. This trend was also observed at the end of incubation: even if no significant differences were observed between the treatments (*p* = 0.07), sample treated at 90% showed a cell viability of about 2 log CFU/mL higher than the control (6.81 log CFU/mL and 4.87 log CFU/mL, respectively). In the case of *T. delbrueckii*, US seemed to activate microbial cells and the reduction in cell viability observed might be mostly related to a natural autolysis occurrence. This was also confirmed by considering the amount of amino acids released during incubation (figure 11).

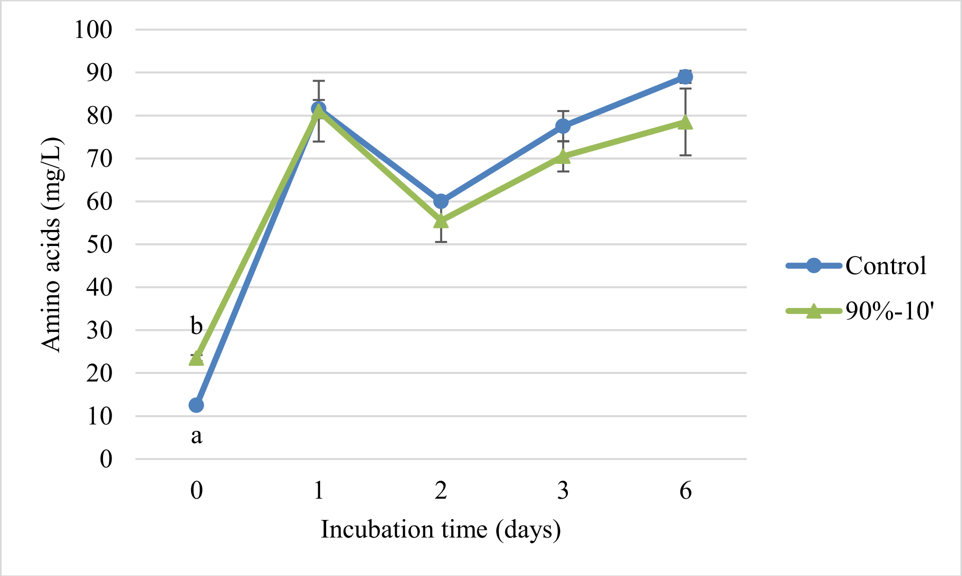


Figure 11. Release of free amino acids (in mg/L) determined on suspensions of *T. delbrueckii* during six days of incubation at 45°C. Data were means and standard deviations of two replicates. Control (untreated sample), 90%-10’ (amplitude of 90% for 10 min). Different letters marked significant differences among treatments at each sampling time, according to ANOVA and Tukey HSD test at $p < 0.05$.

Except at time 0 - just after the treatment - no significant differences were observed at each sampling time between treated and untreated samples; furthermore, the control showed the highest mean content at the end of incubation, thus confirming that for this yeast strain the conditions tested were not suitable for inducing autolysis. It was reported that US might stimulate the growth during the rehydration phase of active dry yeast preparations: the formation of cavitation sites at level of cell aggregates and, consequentially, the localized stress caused by the acoustic waves, seemed to favor the cell division [123], also depending on treatment time and intensity [124].

Based on these preliminary results, to achieve yeast inactivation, US was performed at the same processing conditions (amplitude of 90% for 10 min) in repeated cycles of sonication, up to 5 times. The effect of the repeated US treatment on cell viability of *S. cerevisiae* and *T. delbrueckii* was reported in figure 12 (a and b, respectively).

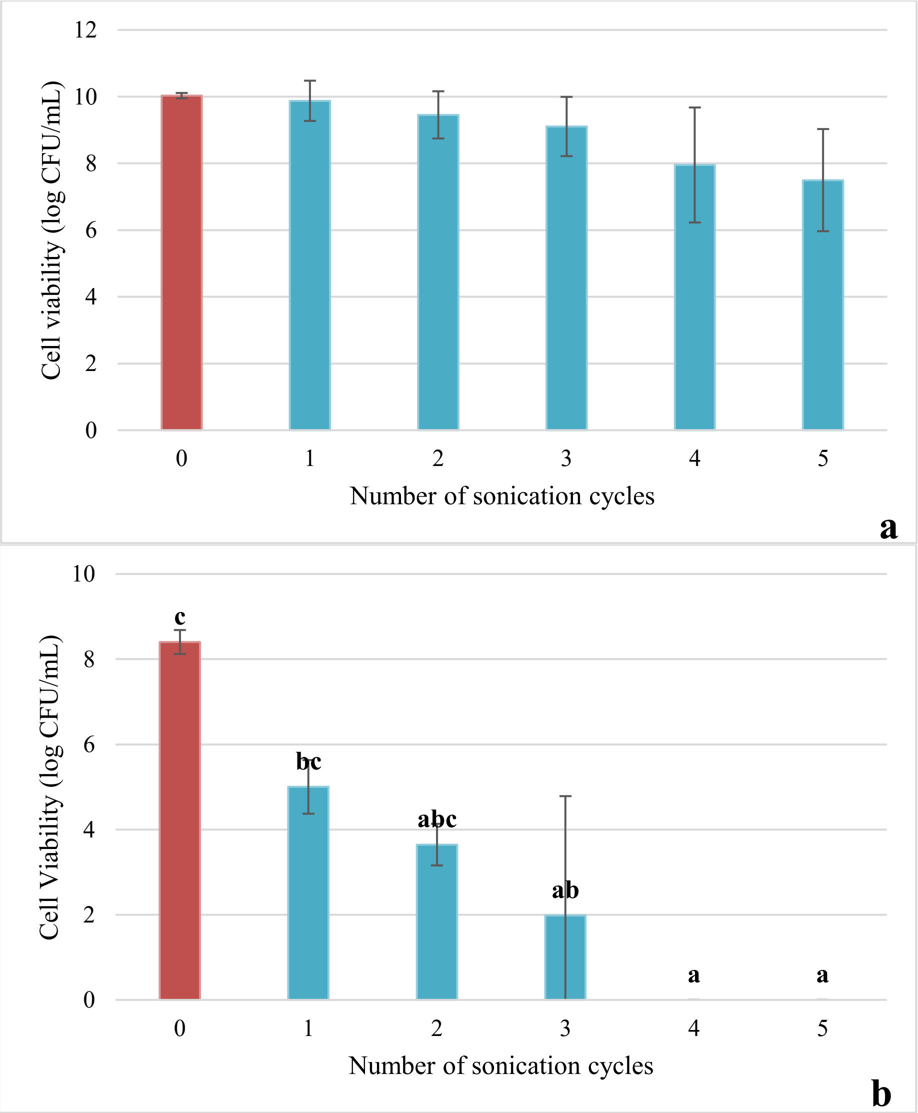


Figure 12. Cell viability (as log CFU/mL) determined on suspensions of *S. cerevisiae* (**a**) and *T. delbrueckii* (**b**) after repeated US treatment (amplitude of 90% for 10 min, 5 cycles. Data were means and standard deviations of two replicates. Different letters marked significant differences among number of sonication cycles, according to ANOVA and Tukey HSD test at $p < 0.05$.

By performing repeated US treatment, the effect on cell viability was quite different from the preliminary tests, with results that were strongly dependent on yeast strains. In the case of *S. cerevisiae* (fig. 12 a), no significant differences were observed and the reduction on the number of viable cells was only of 2.53 log CFU/mL after five cycles of treatment; on the other hand, complete inactivation of *T. delbrueckii* was reached after 4 cycles of sonication with cell viability lower than 100 CFU/mL (fig. 12 b), according to OIV specifications for the maximum cell viability admitted in yeast derivatives [125].

The effect of US treatments on cell viability seemed to be dependent on several parameters. First, low-intensity treatment (frequency of 28 kHz, power of 120W) seemed to enhance the growth of *S. cerevisiae* due to a higher cell membrane permeability, resulting in increase of biomass when the treatment was performed at the end of the lag phase [126]; the effect on growth and consequently on biomass increment seemed to be also dependent on cell growth phase (lag, exponential or stationary phase), power, treatment time and total duration of treatment [127]. In addition, low intensity treatment during the first 12 hours of the alcoholic fermentation led to an enhanced uptake of nutrients due to permeabilization of cell membranes, consequentially determining faster glucose consumption and ethanol production and reducing the fermentation length [128,129].

On the other hand, the application of high intensity treatments might be useful to inactivate microorganisms. Frequency of 850 kHz determined an irreversible damage caused by cavitation and local increase of temperature and pressure, leading to severe breakage of cell walls and membranes, coupled with pH modification and formation of free radicals [130]; furthermore, high ultrasonic power treatment (above 100 W) seemed to be useful for reducing the population of spoilage microorganisms in grape juice and wine [131]. Nevertheless, the inactivation level was also dependent on several other processing parameters, *i.e.*, cell concentration, pH, temperature, amplitude, and power [132–134], as well as matrix and strains [130,135,136]. Even if in the present study lower frequency was used (20kHz), the application of repeated sonication cycles (for a total duration of 50 min), together with an amplitude of 90% and nominal power output of 200W might have determined severe damages to microbial cells, similarly to high intensity treatments.

4.2.Chemical composition of yeast suspension after US treatment and incubation

The suspensions of *S. cerevisiae* and *T. delbrueckii* obtained after US treatment as reported in the previous section (90% of amplitude, 10 min of treatment time, 5 sonication cycles), were divided in two aliquots, of whom one was also subjected to an incubation step for allowing yeast autolysis (45°C for 24 h). The suspensions were then characterized in terms of cell viability, nitrogen compounds (free amino acids and soluble proteins), thiol compounds (determined both on the soluble and insoluble fraction) and total soluble colloids. The suspensions were also analyzed before treatment and used as reference (control) and the results were reported in table 13.

Table 13. Cell viability and chemical composition of yeasts suspensions determined before and after treatments. Control: untreated sample, before US treatment; US: samples treated with US (amplitude of 90% for 10 min, 5 cycle), US + incub: samples treated with US (amplitude of 90% for 10 min, 5 cycle) and incubated at 45°C for 24 h. Data were means and standard deviations (SD) of two replicates. The concentrations were standardized to gram of biomass. Different letters within the same column marked significant differences among treatments for each strain, according to ANOVA and Tukey HSD test at $p < 0.05$.

Yeast	Treatment	Cell viability (log CFU/mL)				Free amino acids (mg/g)				Soluble proteins (mg/g)				Thiol compounds soluble fraction (µmol/g)				Thiol compounds insoluble fraction (µmol/g)				Total soluble colloids (mg/g)			
		Mean		SD		Mean		SD		Mean		SD		Mean		SD		Mean		SD		Mean		SD	
	Control	9.90	±	0.10	b	2	±	1	a	21	±	11	a	0.78	±	0.60	a	1.0	±	0.1		2	±	0	a
S. cerevisiae	US	7.50	±	1.53	ab	9	±	1	b	77	±	5	b	3.38	±	0.01	b	0.8	±	0.8		107	±	8	b
	US + incub	2.39	±	1.96	a	16	±	0	c	117	±	1	c	4.11	±	0.27	b	0.1	±	0.0		147	±	4	c
	Control	8.30	±	0.02	b	2	±	0	a	22	±	1	a	0.43	±	0.18	a	1.4	±	0.1	c	17	±	1	a
T. delbrueckii	US	0.00	±	0.00	a	3	±	0	b	61	±	3	b	1.84	±	0.20	b	nd*			a	169	±	7	b
	US + incub	0.00	±	0.00	a	6	±	0	c	122	±	11	c	1.22	±	0.11	b	0.5	±	0.0	b	189	±	6	b

[*] nd: not detected.

As concerns cell viability, the incubation step performed after US treatment allowed to a further decrease in the number of viable cells of *S. cerevisiae*, reaching a value of 2.39 log CFU/mL compared to 7.50 log CFU/mL, detected on yeast suspension immediately after the treatment.

By considering the chemical composition of autolyzed suspensions, US determined a significant release of almost all the compounds evaluated - especially amino acids, soluble proteins and colloids – compared to the respective controls. After US, the suspensions obtained by *S. cerevisiae* showed the highest mean content of amino acids (9 mg/g), soluble proteins (77 mg/g), thiol compounds detected in the soluble fractions (3.38 μmol/g), and soluble colloids (107 mg/g), thus resulting significantly different from the respective control. After the incubation, the autolytic effect induced by US was enhanced, probably due to the activity of endogenous enzymes on intracellular and cell wall components; this allowed a further increase in the amounts of abovementioned compounds, in particular amino acids (+7 mg/g), proteins (+40 mg/g), and soluble colloids (+40 mg/g). Similar trend was observed for *T. delbrueckii*; this strain also showed the highest mean concentration of soluble colloids after US treatment (169 mg/g), that increased to 189 mg/g after the incubation.

The increased content of soluble compounds detected after the period of incubation might allow to hypothesize an increase in the activity of enzymes that are involved in cell wall degradation [137], thus allowing a further release of intracellular components. Furthermore, the release of polysaccharides and proteins by ultrasounds was previously observed by Zhang et al. [138], strongly affected by intensity of the treatment and cell concentration, sonication time, pH, and processed volume. Interesting results were also reported by processing fermentation lees by US, leading to faster and greater release of soluble colloids and proteins and allowing to reduce the conventional period of aging on lees [111–113,139]; however, few scientific evidences were found about the autolytic effect on non-*Saccharomyces* strains, as well as the potential use of this technology as tool for producing yeast derivatives.

About the influence of yeast strains on derivatives composition, it is worth noting the tendential, higher mean content of soluble colloids released by *T. delbrueckii*, about +62 mg/g after US treatment and +42 mg/g after incubation, compared to *S. cerevisiae*. The amounts detected as well as the different behavior of the two strains in relation to the release of soluble colloids agreed with what observed by Giovani et al. [13] under winemaking conditions; the authors reported a polysaccharides content of about 127 mg/g of dry cell weight for *T. delbrueckii*, higher than *S. cerevisiae* (90 mg/g), with a further increase when cells lost viability at the end of alcoholic fermentation.

4.3.Conclusion

The induction of autolysis by ultrasounds was confirmed and applied for the first time with the purpose of producing yeast derivatives; however, the effects were strongly dependent on the strain and processing parameters. *S. cerevisiae* was not completely inactivated, but the further incubation period allowed to reduce the number of viable cells of about 7 log CFU/mL compared to the starting concentration; complete inactivation was reached in the case of *T. delbrueckii,* according to OIV specification. Low intensity treatment (*i.e.*, 30 % for 10 min) may be useful for activating yeasts to be used as fermentation starters, whereas a more intense treatment (90 % for 10 min, 5 cycle of sonication) may be successfully applied as tool for producing yeast derivatives. *T. delbrueckii* spp. demonstrated a good aptitude to produce yeast derivatives, especially as concerns the amount of soluble colloids released after treatment and incubation (189 mg/g), higher than *S. cerevisiae* (147 mg/g).

Considering the interesting results obtained in the present study, a more complete experimental plan was set up and better described in the following section (Part III): US was compared to traditional methods and high hydrostatic pressure for evaluating their impact on the chemical composition of yeast derivatives; to give continuity to the experimental plan, the same yeast strains were used.

***Part III*: Effect of yeast strains - S. cerevisiae and T. delbrueckii - and lysis treatments on inactivation and chemical composition of yeast derivative powders**

The aim of the present work was to compare methods commonly employed for producing yeast derivatives with emerging technologies, with the purpose of both evaluating the impact of the production process on the chemical composition, and the possibility to use emerging technologies as alternatives to the traditional methods. In this regard, thermal inactivation, β-glucanase addition, ultrasounds and high hydrostatic pressure (hereafter referred to as THERM, ENZ, US and HHP, respectively) were performed as previously described in Materials and methods, section 3.3.

The chemical characterization of yeast derivatives was carried in model solution in order to preliminarily assess the potential release of molecules into the wine; the evaluations concerned cell viability and release of compounds of enological interest, *i.e.*, nitrogen compounds, antioxidant molecules and soluble colloids, both in commercial *S. cerevisiae* and *T. delbrueckii* yeasts (hereafter referred to as S and T). The results for each parameter evaluated were discussed in depth in their respective, following sections.

4.1 Cell viability and yeast inactivation

Cell viability, expressed as logarithm of colony forming units, was determined on suspensions of S and T before and after each treatment and the results were reported in figure 13 (a and b, respectively).

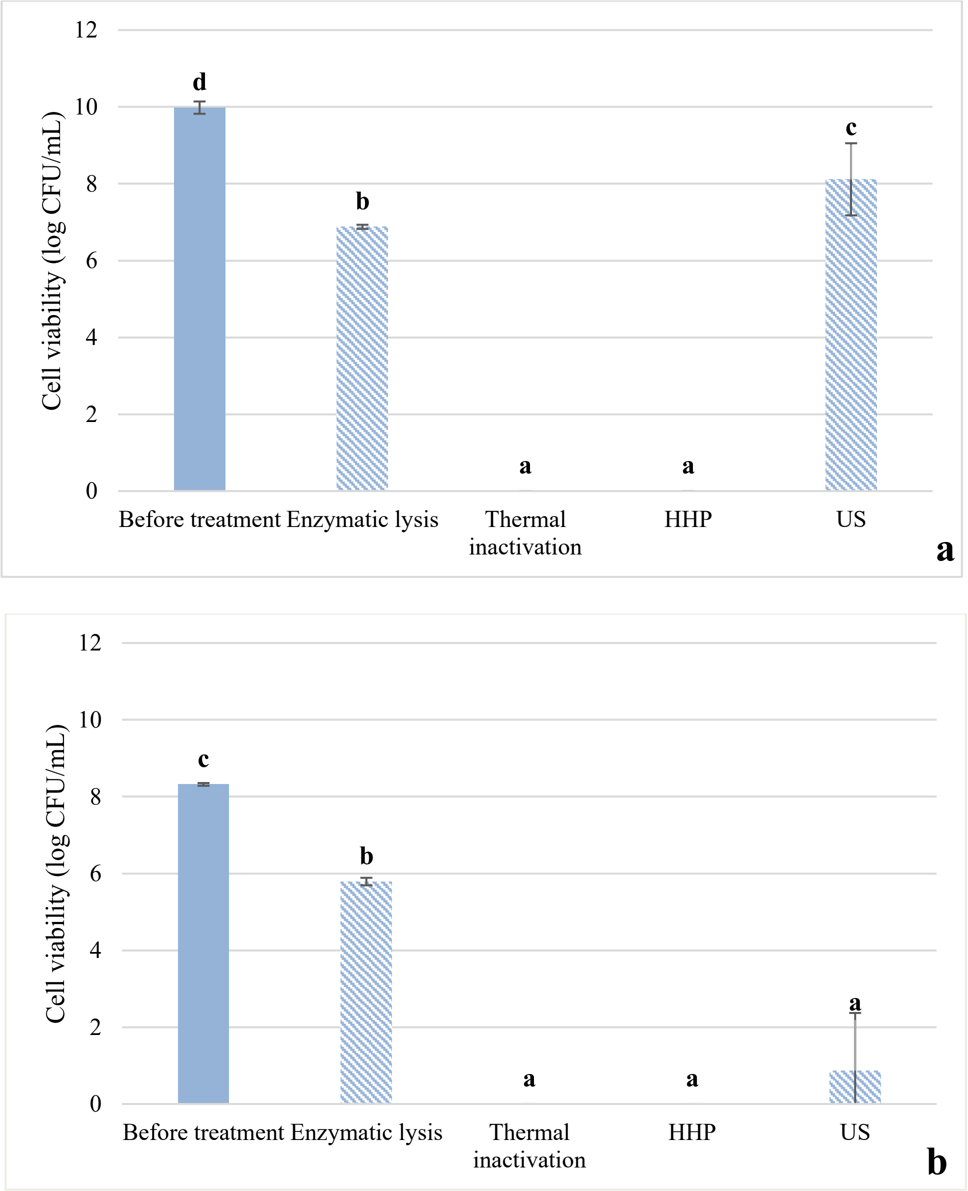


Figure 13. Cell viability (as log CFU/mL) determined before and after treatments on suspensions of *S. cerevisiae* (**a**) and *T. delbrueckii* (**b**). Data were means and standard deviations of three replicates. Different letters marked significant differences among treatments, according to ANOVA e Tukey HSD test at $p < 0.05$. ENZ: enzyme addition; THERM: thermal inactivation; HHP: high hydrostatic pressure: US: ultrasound.

The starting cell concentration was 9.98 and 8.32 log CFU/mL for S and T, respectively. A reduction in cell viability was observed, with significant differences compared to the starting concentration and among treatments; for both the yeast strains, THERM and HHP determined a complete yeast inactivation, whereas the reduction in the number of viable cells after ENZ was of 3.1 and 2.53 log CFU/mL for S and T, respectively. Regarding US, the different effect due to this treatment on yeast inactivation was confirmed, with results that were dependent on the strain: even if a significant difference was observed after treatment, the reduction of cell viability in S_US was only of 1 log CFU/mL compared to the starting concentration, whereas T was almost completely inactivated (0.87 log CFU/mL). However, the following incubation step determined a further reduction of cell viability in S_US until 3.25 log CFU/mL, highlighting the efficacy of US treatment on inducing yeast autolysis, as discussed above (Results - Part II of the present chapter).

The effect of high temperature on microbial inactivation is well known since thermal treatments (*i.e.*, sterilization and pasteurization) are used for food preservation against spoilage microorganisms [140], as well as enzyme addition allowed to accelerate autolytic process, also reducing cells viability [141]. On the other hand, high pressure treatments have been recently introduced in food industry as alternative to traditional pasteurization for inactivating microbial cells [142,143], thanks to the effect of pressurization on cell wall breakage and following cytoplasm exposure [144].

4.2 Chemical composition of yeast derivatives

The chemical characterization of yeast derivatives powders was carried out in model solution, both on soluble and insoluble fractions. Means and standard deviations were calculated, and the results were reported in Annex 4. All the parameters evaluated were subjected to factorial analysis and the results of the Principal Component Analysis (PCA) were reported in figure 14 (a and b).

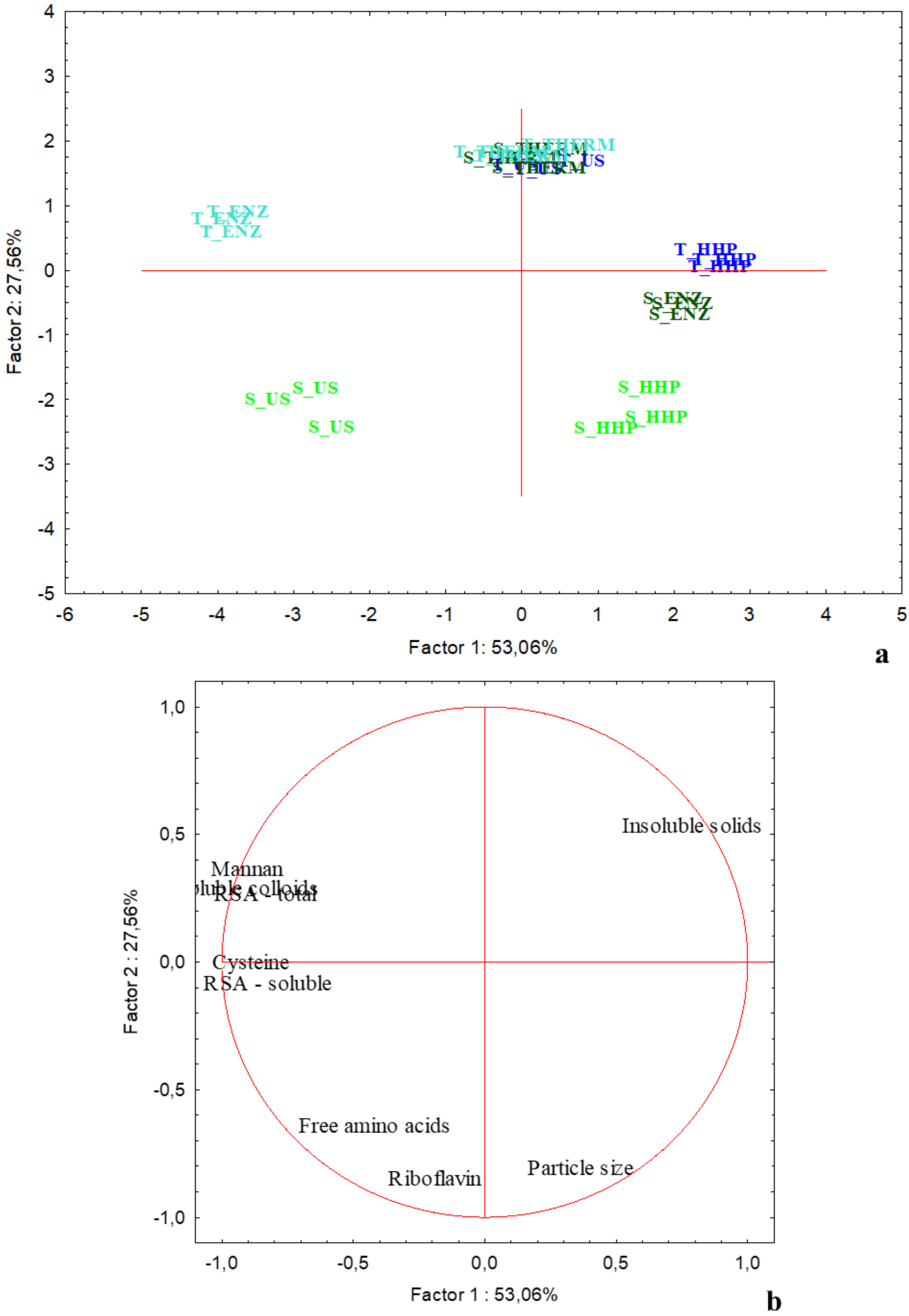


Figure 14. Results of PCA carried out on the compounds detected in yeast derivative powders. Projection of case (samples) (**a**) and variables (volatile compounds) (**b**) on the factor-plan were reported. Factor Loadings (FL) were calculated by Factorial Analysis, and the most relevant variables were selected for marked FL > 0.7. RSA: radical scavenging activity; RSA – total: radical scavenging activity determined on whole autolysate; RSA – soluble: radical scavenging activity determined on soluble fraction of autolysate. S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; HHP: high hydrostatic pressure: US: ultrasound.

Considering the results of PCA, the chemical composition of derivatives seemed to be mostly affected by the treatments applied; for both the yeasts, high-pressure treatment and thermal inactivation seemed to determine a higher release of insoluble solids, together with a higher mean size of soluble particles. On the other hand, the effect of ultrasound and enzyme addition on the chemical composition of the resulting derivatives seemed to be dependent on the strains: T_ENZ showed a tendential higher radical scavenging activity, together with a tendential higher content of polysaccharides (both mannan and total soluble colloids) and cysteine, whereas amino acids and riboflavin seemed to be mostly characterizing S_US.

The effect of yeast strain and treatment on the chemical composition of the resulting derivatives was reported in table 14; due to non-homogeneity of the variances, statistical differences were evaluated per groups (yeast and treatment) according to Kruskall-Wallis ANOVA and the results were discussed in terms of median values.

Table 14. Effect of yeast and treatment on the chemical composition of derivative powders in model solution. Data were the medians calculated for groups (yeast and treatment) and the concentrations were expressed per gram of powder. Different letters within the same row marked significant differences among groups, according to Kruskal-Wallis ANOVA and Bonferroni adjusted Mann-Whitney U test ($p < 0.05$). RSA: radical scavenging activity; RSA (total): radical scavenging activity determined on the whole derivative; RSA (soluble): radical scavenging activity determined on the soluble fraction of derivative; RPC: reducing proteins containing cysteine-residues. S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; HHP: high hydrostatic pressure: US: ultrasound.

	Effect of yeast				Effect of treatment							
	S		T		ENZ		THERM		US		HHP	
	Median		Median		Median		Median		Median		Median	
Free amino acids (mg/g)	10	b	4	a	10		4		16		6	
Soluble proteins (mg/g)	61		48		56	ab	55	a	142	b	40	a
Mannan (mg/g)	62		74		96	ab	86	b	94	b	15	a
Soluble colloids (mg/g)	147		164		218	ab	171	b	187	b	119	a
Insoluble solids (mg/g)	571		639		463		674		399		694	
Particle size (nm)	1340	b	516	a	1078	bc	514	a	674	ab	1674	c
Riboflavin (µg/g)	14		11		13	b	7	a	13	b	15	b
RSA total (µmol/g)	39.9		44.7		43.3	ab	49.1	b	45.0	ab	33.0	a
RSA soluble (µmol/g)	28.3		30.4		32.7		33.3		34.1		20.5	
Cysteine (µmol/g)	1.7		2.0		2.3	ab	1.7	a	2.6	b	1.6	a
Glutathione (µmol/g)	4.5		3.7		5.5		4.2		3.8		6.0	
RPC (µmol/g)	46.9	b	0.4	a	33.0		16.0		18.3		42.8	

As concerns the differences among all the samples, the results were reported in table 15. All the parameters evaluated were discussed in depth in their respective, following sections.

Table 15. Chemical composition of yeast derivative powders in model solution. Data were means of three replicates; the concentrations were expressed per gram of powder. Different letters within the same row marked significant differences among samples, according to ANOVA and Tukey HSD test at $p < 0.05$.

	S_ENZ		S_THERM		S_US		S_HHP		T_ENZ		T_THERM		T_US		T_HHP	
Parameter	Mean		Mean		Mean		Mean		Mean		Mean		Mean		Mean	
Free amino acids (mg/g)	10	a	5	a	32	b	9	a	5	a	3	a	4	a	5	a
Soluble proteins (mg/g)	58	a	62	a	215	b	53	a	71	a	49	a	87	a	33	a
Mannan (mg/g)	17	a	117	c	116	c	11	a	178	d	62	b	84	b	21	a
Soluble colloids (mg/g)	111	a	187	cd	215	d	104	a	327	e	157	bc	171	c	133	ab
Insoluble solids (mg/g)	616	cd	678	de	289	a	492	bc	350	ab	659	d	619	cd	823	e
Particle size (nm)	1495	f	657	c	1184	d	2068	g	662	c	358	b	168	a	1276	e
Riboflavin (μg/g)	11.2	bcd	6.8	ab	18.2	ef	19.4	f	14.2	de	6.3	a	8.8	abc	13.2	cd
RSA total (μmol/g)	29.4	a	46.8	bcd	51.1	cd	36.0	abc	60.6	d	51.9	cd	36.1	abc	31.3	ab
RSA soluble (μmol/g)	24.6	a	28.5	ab	41.6	c	28.2	ab	41.1	c	37.6	bc	22.6	a	18.7	a
Cysteine (μmol/g)	1.6	a	1.8	a	2.9	bc	1.7	a	3.1	c	1.7	a	2.5	b	1.6	a
Glutathione (μmol/g)	4.0	ab	5.0	b	3.6	ab	9.0	d	7.2	c	3.5	a	4.2	ab	3.3	a
RPC (μmol/g)	66.5	d	33.9	b	40.5	b	49.8	c	1.3	a	nd*	a	nd	a	35.7	b

4.2.1 Free amino acids and soluble proteins, soluble colloids and insoluble solids

The release of amino acids was mostly affected by yeast, with S statistically different from T; no effect of the treatment was observed by the statistical analysis, even if US seemed to be the most effective in releasing higher amounts of such compounds (table 14). By considering all the samples, the content of amino acids was quite similar among all the treatments, with a concentration ranging from 3 to 5 mg/g for derivatives from T; higher mean amounts of such compounds was found in S, with values of 5, 9 and 10 mg/g detected in S_THERM, S_HHP and S_ENZ respectively, whereas S_US showed the highest mean content, with a value of 32 mg/g, thus resulting the only statistically different from all the other samples. Concerning soluble proteins, no effect of the yeast was observed - even if a tendential, higher median content of 61 mg/g was detected in S compared to T (48 mg/g) – whereas the treatments mostly affected the release of these compounds, with US statistically different from HHP and THERM (with a median content of 142, 40 and 55 mg/g, respectively). By considering all the samples, the highest value was found in S_US with a content of 215 mg/g, resulting the only statistically different from all the other samples, followed by T_US (87 mg/g) in terms of mean values (table 15).

A similar trend was observed for mannan content and total soluble colloids: the effect of yeast did not result significant, even if the higher amounts were detected in T with 74 mg/g of mannan and 164 mg/g of total soluble colloids compared to S. On the other hand, HHP determined the lowest release of mannan (15 mg/g) and colloids (119 mg/g), thus resulting statistically different from THERM and US (table 14).

However, the highest concentration of mannan and soluble colloids was detected in T_ENZ (178 mg/g and 327 mg/g, respectively), resulting the only significantly different from all the other samples, followed by S_US (116 mg/g of mannan and 215 mg/g of total soluble colloids). By comparing all the samples, US and THERM seemed to be more effective in favoring the release both of mannan and total soluble colloids in S, whereas ENZ gave the better results in the case of T, probably depending on a different susceptibility of the strain to the different treatments performed, coupled with a different cell wall composition and structure, concentration of yeast suspension or cell size.

The higher permeability of cell walls and membranes induced by enzyme addition [145] and by ultrasound [139,146] may enhance the release of intracellular components and the activity of endogenous enzymes, thus inducing autolysis and higher degradation rate, and increasing the concentration of soluble molecules. Furthermore, in a study carried out by Cacciola et al. [111] focused on the effect of US on processing yeast lees for winemaking use, the authors observed how the release of soluble colloids and proteins was mostly affected by US parameters (*i.e.*, amplitude and time of sonication) and how, during wine aging, the content of soluble proteins released from lees after US treatment was comparable to those obtained by enzyme addition. These results might explain the higher mean content of nitrogen compounds detected in S_US and T_US and of polysaccharides in T_ENZ and S_US.

The content of soluble proteins in S_ENZ and T_ENZ was quite similar to those detected in commercial yeast extracts and autolysates, also obtained by enzyme-assisted lysis, with an amount ranging from 33 to 78 mg/g of powder [147]; furthermore, greater concentrations of soluble colloids in autolysates obtained by *Saccharomyces* spp. after thermal treatment was obtained, higher than those detected after enzyme addition and mechanical disruption [94], thus confirming the highest content here detected in S_THERM compared to S_ENZ and S_HHP.

Insoluble solids content was not affected by yeast and treatment as resulted by statistical analysis (table 14), even if higher median concentration were detected in T (639 mg/g) compared to S (571 mg/g), and in THERM and HHP (674 mg/g and 694 mg/g, respectively) compared to ENZ and US (463 mg/g and 399 mg/g, respectively). However, by considering all the samples (table 15), the highest content was observed for S treated by ENZ, THERM and HHP, with an amount of about 616, 678 and 492 mg/g respectively. In the case of T, the product obtained by HHP showed the highest insoluble solids content (823 mg/g), followed by THERM and US (659 and 619 mg/g), the former resulted significantly different from all the other samples, except for S_THERM. As expected, the yeast derivatives that showed the highest mean content of soluble colloids also showed the lowest amount of total insoluble fractions, with values of 289 mg/g and 350 mg/g for S_US and T_ENZ, probably due to a greater and faster cell degradation. Thermal inactivation is generally employed for extracting cell wall components, especially mannoproteins [93], as well as high-pressure treatments allow a high cell disruption, but pressure above 600 MPa might cause inactivation of intracellular enzymes, thus decreasing or preventing the induction of autolytic process [118]; this might explain the highest mean content of insoluble solids tendentially observed in the derivatives obtained by THERM and HHP for both the yeast strains.

4.2.2 Particle size distribution

In figure 15, particle size distribution of derivatives from S (**a** and **b**) and T (**c** and **d**) was reported.

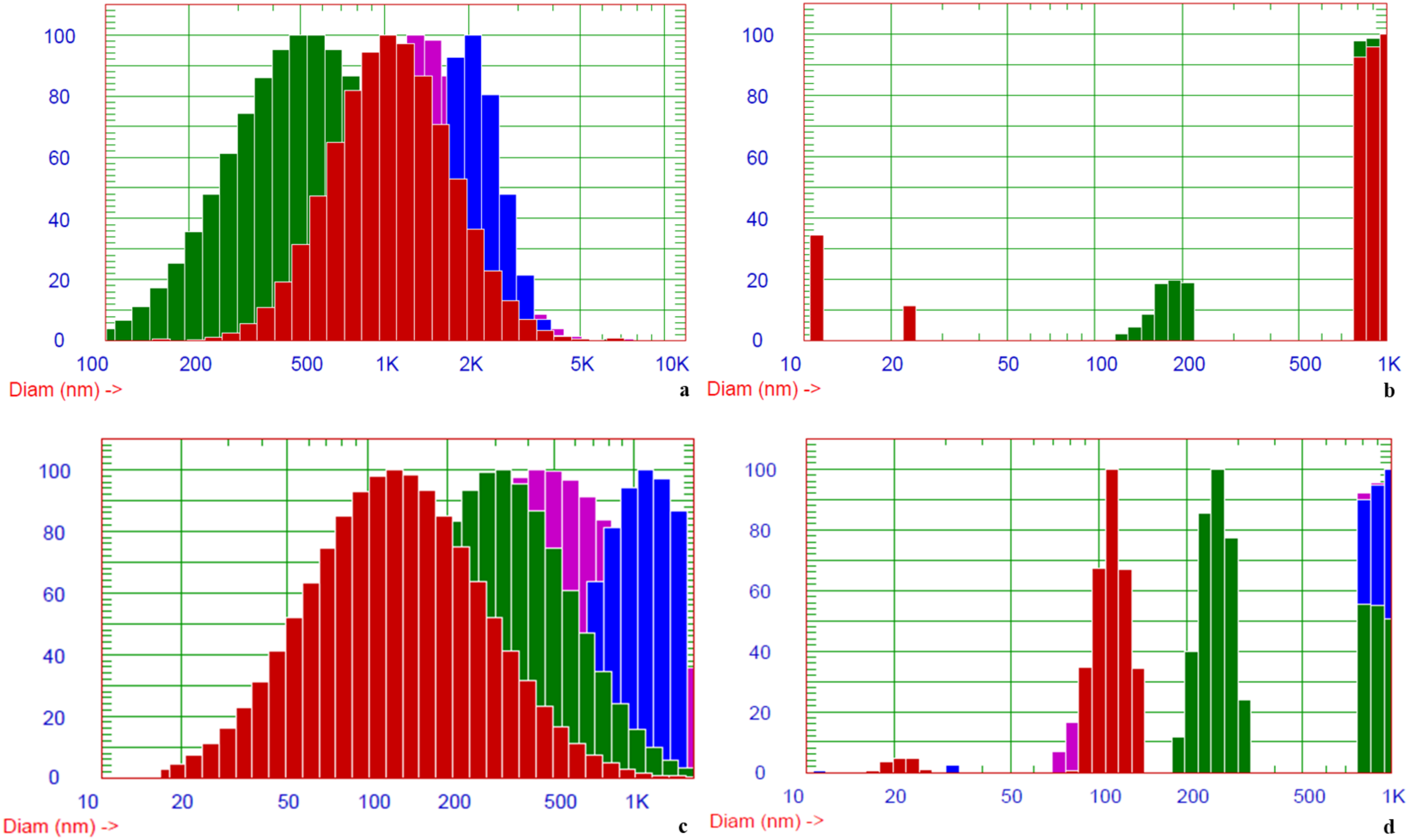


Figure 15. Particle size distribution of derivatives of *S. cerevisiae* (**a** and **b**) and *T. delbrueckii* (**c** and **d**). Mean particles diameter (**a** and **c**) and distribution of particles with mean size below 1 µm (**b** and **d**) were reported. Red: US; blue: HHP; green: THERM; violet: ENZ.

The particle size, as mean particle diameter expressed in nanometers, was affected both by yeast and treatment performed, as resulted by the statistical analysis (table 14); higher mean diameter was observed for S, with a median value of 1340 nm, compared to T (516 nm), whereas HHP showed the highest median value (1674 nm), thus resulting significantly different from THERM and US (514 nm and 674 nm, respectively). Among all the samples, the highest values were detected for S_HHP, S_ENZ and T_HHP (2068, 1495 and 1276 nm, respectively), whereas T_US and T_THERM showed the lowest ones (168 nm and 358 nm, respectively). This trend was confirmed by comparing the mean distribution of particles (fig. 15 a and c): HHP in fact showed the highest mean value for both the yeasts, compared to the other treatments. On the other hand, by considering the distribution of particles with mean diameter below 1 μm (fig. 15 b and d), S derivatives seemed to be most characterized by particles with mean diameter above 1 μm, but small fractions with a mean diameter around 200 nm and around 10 and 20 nm were also detected in THERM and US, respectively. The effect of these two treatments in reducing the particles size was more evident in the case of T, together with ENZ. By considering as observed till now, the higher degradation rate determined by US and ENZ might explain not only the higher content of soluble molecules but also the presence of colloidal substances with smaller size, whereas the only cell disintegration might occur in THERM and, especially, in HHP might explain the presence of colloids with mean diameter greater than 1 μm. However, the efficacy of US on reducing mean particles size was previously reported by Natolino & Celotti [148] that observed how colloidal status of wine was affected by US treatment and how sonicated samples showed a reduced mean particle size compared to the untreated controls.

4.2.3 Riboflavin content

The concentration of riboflavin (table 14) was mostly affected by treatment, with the highest amount observed in HHP (15 µg/g) whereas THERM determined the lowest release (7 µg/g), probably due to thermal degradation. However, the highest content was detected in derivatives obtained from S by emerging technologies (19.4 and 18.2 µg/g for HHP and US, respectively), and in T_ENZ and T_HHP (14.2 and 13.2 µg/g, respectively) (table 15). Wines contain certain amount of riboflavin because of its presence into the grapes [149] and the concentration might increase during alcoholic fermentation due to yeast metabolism, reaching a final concentration even higher than 100 µg/L in relation to the strains used [70]. In the case of bottled white wine, in certain condition that include the presence of riboflavin (at least 50 µg/L) but also methionine (at least 1.5mg/L), the former may be involved in light-induced reaction thus determining the appearance of the so-called light-struck defect [74]. Even if statistical differences emerged, from the practical point of view, if derivatives are added at a concentration of 200-400 mg/L, the amount of riboflavin released into the wine might be considered negligible, since it is much lower than the threshold level necessary to trigger the light-induced reactions.

4.2.4 Antioxidant properties

Regarding antioxidant properties, no statistical differences were observed between the strains in the radical scavenging activity (RSA) determined by DPPH assay and expressed as µmol of glutathione per gram of powder (table 14). On the other hand, RSA determined on whole derivative (RSA total) was affected by treatment, with HHP and THERM showing the lowest and the highest values, respectively, thus resulting statistically different.

Among the samples (table 15), the highest mean values were detected in the derivatives obtained by traditional methods, ENZ and THERM for T (60.6 µmol/g and 51.9 µmol/g, respectively), and by US and THERM for S (51.1 µmol/g and 46.8 µmol/g, respectively). In addition, the antioxidant property seemed to be mainly related to the compounds present in the soluble fraction of the powders, since it represented from 60% to 80% of the total radical scavenging activity.

Regarding cysteine content, yeasts did not affect the release, whereas the impact of treatment on its concentration was observed: the lowest amount was detected in HHP and THERM (about 1.7 µmol/g), resulting significantly different from US (2.6 µmol/g), that showed the highest median content, followed by ENZ (2.3 µmol/g); however, the concentrations found were very low, and the statistical differences observed might be considered negligible from the practical point of view. The amounts detected were quite similar among all the samples, ranging from 1.6 to 2.9 µmol/g for derivatives obtained from S, and from 1.6 to 3.1 µmol/g for T; however, T_ENZ, S_US and T_US resulted significantly different from all the other samples, with a mean content of 3.1 µmol/g, 2.9 µmol/g and 2.5 µmol/g, respectively.

The content of glutathione was not affected neither by yeast nor by treatment, with a tendential higher median content observed for S (4.5 µmol/g) and for HHP (6.0 µmol/g). However, the highest amount was detected in S_HHP (9 µmol/g), followed by T_ENZ (7.2 µmol/g), both resulting statistically different between them and from all the other samples.

The amount of reducing proteins containing cysteine-residues (RPC) - linked to the cell wall fractions - was only affected by the yeast strains with a content of 46.9 µmol/g detected for S, about five times greater than T (0.4 µmol/g); by considering the treatment, even if no statistical differences emerged, HHP and ENZ seemed to be the most suitable treatments for preserving such protein fractions, with amounts of 42.8 µmol/g and 33.0 µmol/g, respectively, compared to US (18.3 µmol/g) and THERM (16.0 µmol/g). However, RPC were detected in all the derivatives obtained from S; the amounts ranged from 33.9 to 66.5 µmol/g in S_THERM and S_ENZ, respectively; the latter resulted statistically different from all the other samples, followed by S_HHP (49.8 μmol/g). Regarding T, only HHP allowed to preserve the content of reducing proteins in derivatives obtained starting from this strain (35.7 μmol/g).

The presence of mannoproteins or other compounds containing thiol groups may be useful in removing reduced odors by formation of disulfide bonds [150], but also in protecting wine against oxidation. The antioxidant activity of yeast derivatives seemed to be not only related to the content of glutathione, but also to the presence of other compounds. Jaehrig et al. [151] reported the ability of cell wall proteins containing cysteine residues to exhibit antioxidant activity. Furthermore, other authors reported how inactive yeast preparations and yeast lees showed a good radical scavenging activity [16] in comparison to glutathione and sulfur dioxide, as well as a non-negligible protection against polyphenol oxidation was also observed [152], probably due to the presence of thiol groups linked to cell wall fractions [153].

The differences observed between the two strains might be related to different sulfur metabolism linked to glutathione production [18], as well as different behavior in the synthesis of proteins linked to cell walls [120]. However, by characterizing several commercial yeast derivatives, that included mannoproteins, cell hulls and lysates, Tirelli et al. [32] observed how the occurrence of Maillard reaction during manufacturing process of yeast derivatives may lead to a lack of sulfur-containing compounds; this might explain the lower concentration of such molecules in the derivatives obtained by thermal inactivation. On the other hand, the potential secondary activity of the exogenous enzymes or the possible enhanced proteolytic activity caused by US might lead to a higher protein hydrolysis with following release of amino acids and small peptides that seemed to exhibit antioxidant properties [154,155]; this might in part explain the highest mean values in terms of radical scavenging activity observed in T_ENZ and in S_US.

4.2.5 Volatile profile of autolysate powders

The volatile profile of the yeast derivative powders was determined by SPME-GC-MS. A total of 28 volatile compounds were tentatively identified and listed in Annex 5.

The results of semi-quantitative analysis carried on the volatile compounds detected in the headspace of yeast derivative powders, with means and standard deviations were reported in Annex 6.

The effect of yeast and treatment on the volatile profile of powders and the differences among the samples were reported in table 16.

Table 16. Effect of yeast and treatment on the volatile profile of yeast derivative powders and differences among the samples. Different letters within the same row marked significant differences among groups (yeast and treatment) and among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$.

	S	T	ENZ	THERM	US	HHP	S_ENZ	S_THERM	S_HHP	S_US	T_ENZ	T_THERM	T_HHP	T_US
Compound	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean
acetic acid	27061 b	16106 a	36515	758	43898	5163	43218 cd	945 a	6736 ab	57344 d	29812 bc	570 a	3589 a	30453 bc
propanoic acid	383 a	652 b	737 b	nd a	1182 c	152 a	395 ab	nd a	215 a	923 bc	1078 c	nd a	90 a	1442 c
2-methylpropanoic acid	20867 a	37420 b	45134	1902	66669	2869	22729 b	1502 a	3393 a	55844 c	67539 d	2303 a	2345 a	77494 e
butanoic acid	1018	4258	4296	168	5709	380	1086 b	114 a	363 a	2510 c	7507 d	223 a	398 ab	8907 e
3-methylbutanoic acid	12092 a	20541 b	26640	760	36520	1344	14327 b	612 a	1208 a	32218 c	38953 d	907 a	1480 a	40822 d
pentanoic acid	87	50	39 ab	nd a	175 b	59 ab	nd	nd	118	229	79	nd	nd	121
hexanoic acid	239	200	334 b	nd a	496 c	49 a	305 b	nd a	99 a	553 c	362 b	nd a	nd a	439 bc
2-ethylhexanoic acid	116	nd*	151	nd	82	nd	302	nd	nd	163	nd	nd	nd	nd
octanoic acid	67	nd	nd	nd	45	89	nd	nd	178	89	nd	nd	nd	nd
decanoic acid	13	nd	nd	nd	nd	27	nd	nd	53	nd	nd	nd	nd	nd
2-methyl-1-propanol	25100 b	13155 a	42785	362	32771	592	55051 d	724 ab	1184 ab	43441 cd	30518 bcd	nd a	nd a	22101 abc
3-methyl-1-butanol	95486	19169	114650	2384	107782	4494	181811 c	3733 a	7523 a	188879 c	47489 b	1036 a	1465 a	26686 ab
1-hexanol	518 a	654 b	1009	nd	1297	37	966 b	nd a	75 a	1030 b	1053 b	nd a	nd a	1564 c
1-heptanol	431	137	428 b	nd a	707 c	nd a	712	nd	nd	1012	144	nd	nd	402
1-octanol	163	61	54 a	nd a	395 b	nd a	nd	nd	nd	651	108	nd	nd	138
2-phenylethanol	3777	1480	4727	30	5522	236	5529 d	60 a	141 a	9379 e	3925 c	nd a	330 ab	1665 b
2,3-butanediol (levo)	8785 b	4521 a	18625	122	7488	378	22216 c	135 a	319 a	12470 b	15034 b	108 a	437 a	2506 a
2,3-butanediol (meso)	1317 b	915 a	2058	nd	2248	157	1841 bc	nd a	143 a	3284 d	2275 cd	nd a	170 a	1212 b
1,2-propanediol	4130	534	6846	48	2006	428	12223 d	96 a	619 ab	3582 c	1469 b	nd a	237 a	430 a
ethyl lactate	193	444	763 b	nd a	511 b	nd a	nd	nd a	nd a	774 ab	1525 b	nd a	nd a	249 a
ethyl octanoate	70	15	107 b	nd a	64 b	nd a	152 b	nd a	nd a	129 b	62 ab	nd a	nd a	nd a
γ-valerolactone	300	240	258 a	nd a	820 b	nd a	nd a	nd a	nd a	1199 b	517 a	nd a	nd a	441 a
γ-butyrolactone	900	619	1170	26	1772	70	1015 ab	51 a	64 a	2470 c	1326 bc	nd a	76 a	1073 ab
2,5-dimethylpyrazine	53	201	289 c	nd a	219 b	nd a	nd a	nd a	nd a	212 b	577 c	nd a	nd a	225 b
2,6-dimethylpyrazine	99	608	508 b	nd a	907 c	nd a	nd a	nd a	nd a	397 b	1016 c	nd a	nd a	1417 d

(continue)

Table 17. *(continue)*

2, 3, 5-trimethylpyrazine	89	75	183 b	nd a	146 b	nd a	202 b	nd a	nd a	154 b	163 b	nd a	nd a	138 b
2-ethyl-3,6-dimethylpyrazine	147 a	303 b	737 b	nd a	163 a	nd a	479 b	nd a	nd a	108 a	995 c	nd a	nd a	217 ab
methionol	114	89	145 b	nd a	261 b	nd a	nd a	nd a	nd a	455 c	290 b	nd a	nd a	67 a

*nd: not detected

The results PCA carried out on the absolute area/1000 of the volatile compounds detected in the headspace of powders were reported in figure 16 (a and b).

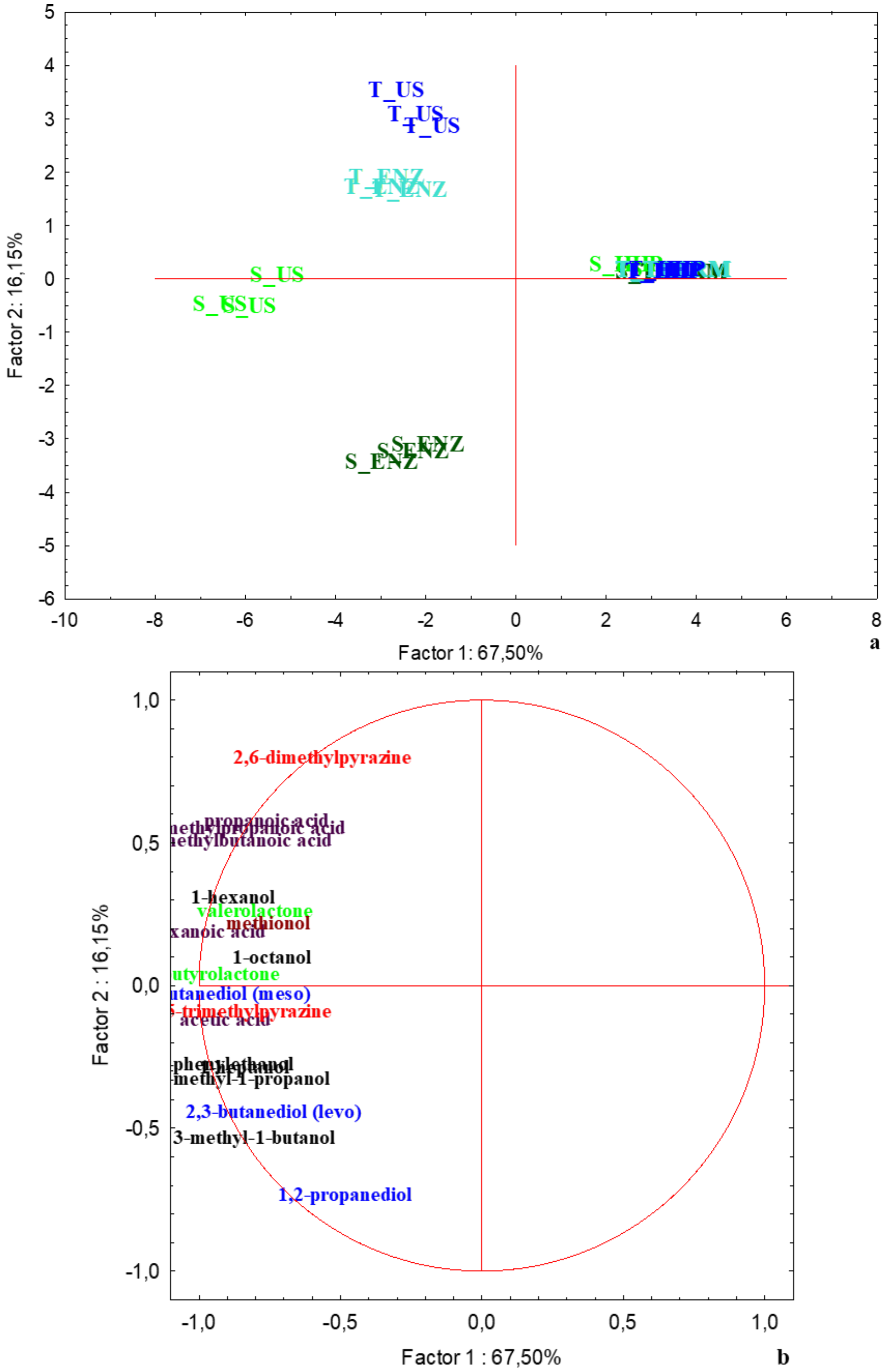


Figure 16. PCA carried out on the absolute area/1000 of volatile compounds detected in the headspace of autolysate powders. Projection of case (samples) (**a**) and variables (volatile compounds) (**b**) on the factor-plan were reported. Factor Loadings (FL) were calculated by Factorial Analysis, and the most relevant variables were selected for marked FL > 0.7. S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; HHP: high hydrostatic pressure: US: ultrasound.

The volatile profile of yeast derivatives seemed to be dependent on the treatment; in general, autolysates obtained by ENZ and US were mainly characterized in terms of aroma compounds for both the yeasts, whereas HHP and THERM allowed to obtain products with potential lower odor impact. The concentration seemed to be related to the treatment, whereas the aromas that mainly characterized the different derivatives seemed to be influenced by the strains: acids mainly characterized T_US and T_ENZ, whereas alcohols, diols, and lactones were mostly present in S_US and S_ENZ. These results agreed with those observed in previous studies, in which the enzyme addition led to a more characterized volatile profile in the resulting autolysate powders [94]; moreover, autolysates obtained by high-pressure treatment showed a poorest volatile profile, comparable to those obtained by thermal treatment and commercial preparations [156].

The trend of the main fatty acids detected in the headspace of powders, acetic acid, 2-methylpropanoic acid and 3-methylbutanoic acid was reported in figure 17.

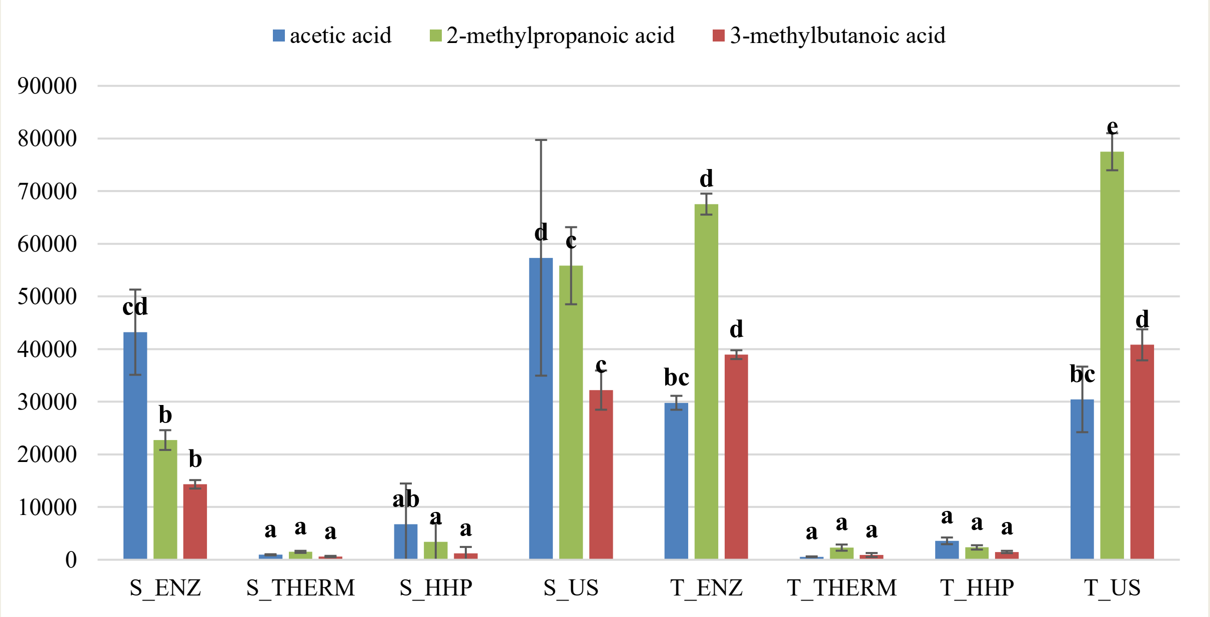


Figure 17. Content of the main fatty acids (absolute area/1000) detected in the headspace of the powders. Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; HHP: high hydrostatic pressure: US: ultrasound.

As previously discussed, concerning PCA results, yeast significantly affected the content of the three acids considered, with T showing the highest concentration of 2-methylpropanoic acid and 3-methylbutanoic acid. Even if no statistical differences were observed, THERM and HHP showed the lowest content of these three acids (table 16). However, as previously observed, these aroma compounds, in particular 2-methylpropanoic acid and 3-methylbutanoic acid manly characterized the derivatives T_US and T_ENZ, thus resulting significantly different from S_US and S_ENZ.

Free fatty acids are detected in inactive yeast preparations [147,157] since they are produced by yeast sugar metabolism [158] and released during autolysis process [88]. Their release into the wine might negatively affect the volatile profile, since they are describe as characterized by sour, pungent and cheese notes [147]. The results obtained in the present study agreed with those reported in Comuzzo et al. [94], where the products obtained by enzyme addition were mostly characterized by fatty acids compared to the autolysates obtained by thermal and mechanical treatments; furthermore, the lowest amount of these compounds in thermal treatment might be related to degradation of fats [159], even if no derivatives of such reaction (*e.g.*, lactones or aldehydes) were detected in the respective products.

The trend of the main higher alcohols detected in the headspace of derivative powders, 2-methyl-1-propanol, 3-methyl-1-butanol and 2-phenylethanol was reported in figure 18.

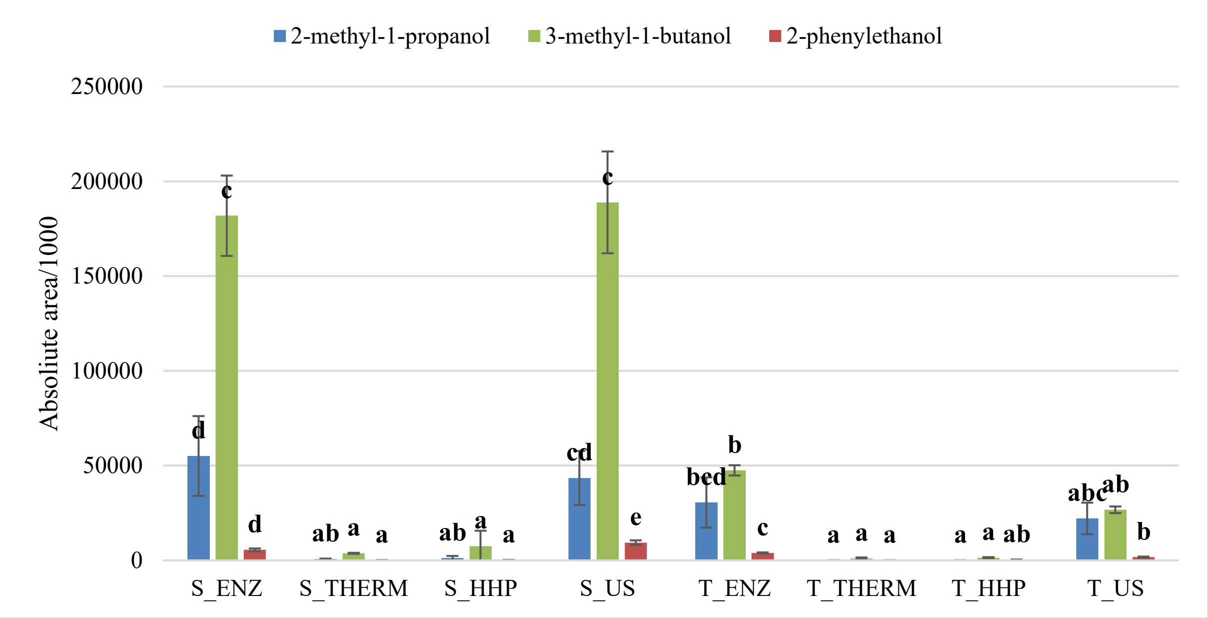


Figure 18. Content of the main higher alcohols (absolute area/1000) detected in the headspace of the powders. Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; HHP: high hydrostatic pressure: US: ultrasound.

Yeast tendentially affected the concentration of alcohols. Derivatives obtained starting from S showed a tendential higher content compared to T, as previously observed (figure 16); however, the only statistical difference was observed for 2-methyl-1-propanol. Regarding the treatments, even if no statistical differences were observed for the three compounds here examined, US and ENZ tendentially allowed to obtain derivatives mostly characterized by such volatile compounds (table 16). However, S_ENZ and S_US showed the highest mean content of 3-methyl-1-butanol and 2-phenylethanol compared to T_ENZ and T_US, thus resulting significantly different. Higher alcohols are produced during fermentation by amino acids metabolism, with valine, leucine, and phenylalanine as precursors of 2-methyl-1-propanol, 3-methyl-1-butanol and 2-phenylethanol, respectively [158]. Consequently, yeast derivatives contain such compounds [147] that may confer from floral or honey-like notes (*e.g.*, 2-phenylethanol) to pungent, malty, or solvent-like attributes (*e.g.*, 3-methyl-1-butanol) [160]; the trend observed was in line with those reported by Comuzzo et al. [94] with autolysates obtained by thermal treatment and mechanical disruption the poorest in terms of higher alcohols content.

The trend of the main alkylpyrazines detected in the headspace of yeast derivative powders, 2,6-dimethylpyrazine and 2,3,5-trimethylpyrazine was reported in figure 19.

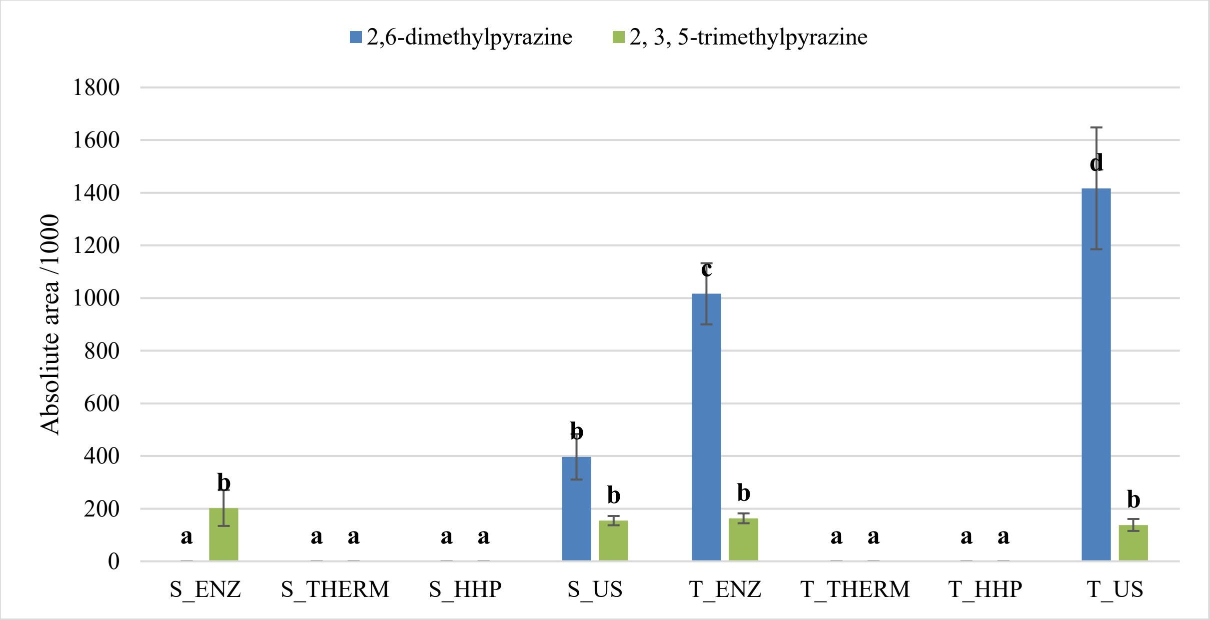


Figure 19. Content of the main alkylpyrazines (absolute area/1000) detected in the headspace of autolysate powders. Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; HHP: high hydrostatic pressure: US: ultrasound.

The concentration of pyrazines was mostly affected by the treatment, since they are not yeast by-products, but the formation occurs during manufacturing process due to the Maillard reaction [95]. In general, the highest mean values were observed in ENZ and US derivatives (table 16).

Yeast derivatives are characterized by such compounds, as previously reported by other authors [147,157], with tendential higher amounts in products obtained by enzymatic treatments [94]; furthermore, they might negatively impact on wine aroma profile, since they are described with potato [147] and pungent notes [160].

Derivatives obtained starting from both the yeasts treated by US and ENZ showed a tendential, higher mean concentration of pyrazines; this might be probably related to the fact that such products were also characterized by the higher mean content of nitrogen compounds, thus possibly determining the occurrence of Maillard reaction, at least at the early stages.

4.3 Conclusion

The chemical composition of yeast derivatives was affected both by strain and applied treatment. Concerning the effect of yeast, derivatives obtained by *S. cerevisiae* tendentially showed higher concentration of nitrogen compounds, riboflavin, glutathione and reducing proteins, whereas products obtained by *T. delbrueckii* had higher amount of total soluble colloids, together with higher cysteine content and greater radical scavenging activity. On the other hand, a relevant effect of treatment was also observed: US and ENZ tendentially determined higher content of soluble molecules and higher concentration of volatile compounds, whereas THERM and HHP allowed to obtain derivatives with higher amounts of insoluble solids, higher mean particles size and the poorest volatile profile; the latter seemed to be the most suitable technology for preserving the content of antioxidant molecules (*e.g.*, glutathione and reducing proteins).

T. delbrueckii is a good candidate for the production of yeast derivatives, as well as emerging technologies a good alternatives to the traditional methods.

US may be useful for producing yeast extracts or autolysates to be used both as fermentation and wine quality enhancers, thanks to the highest amounts of soluble molecules (*e.g.*, nitrogen compounds and polysaccharides), possibly replacing the enzyme-assisted lysis. High hydrostatic pressure allows to obtain products with chemical composition and aroma profile similar to thermal inactivation; these characteristics, together with the highest amount of reducing proteins linked to cell walls, make this technology suitable for producing yeast hulls or walls, possibly less impacting or, even improving the volatile profile of added wines.

In order to evaluate the real impact of the different derivatives in winemaking condition, these products were added to a white wine and the effect on the chemical composition and volatile profile during aging was assessed (Chapter 4).

Chapter 4

Effect of the addition of yeast derivatives on white wine evolution and volatile profile during aging

1. Introduction

Yeast derivatives are used during winemaking process with several applications, first as fermentation enhancers; the presence of amino acids and proteins, polysaccharides, vitamins [77], fatty acids and sterols [161] as growth factors allows better adaptation to the medium and reduces the occurrence of stuck fermentation [162]. The use of these additives during wine aging as quality enhancer is also widespread; by simulating the effect of aging on lees, an improvement of wine quality and stability in shorter time have been reported [163–165], possibly reducing the conventional aging time.

Among yeast derivatives, mannoproteins have been widely studied and well-known for their positive effect towards wine chemical stability, such as the improvement of protein stability [30,166] and the inhibition of tartrate salts crystallization [167].

Adsorption phenomena and formation of stable complexes between phenolic compounds and mannoproteins was also reported [163,164], consequentially impacting on wine astringency and color [165,168,169]; nevertheless, a destabilizing effect of mannoproteins was also observed, with the formation of aggregates that, precipitating, may lead to a lack of color [170]. Moreover, this potential interaction with phenolic fraction may reduce wine predisposition to browning [166], and the release of antioxidants molecules (*i.e.*, glutathione) [171,172] or the presence of thiol compounds linked to cell walls [32,151,153] may positively enhance the protection of wine against oxidation, in some cases comparable to other additives [16,152].

Another relevant aspect related to the addition of yeast derivatives concerns the impact on wine aroma profile and consequentially sensory perception [163,164,166,173], due to the interaction between volatile compounds and mannoproteins [174,175]. However, this modulation effect is dependent on the type of yeast derivative preparation, being mainly related to their composition and manufacturing process [94]; these interactions seem to be also dependent on pH, temperature, type of aroma compound [175,176] and contact time [165,177]. Nevertheless, some odor-active compounds - resulting from yeast metabolism or produced during manufacturing process - may be released into the wine causing the formation of *off-flavors* [157,177,178], but the occurrence seems to be mainly dependent on the type of product and dosage [147].

In relation to their composition and manufacturing process, yeast derivatives release a certain amount of soluble molecules, *e.g.*, cell wall polysaccharides and glycoproteins together with intracellular components (*e.g.*, proteins) that might impact on the wine colloidal state. Wine is a complex system defined by different molecules, both originating from grapes and released by yeasts during fermentation and aging [179,180]; similarly, the addition of yeast derivatives, with subsequent release of soluble molecules and formation of aggregates with wine substances [30,170,181] may impact on wine colloidal state. During aging, these particles are also subjected to several modifications that concern molecular weight [179], particle size and charge also in relation to treatment performed for inducing yeast autolysis [111]; these modifications may consequentially condition the filterability of the wine [182].

2. Aim of the work

As reported above (Chapter 3), yeast strain and methods for inducing autolysis may affect the chemical composition of the resulting derivatives; the previous characterization in model solution allowed to understand the potential release of compounds of enological interest (*e.g.*, antioxidants and soluble colloids), together with the aroma profile of the powders, but it was also necessary to assess their impact in real conditions. For this reason, the aim of the present work was to evaluate the effect of the addition of yeast derivatives on the chemical composition and volatile profile of white wine during aging.

The impact and the comparison among yeast derivatives obtained starting from two different strains - *S. cerevisiae* and *T. delbrueckii* – and produced by different technologies were assessed, also in relation to the period of contact, shorter (two months) and longer (six months); the evaluations mainly concerned the protection against wine oxidation, the release of soluble colloids and the impact on wine volatile profile, also in comparison with the starting composition of the respective yeast derivatives.

3. Materials and methods

3.1 Reagents

Ethanol (96% v/v), methanol (HPLC grade), *p*-dimethylaminocinnamaldehyde (DAC), (+)-catechin hydrate (≥ 98%, HPLC grade), mannan from *S. cerevisiae* and ethyl heptanoate were purchased from Sigma-Aldrich (St. Louis, MO, USA); sodium chloride was purchased from Fluka (Honeywell, NC, USA), hydrochloric acid (37% v/v) from Carlo Erba Reagents (Milan, Italy) and hydrogen peroxide (30% v/v) from VWR Chemicals (Milan, Italy). MilliQ water was produced by MilliQ Advantage A10 apparatus (Merck Millipore, Billerica, MA, USA) and microfiltered at 0.22 μm before use. Commercial β-glucanase preparation and commercial active dry yeasts (ADY) *S. cerevisiae* and *T. delbrueckii* were from Enologica Vason S.p.A. (San Pietro in Cariano, VR, Italy). A table white wine was supplied by a local producer (Friuli Venezia-Giulia, North-East Italy).

3.2 Wine aging on yeast derivatives

Yeast derivative powders were obtained as reported in Chapter 3, section 3.3. Briefly, active dry yeast preparations of *S. cerevisiae* (hereafter referred to S) and *T. delbrueckii* (hereafter referred to T) were subjected to thermal inactivation, β-glucanase addition, ultrasounds and high hydrostatic pressure (hereafter referred to as THERM, ENZ, US and HHP, respectively).

The initial chemical composition of the white wine used for the experimental trials was as follows: pH 3.14, total acidity 4.95 g/L (as tartaric acid), free sulfur dioxide 24 mg/L, color (absorbance 420 nm) 0.06, POM-test 117%, TPI 5.8 and catechins content 7.1 mg/L. Doses of 0.3 g/L of yeast derivative powders were added to wines, bottled in 100 mL white glass bottles and manually sealed with crown caps after nitrogen blowing in the headspace; for aging, the wine samples were stored at a temperature of 20±1 °C for six months, and homogenized weekly. Three independent replicates were prepared, and the chemical characterization of wines were carried out after two and six months after the addition. Wines added with sulfur dioxide up to a final concentration of 50 mg/L (of free sulfur dioxide) was used as reference (hereafter referred to CON). Except for wine aroma compounds, the samples were centrifuged (3000 rpm for 15 minutes) and subjected to the chemical characterization, as described in the following sections.

3.3 Wine color and browning assay (POM-test), total phenolic index and catechins content

The evaluation of wine color (abs 420 nm) and the predisposition of wine towards browning (POM-test) was performed as reported by Comuzzo et al. [16]. CieLab parameters (L*, a*, b*) were also evaluated by UV/VIS spectrophotometer, model V-530 (Jasco Inc, USA) in 10 mm path length quartz cuvettes.

Total polyphenols index (TPI) was evaluated as reported by Comuzzo et al. [183]; the TPI was calculated multiplying the absorbance measured at 280 nm by the dilution factor (x10). The amount of catechins was determined following the method described by Zironi et al. [184]; briefly, 0.5 mL of wine were added with 2.5 mL of DAC solution, previously prepared by dissolving 0.1g in 75 mL of methanol and 25 mL of hydrochloric acid at 37% v/v; after 5 minutes, the absorbance was measured in disposable 10 mm optical path-length PMMA cuvettes (Kartell S.p.A. Labware Division, Noviglio, MI, Italy) against a blank prepared by replacing the sample with ethanol (10% v/v). The concentrations were calculated in relation to a calibration curve prepared with standard solutions of catechin (0-25 mg/L) and expressed in mg/L.

3.4 Total soluble colloids (SE-HPLC), particle size distribution (PSD) and filtration assay

Total soluble colloids were evaluated on wine samples after aging by alcoholic precipitation and SE-HPLC as described in Chapter 1, section 3.7, whereas particle size distribution as reported in Chapter 3, section 3.8. The filtration assay was carried out by measuring the time necessary to filter 20 mL of centrifuged wines on 0.45 μm pore size cellulose membranes, 47 mm diameter (Artiglass, PD, Italy); filtration flow rate was calculated and expressed in mL/min/cm^2.

3.5 Wine volatile profile

Ten milliliters of wine were introduced in 20 mL glass vials sealed with PTFE/silicone septa containing 3 g of sodium chloride and added with 50 μL of ethyl heptanoate (0.0984 g/L in ethanol 96 % v/v) used as internal standard. The volatile profile was then analyzed by SPME-GC-MS following the operating conditions described in Chapter 3, section 3.11. The identification of volatile compounds was tentatively carried out by comparing their mass spectrum with those of external standards and 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. The semi-quantitative analysis was based on the internal standard method, considering a response factor equal to 1.00.

3.6 Statistical analysis

Means and standard deviations, analysis of variance, factorial analysis and principal component analysis (PCA) were carried out as reported in Chapter 3, section 3.12. Relationships among the parameters were investigated using Pearson correlation coefficient and the statistical significance was fixed at $p < 0.05$.

4. Results and discussion

The chemical composition of wine aged on yeast derivatives was analyzed after two and six months and the results were summarized in table 17 and 18, respectively: means and standard deviations at each sampling time were calculated and the statistical differences among the samples were reported. The chemical parameters analyzed were discussed in depth in their respective, following sections.

Table 18. Effect of yeast derivatives addition on the chemical composition of wines after two months of aging. Data were means and standard deviations (SD) of three replicates. Different letters within the same column marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. CON: Control; S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

Treatment	Color	L*	a*	b*	POM-test	TPI	Catechins (mg/L)	Mannan (mg/L)	Total soluble colloids (mg/L)	Particle size (nm)	Filtration flow (mL/min/cm^2)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
CON	0.05 ± 0.01 a	99.5 ± 0.5 b	-0.6 ± 0.0 a	3.9 ± 0.1 ab	126 ± 39 b	5.7 ± 0.1	7.0 ± 0.1 ab	226 ± 24 abc	1280 ± 123	801 ± 46 a	19.0 ± 4.6
S_ENZ	0.06 ± 0.01 ab	98.7 ± 0.6 b	-0.5 ± 0.0 abc	3.7 ± 0.1 a	103 ± 29 ab	5.6 ± 0.1	7.0 ± 0.4 ab	260 ± 10 c	1322 ± 190	971 ± 156 ab	18.8 ± 3.1
S_THERM	0.07 ± 0.00 ab	98.2 ± 0.3 ab	-0.5 ± 0.0 abc	3.7 ± 0.0 a	52 ± 12 a	5.7 ± 0.1	8.1 ± 0.3 ab	296 ± 23 c	1252 ± 159	1265 ± 334 abcd	18.4 ± 2.5
S_US	0.07 ± 0.00 abc	98.2 ± 0.3 ab	-0.5 ± 0.0 bc	4.7 ± 0.0 e	71 ± 15 ab	5.2 ± 0.2	6.8 ± 1.2 ab	268 ± 11 c	1254 ± 117	1021 ± 145 abc	21.1 ± 3.3
S_HHP	0.08 ± 0.01 bc	97.6 ± 1.1 ab	-0.6 ± 0.0 ab	3.9 ± 0.1 a	69 ± 22 ab	5.9 ± 0.3	6.9 ± 0.4 ab	128 ± 76 a	1731 ± 575	1874 ± 256 cd	12.8 ± 4.6
T_ENZ	0.08 ± 0.00 bc	97.6 ± 0.3 ab	-0.4 ± 0.0 c	4.5 ± 0.2 de	69 ± 16 ab	5.5 ± 0.3	6.4 ± 0.8 a	273 ± 25 c	1379 ± 68	1743 ± 134 bcd	19.3 ± 4.0
T_THERM	0.07 ± 0.01 ab	98.3 ± 0.6 ab	-0.5 ± 0.0 abc	4.2 ± 0.1 cd	74 ± 16 ab	5.8 ± 0.1	8.3 ± 0.3 b	307 ± 8 c	1221 ± 99	1008 ± 367 abc	19.5 ± 4.5
T_US	0.06 ± 0.01 ab	98.8 ± 0.4 b	-0.6 ± 0.0 ab	4.2 ± 0.1 bc	99 ± 12 ab	5.7 ± 0.6	7.0 ± 0.9 ab	237 ± 29 bc	1112 ± 125	2014 ± 341 d	18.5 ± 0.7
T_HHP	0.09 ± 0.02 c	96.5 ± 1.2 ab	-0.4 ± 0.1 c	4.2 ± 0.1 cd	46 ± 17 a	5.1 ± 1.2	6.8 ± 0.4 ab	138 ± 53 ab	1264 ± 446	1842 ± 601 bcd	14.6 ± 4.4

Table 19. Effect of yeast derivatives addition on the chemical composition of wines after six months of aging. Data were means and standard deviations (SD) of three replicates. Different letters within the same column marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. CON: Control; S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

Treatment	Color	L*	a*	b*	POM-test	TPI	Catechins (mg/L)	Mannan (mg/L)	Total soluble colloids (mg/L)	Particle size (nm)	Filtration flow (mL/min/cm^2)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
CON	0.07 ± 0.01	98.6 ± 0.6	-0.6 ± 0.0	4.5 ± 0.1 c	91 ± 35	5.3 ± 0.3	8.1 ± 0.5	132 ± 16	1697 ± 384	1211 ± 500 ab	5.8 ± 0.6
S_ENZ	0.06 ± 0.00	98.7 ± 0.1	-0.6 ± 0.0	3.9 ± 0.0 a	98 ± 6	4.9 ± 0.7	7.6 ± 0.9	138 ± 116	1273 ± 460	1821 ± 218 ab	5.6 ± 0.3
S_THERM	0.06 ± 0.00	99.0 ± 0.1	-0.7 ± 0.0	4.0 ± 0.1 a	76 ± 13	5.3 ± 0.2	8.3 ± 0.4	174 ± 63	1214 ± 354	1550 ± 417 ab	6.5 ± 0.8
S_US	0.07 ± 0.00	98.1 ± 0.2	-0.6 ± 0.0	4.9 ± 0.1 d	73 ± 13	5.8 ± 0.2	8.2 ± 0.3	178 ± 47	1963 ± 272	978 ± 214 ab	5.7 ± 0.3
S_HHP	0.06 ± 0.00	98.7 ± 0.2	-0.6 ± 0.0	4.1 ± 0.1 ab	81 ± 8	5.0 ± 0.6	8.5 ± 0.4	178 ± 13	1417 ± 618	769 ± 219 a	6.1 ± 0.5
T_ENZ	0.07 ± 0.00	98.1 ± 0.4	-0.6 ± 0.0	4.5 ± 0.1 c	88 ± 15	5.6 ± 0.3	8.0 ± 0.3	205 ± 13	2008 ± 473	1201 ± 144 ab	5.9 ± 0.4
T_THERM	0.06 ± 0.00	98.9 ± 0.1	-0.7 ± 0.0	4.2 ± 0.1 abc	80 ± 7	5.9 ± 0.7	8.4 ± 0.4	187 ± 81	1231 ± 569	796 ± 424 ab	6.1 ± 0.6
T_US	0.06 ± 0.00	98.4 ± 0.2	-0.7 ± 0.0	4.5 ± 0.1 c	92 ± 24	4.6 ± 1.9	8.1 ± 1.4	197 ± 77	1907 ± 1116	1512 ± 619 ab	6.0 ± 0.2
T_HHP	0.07 ± 0.01	98.3 ± 0.9	-0.6 ± 0.0	4.3 ± 0.3 bc	72 ± 25	5.5 ± 0.2	7.9 ± 0.7	56 ± 74	651 ± 445	800 ± 147 ab	6.7 ± 0.3

4.1 Effect of yeast derivatives addition towards wine color, browning potential and phenolic fraction after two and six months of aging

In figure 20, the effect of yeast derivatives addition on wine color (**a**), browning potential (POM-test) (**b**) and phenolic fraction, both TPI (**c**) and catechins content (**d**) after two months of aging was reported.

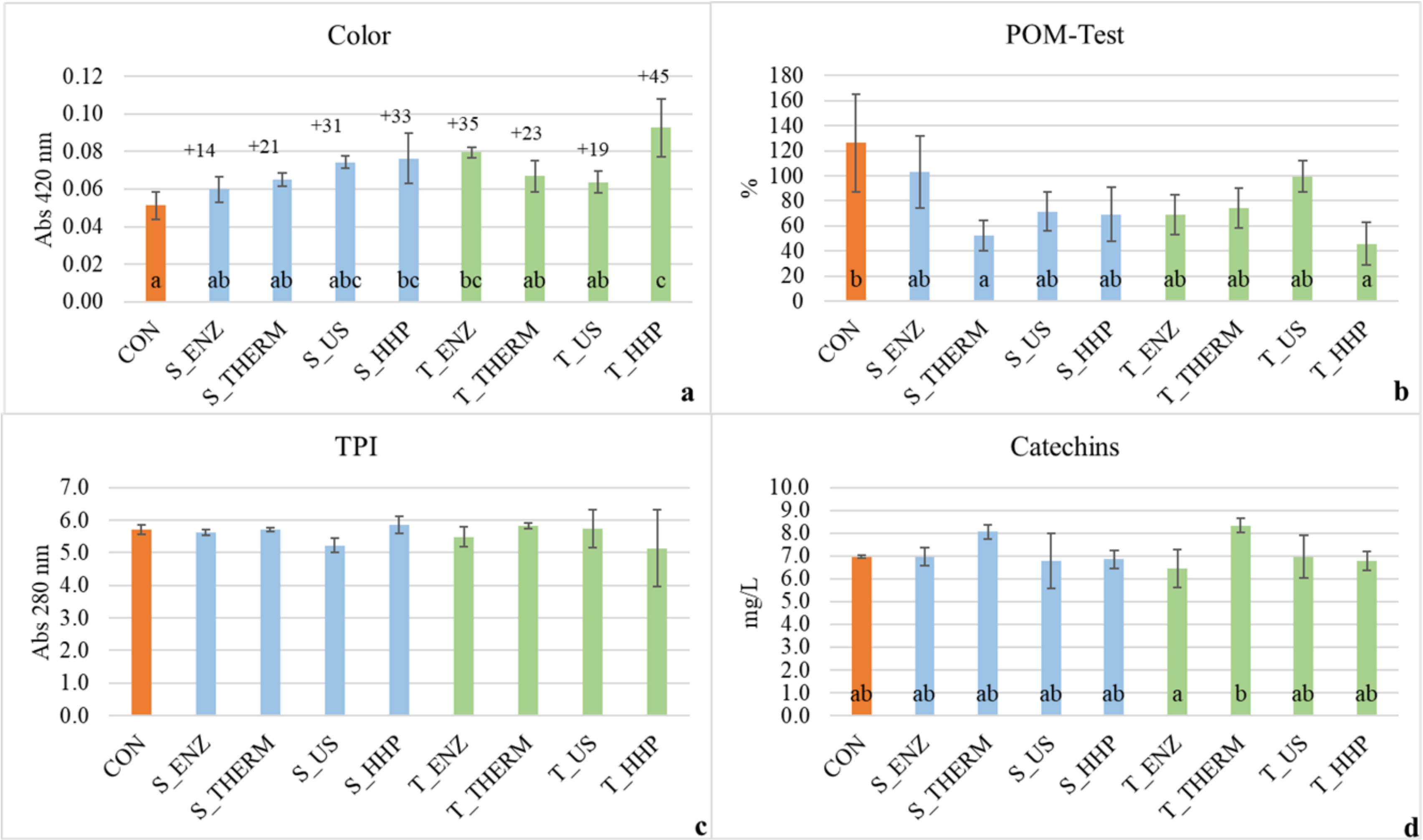


Figure 20. Wine color (**a**) and browning potential (POM-test) (**b**), TPI (**c**) and amount of catechins (**d**) after two months of aging on yeast derivatives. Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. CON: control; S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

The addition of yeast derivatives determined an increase in wine color after two months of aging in all the samples; this trend was particularly evident in the case of wines added with T_HHP, T_ENZ and S_HHP, with an increase of 45%, 35% and 33% respectively, resulting significantly different from the control. Observing the results of CieLab analysis reported in table 17, these values correspond to lightness (L*), red (+a*), green (-a*), yellow (+b*) and blue (-b*); the more pronounced increase in wine color observed in T_HHP, T_ENZ and S_HHP was confirmed by the lowest L* value (96.5, 97.6 and 97.6, respectively); T_HHP and T_ENZ also showed tendential lower intensity of green (-a*), with less negative values compared to CON (-0.6), and higher intensity in yellow components (4.2 and 4.5, respectively) compared to CON (3.9). By considering the results about browning potential, this index is related to the potential oxidizability of wine, with lower values corresponding to lower oxidizability. In general, all the added wines showed lower oxidizability indexes compared to the control. Wines added with S_THERM and T_HHP showed the lowest POM-test values, thus resulting the only statistically different from the control, whereas for all the remaining samples no significant differences emerged. However, the increase in wine color coupled with a tendential lower POM-test, and lower TPI and catechins content detected in almost all the wines added with yeast derivatives might in part due to the occurrence of oxidation process. This trend was particularly evident - at least in terms of mean values - for T_HHP and T_ENZ that in fact showed the lowest mean TPI (5.1 and 5.5 respectively) and the lowest mean amount of catechins (6.8 mg/L and 6.4 mg/L, respectively) compared to the control (with TPI value of 5.7 and catechins content of 7 mg/L); however, no statistical differences were found and these slight differences may be also considered negligible from the practical point of view.

On the other hand, wines added with S_ENZ and T_US showed a similar behavior towards wine oxidation compared to the control; the color increase was lower (+14% and +19%

respectively), as well as they showed the highest mean values of POM-test (103% and 99%, respectively) compared to all the other added wines, at least in terms of mean values; lastly, the TPI and the amount of catechins were quite similar to the control.

After one month from the addition of yeast derivatives, an increase in wine color and a decrease in POM-test value was observed in our previous experiment, with sulfur dioxide the most effective in protecting wine color and in determining lower browning [156]; this confirmed the results here obtained, especially as concern wines added with T_HHP, T_ENZ and S_HHP after two months of aging.

In figure 21, the effect of yeast derivatives addition on wine color (**a**), browning potential (**b**), TPI (**c**) and catechins content (**d**) after six months of aging was reported.

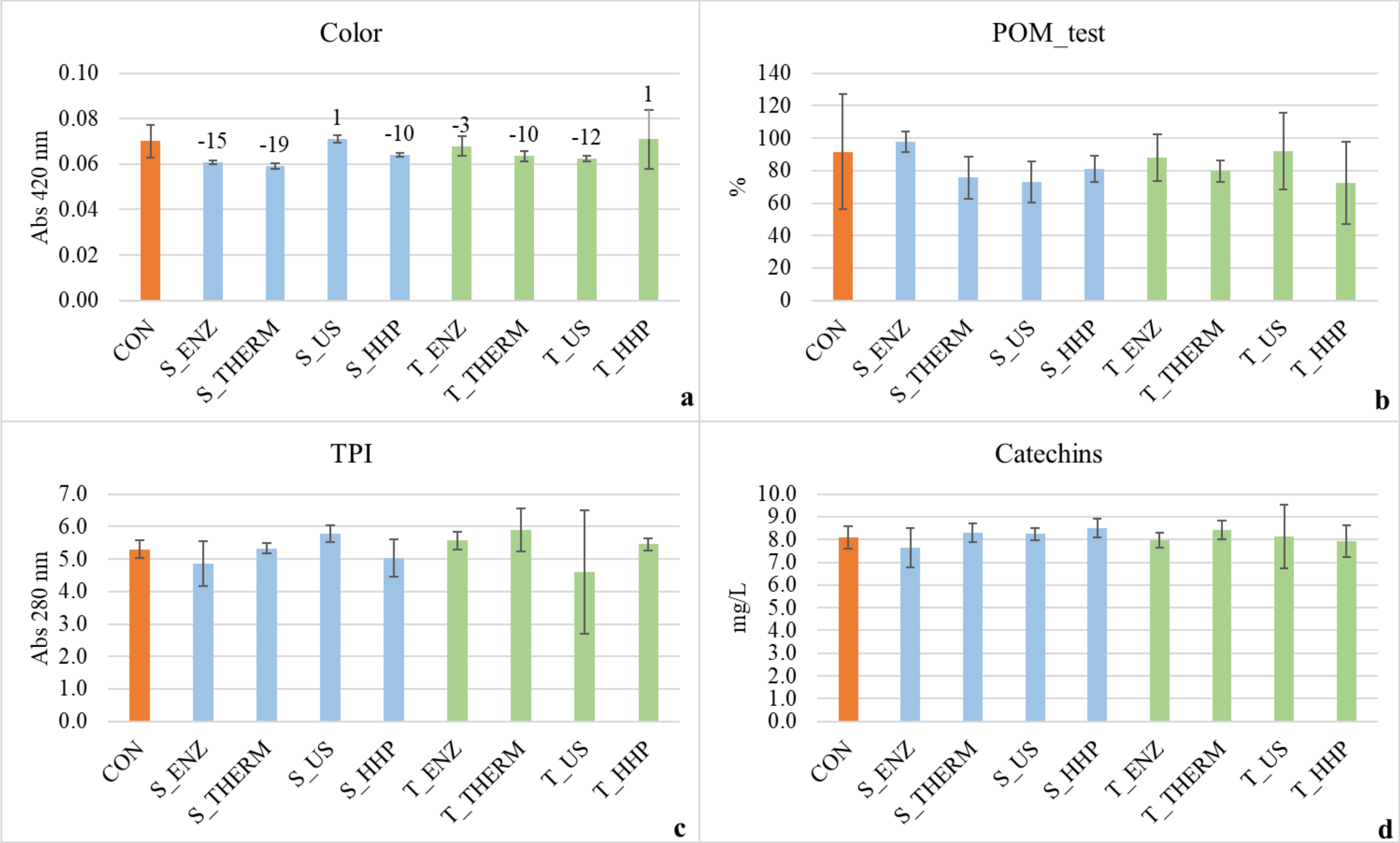


Figure 21. Wine color (**a**) and browning potential (POM-test) (**b**), TPI (**c**) and amount of catechins (**d**) after six months of aging on yeast derivatives. Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. CON: control; S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

By considering the results obtained after six months of aging, no differences were reported among all the treatments for all the chemical parameters analyzed. A slight color loss was observed in almost all the wines added with yeast derivatives compared to the control, except for S_US and T_HHP. This trend was confirmed by CieLab analysis (table 18): no differences were observed as concern L* values; the lowest intensity of yellow color (b*) was detected in S_ENZ, S_THERM and S_HHP (3.9, 4.0 and 4.1, respectively) whereas the highest value was found in S_US (4.9), all resulting statistically different from the control (4.5). Lastly, no differences were observed among the samples neither for POM-test nor for TPI and catechins content compared to the sulfite samples.

However, S_ENZ and T_US showed a tendential higher oxidizability potential (98% and 92%, respectively) compared to the control (91%); in both the samples, the higher color decrease was also coupled with lower values of TPI (4.9 and 4.6 in S_ENZ and T_US, respectively) and - only in the case of S_ENZ – slightly lower amount of catechins (7.6 mg/L), compared the control (8.1 mg/L), at least in terms of mean values. These results agreed with those reported by del Barrio-Galan et al. [164] who observed that, after the addition of yeast derivatives, a decrease in color intensity and total polyphenols were observed after six months of aging. A similar trend was also observed by Rodriguez-Nogales et al [167]: the authors reported that, after the addition of different yeast derivatives (both cell walls, autolysates and mannoproteins) for sparkling wine production, an increase in wine color after the first three months occurred, followed by a decrease after nine months, without a significant reduction in polyphenols content. The authors also hypothesized that mannoproteins released by yeast derivatives protected polyphenols towards oxidation; similarly, the soluble colloids and mannans released into the wines after the addition of derivatives, as better discussed in the following section, might have contribute to protect the phenolic fraction, especially after six months of aging.

However, at both sampling times, the best results were in general obtained for wines added with S_ENZ.

The protective effect of yeast lees and derivatives towards wine oxidation in comparison to other antioxidant additives was previously reported [16,152], depending not only on the amount of glutathione [155,171] but also on other peptides exhibiting antioxidant properties[172] and on compounds linked to cell wall fractions [32,151]. Furthermore, the antioxidant activity tended to decrease during time, also depending on the starting concentration of antioxidant molecules: the higher the concentration, the faster the consumption of such compounds [153,171].

Moreover, by considering the overall chemical composition of the respective yeast derivatives as discussed above (table 15 in Chapter 3, Part III, section 4.2), S_ENZ showed a mean content of glutathione of about 4 µmol/g, lower than the highest amount detected in S_HHP (9 µmol/g) and in T_ENZ (7.2 µmol/g). The occurrence of a gradual, slow release of glutathione or other antioxidant molecules during wine aging together with the highest amount of reducing proteins detected in S_ENZ (66.5 µmol/g) might at least in part explain its better behavior towards wine color evolution and protection against oxidation.

The potential adsorption phenomena of phenolic compounds on the insoluble fraction of yeast lees [153] and of yeast derivatives [164,165] was proposed as other mechanism involved in preserving polyphenols from oxidation. The non-negligible content of total insoluble solids (about 600 mg/g) detected in S_ENZ derivative (table 15 in Chapter 3, Part III, section 4.2) might explain the lowest TPI and lowest amount of catechins detected in the resulting wines, thus confirming the better behavior and the good protection against wine oxidation of such derivative, whose performance was quite comparable to sulfur dioxide.

4.2 *Effect of yeast derivatives addition on the amount of mannans and total soluble colloids after two and six months of aging*

The amount of mannans detected into the wines after two and six months of aging was reported in figure 22.

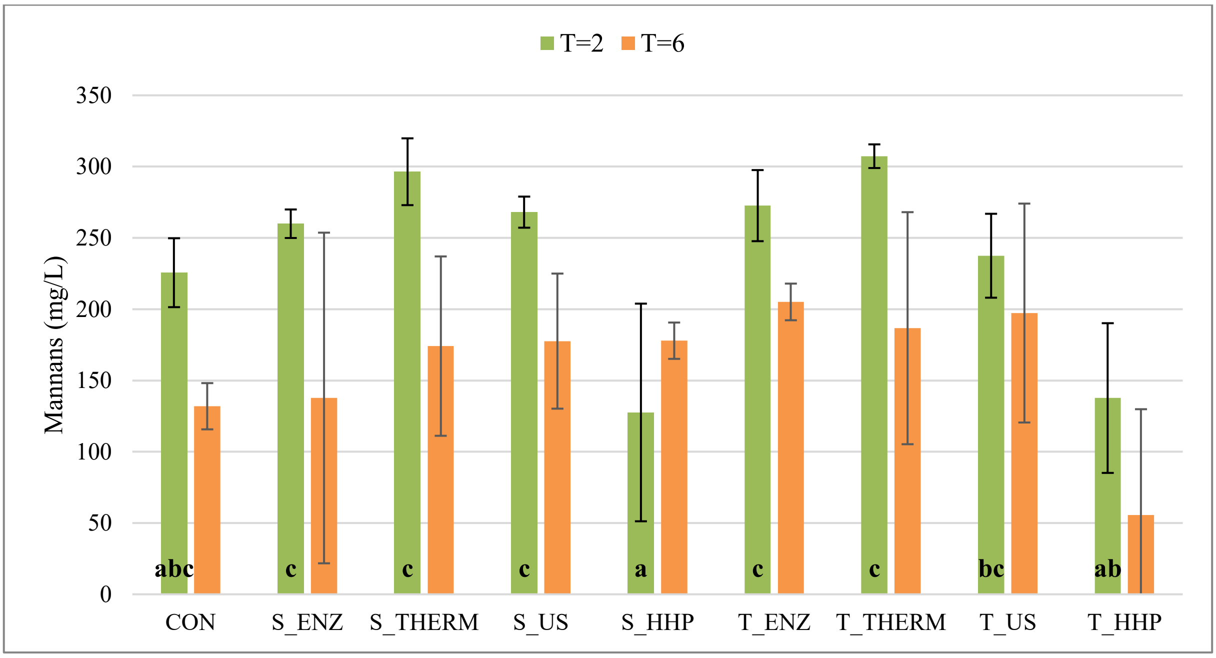


Figure 22. Amount of mannans (mg/L) detected into the wines after two and six months of aging. For each sampling time, different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$, CON: control; S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

After two months, the amount of mannans tendentially increased in almost all the added wines, except for the samples added with derivatives obtained by HHP treatment, with the lowest mean content (128 mg/L and 138 mg/L for S_HHP and T_HHP, respectively). Even if no significant differences emerged compared to the control, the highest mean amounts were detected in wines added with derivatives obtained from both the yeasts by traditional methods, in particular thermal inactivation, followed by enzyme addition and ultrasounds. After six months of aging, the amount of mannans tended to decrease in almost all the samples, except for wines added with S_HHP that, on the contrary, showed a slight release of such compounds during the further four months of aging (final concentration of 178 mg/L). Even in this case, no significant differences emerged among the samples; in general, wines that showed higher contents of mannans after two months, *i.e.*, wines added with THERM, ENZ and US derivatives from both the yeasts, were also the most characterized in such compounds at the end of aging.

By considering the amount of mannans detected in yeast derivative powders (table 15 in Chapter 3, Part III, section 4.2), it would be said that in general yeast derivatives mostly characterized by the highest content of mannans also determined the highest release of such compounds into the wine at both sampling times, despite of the decrease observed at the end of aging in almost all the samples. Furthermore, the poorest amounts of mannans detected in wines added with S_HHP and T_HHP might be explained by the lowest amount of such polysaccharidic fraction in their respective derivatives (11 mg/g and 21 mg/g for S_HHP and T_HHP, respectively).

Lastly, as concern S_ENZ, even if the mannans content in the respective derivative (17 mg/g) was lower compared to the other products, the amounts detected into the wines after aging were comparable to the other samples.

The amount of total soluble colloids detected into the wines after two and six months of aging was reported in figure 23.

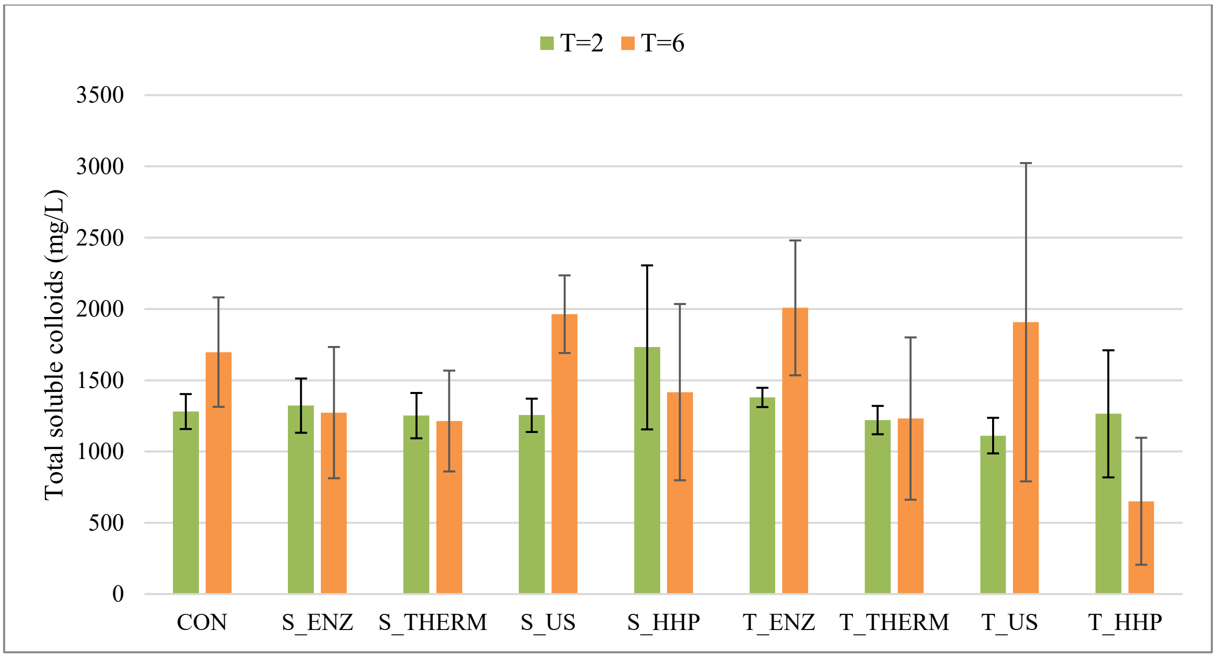


Figure 23. Amount of total soluble colloids (mg/L) detected into the wines after two and six months of aging, CON: control; S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

By considering the content of total soluble colloids after two months, the sample that showed the lowest amount of mannans - S_HHP - conversely presented the highest mean content of soluble colloids, with a mean value of 1731 mg/L; however, no differences were found among all the samples. After six months of aging, a tendential increase in colloidal substances were observed in control wine, and in wines added with S_US, T_US and T_ENZ, whereas wines added with S_HHP and T_HHP showed a reduction; for the remaining samples the content was quite similar to those detected after two months.

The release of soluble colloids from yeast derivatives into the wines followed in part the trend previously observed for mannans, with a more evident effect at the end of aging. As discussed above, wines added with S_US, T_US and T_ENZ tendentially showed the highest mean amounts of total soluble colloids after six months (1963 mg/L, 1907 mg/L and 2008 mg/L, respectively); the respective derivatives (table 15 in Chapter 3, Part III, section 4.2) were in fact the most characterized (215 mg/g, 171 mg/g and 327 mg/g, respectively), together with S_THERM (187 mg/g), for which the good content of such compounds did not correspond to a higher release in the resulting wines. Lastly, wines added with S_HHP and T_HHP showed the poorest content of soluble colloids at the end of aging (1417 mg/L and 651 mg/L, respectively), as it was for their respective derivatives (104 mg/g and 133 mg/g). The trend was confirmed by considering the relationship between the chemical composition of yeast derivatives and the chemical parameters evaluated in the wines after two and six months of aging: a slight positive correlation was found between mannans detected in yeast derivatives and in wines at both sampling time ($r = 0.61$ at time 2, and $r = 0.58$ at time 6) and between total soluble colloids detected in yeast derivatives and in wines after six months of aging ($r = 0.64$). On the other hand, the negative correlation found between the amounts of insoluble solids (detected in yeast derivatives) and the content of soluble colloids in the wines after six months of aging ($r = - 0.84$, $p < 0.01$) might explain the lowest release of such compounds into the samples added with THERM and HHP derivatives; in fact, these products were characterized by the highest content of insoluble solids (table 15 in Chapter 3, Part III, section 4.2).

After the addition of yeast derivatives, an initial increase in total polysaccharides content, followed by a reduction at the end of aging was previously reported [164], probably due to the formation of complexes with other wine colloids, that might precipitate [170]. Furthermore, Guadalupe et Ayestaran [179] also observed how during aging, polysaccharides (especially higher molecular weight fractions) may be subjected to degradation phenomena; this scientific evidence might explain the tendential reduction in terms of mannans content observed in all the samples at the end of aging.

Independently on the starting concentration of polysaccharidic fractions in yeast derivatives, residual enzymatic activities on cell wall components potentially occurred in wines added with

ENZ and US derivatives; this might explain on one hand the good release of polysaccharides observed in wines added with S_ENZ after two months of aging, comparable to the richest products and, on the other hand, the further concomitant release of soluble colloids detected in such samples (*i.e.*, T_US, T_ENZ and S_US) at the end of the aging period considered.

During wine aging on lees, the occurrence of autolytic process led to an enrichment of wine in polysaccharides also depending on the strains used, with interesting results obtained by non-*Saccharomyces* yeasts [185,186]; the results here obtained confirmed the good performance and aptitude of *T. delbrueckii* for yeast derivatives production, comparable to *S. cerevisiae*.

As concern manufacturing process, the efficacy of ultrasound treatment in enhancing the release of polysaccharides during wine aging on lees was reported [14,112,113], with results similar to enzyme addition [111]. Furthermore, the amounts of mannans and total soluble colloids released into the wines added with US derivatives were comparable to those detected for the traditional techniques, confirming its potential application for yeast derivatives production.

Lastly, as concerns wines added with HHP derivatives, even if the powders were not characterized by the greatest polysaccharidic fractions, at the end of aging the amount of mannans and total soluble colloids detected into the wines were comparable to the other samples, in a more evident manner for S_HHP wines. It would be possible that, despite pressure above 600 MPa may inactivate endogenous enzymes, possible residual enzymatic activity might be occurred [118], thus favoring the release of cell wall polysaccharides [117]. Moreover, the hydrolysis of high molecular weight fractions [179], together with the fact that higher concentrations of polysaccharides in yeast derivatives not always corresponded to higher release into the wine [164], might explain the trend here observed for wines added with HHP and THERM derivatives.

4.3 Effect of yeast derivatives addition on particle size distribution (PSD) and filtration assay after two and six months of aging

In figure 24 the mean diameter of particles determined in the soluble fraction of wines after two month of aging was reported.

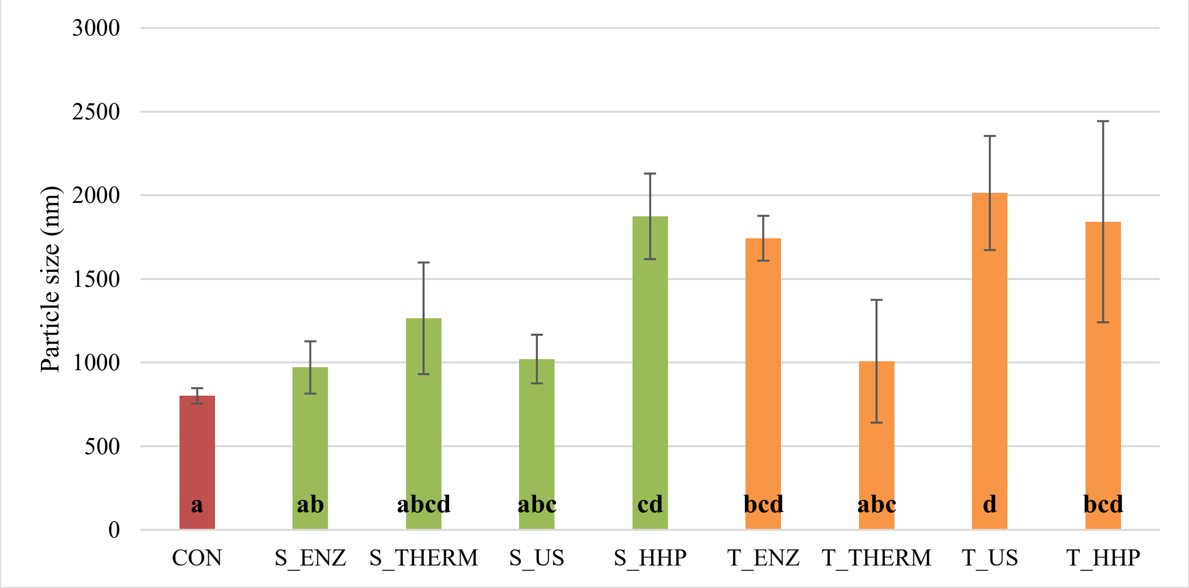


Figure 24. Mean diameter of particles (nm) determined by particle size distribution (PSD) on wines after two months of aging on yeast derivatives. Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$.CON: control; S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

An increase in the mean diameter of colloidal particles was observed in all the added wines, due to the release of both mannans and total soluble colloids from the powders after two months of aging, thus modifying the colloidal state of the wine. Significant differences were found for wines added with S_HHP and T_HHP, T_ENZ and T_US compared to the control. This might be due to the highest mean amount of mannans (for T_ENZ and T_US) and total soluble colloids (for S_HHP and, in less intense manner, for T_HHP) detected into the wines after two months, as discussed in the previous section (section 4.2 of the present chapter); furthermore, for the latter samples, the highest mean particles diameter determined in the respective derivatives (2068 nm and 1276 nm for S_HHP and T_HHP, respectively, as reported in table

15, Chapter 3, Part III, section 4.2) might have also contributed to increase the mean particles size in the resulting wines.

After six months of aging, a change in the mean particles size was observed, as reported in figure 25.

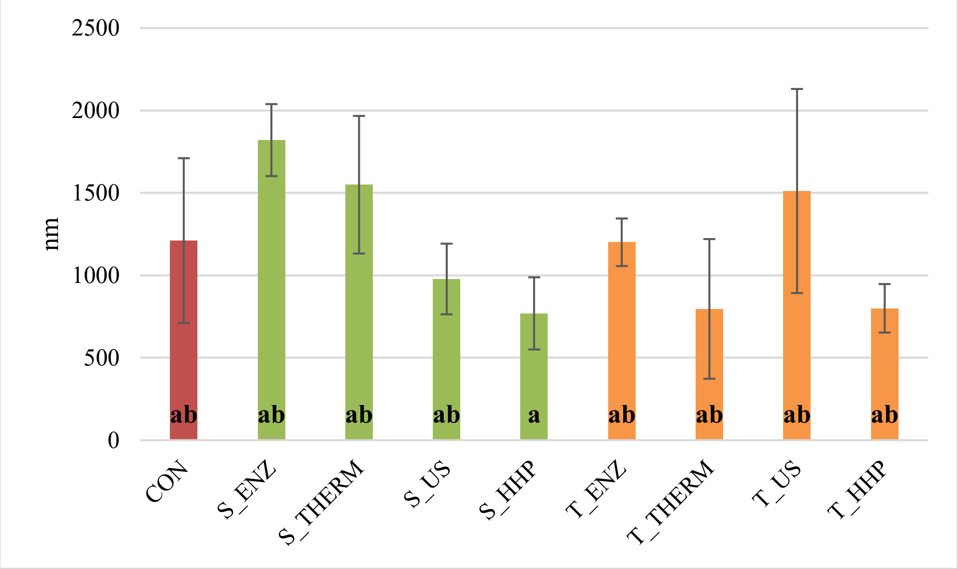


Figure 25. Mean diameter of particles (nm) determined by particle size distribution (PSD) on wines after six months of aging on yeast derivatives. Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. CON: control; S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

No significant differences were observed among the samples, also compared to the control wine; almost the added wines showed a reduction in the mean particles diameter, except for control, S_ENZ and S_THERM, that conversely showed an increase in relation to the previous sampling time (fig. 24).

In figure 26, the distribution of particles with mean diameter below 1 µm was reported.

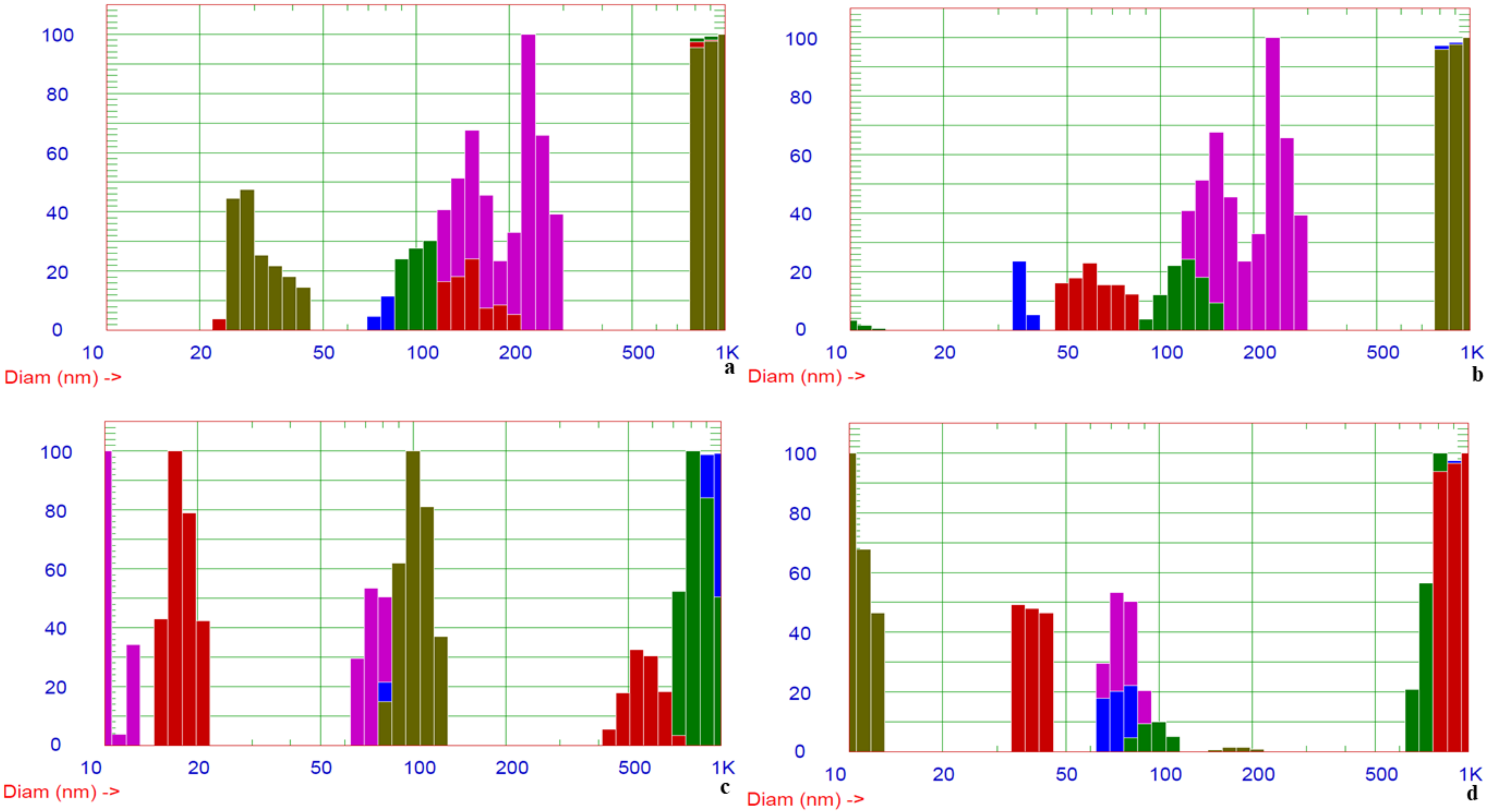


Figure 26. Particle size distribution (particles with mean size below 1 μm) of wines after two (**a** and **b**) and six (**c** and **d**) months of aging. Particles size distribution of control and wines added with derivatives from *S. cerevisiae* (**a** and **c**), and control and wines added with derivatives from *T. delbrueckii* (**b** and **d**). Violet: Control; blue: ENZ; dark green: THERM; red: US; olive green: HHP.

Soluble colloids with small size were present into the wine after two months (fig. 26 a and b); at the end of aging (fig. 26 c and d), a tendential reduction of mean particles size was observed, with slightly higher presence of particles with a mean diameter of 10-20 nm and 50-100 nm; this trend was observed in almost all the samples, whereas higher fractions (around or above 1 μm) were still present in S_THERM, S_ENZ, T_US and T_THERM. As observed by Guadalupe et Ayestaran [179], the degradation of greater polysaccharidic fractions - naturally present into the wine or released by yeasts during fermentation and aging – into smaller molecules, might partially confirm the trend here observed.

Concerning filtration assay, this parameter was calculated since the modification in the amounts of soluble molecules after yeast derivatives addition may impact on the colloidal state and, consequentially, on the filterability of the resulting wines before bottling.

As reported in tables 17 and 18, no differences in wine filterability were observed among all the samples neither after two nor after six months of aging. After two months, the filtration flow ranged from the minimum of 12.8 mL/min/cm^2 (S_HHP) to the maximum of 21.1 mL/min/cm^2 (S_US), whereas after six months all samples showed a reduced filtration flow compared to the previous sampling time. The values found were quite similar among all the samples, with the lowest filtration flow for S_ENZ and S_US (5.6 and 5.7 mL/min/cm^2) and the highest mean value for T_HHP (6.7 mL/min/cm^2); however, the slight differences observed among the samples were considered negligible from the practical point of view.

The release of soluble colloids might have contributed to the further reduction on filtration flow occurred during aging; this might be confirmed by the negative correlation found between the content of soluble colloids present into the wine and filtration flow rate, after two ($r = -0.66$, $p = 0.07$) and after six months of aging ($r = -0.70$, $p = 0.052$).

However, after six months of aging the reduction in the filterability observed in all the samples might be due to the slight, higher presence of smaller colloidal particles or to the greater presence of soluble colloids, compared to the previous sampling time, thus possibly having determined a faster fouling of filtration membranes [182].

Few evidence was reported in literature about the evolution and changes on colloidal particles size during aging on lees, also in relation to the type of treatments applied for inducing yeast autolysis and the consequent effect on wine filterability. In a study carried out by Comuzzo et al. [187], the filtration flow of wine aged for six months both on untreated and treated lees (by enzyme addition and high pressure) was quite similar among them, but lower compared to control wine. On the other hand, the addition of mannoproteins for tartaric stabilization slightly reduced white wine filterability compared to untreated wine, also depending on the dosage used [188]. These results might confirm on one hand the reduction of filtration flow observed in all the samples during aging and, on the other hand, the slight differences compared to the control, for which a reduction in the filterability was also observed.

4.4 Effect of yeast derivatives addition on aroma profile of wines after two and six months of aging

Thirty-eight volatile compounds were tentatively identified in wines after aging (Annex 5); the results of semi-quantitative analysis carried out on the concentration (in µg/L) of volatile compounds detected in the headspace of wines after two and six months of aging were reported in Annexes 7 and 8, respectively; means and standard deviations were calculated, and the results of the statistical analysis were also reported.

After two months, wines volatile profile was not well defined with different behaviors among the samples and even among some replicates, probably because the time considered was not enough to reach a stable state. However, it would be said that in general wines added with T_ENZ, T_HHP and S_ENZ showed the poorest volatile profile, similarly to control wines, whereas wines added with THERM and US derivatives (by both the yeasts) and S_HHP were tendentially more characterized in terms of aroma compounds. These results were in line with those reported by Comuzzo et al. [94] that observed how wines added with yeast derivatives obtained by thermal and mechanical treatments were the most characterized in terms of volatile profile, showing a more intense aroma perception, whereas the addition of derivatives obtained by enzymatic treatment determined a lower odor impact in the wine, even this product was the most characterized in terms of aroma compounds.

At the end of aging, the aroma profile was quite different from the previous sampling time, with a more defined aroma evolution; the differentiation among the samples allowed to better clarify the impact of the different products on the volatile profile of the resulting wines. The results of PCA carried out on the concentration (in µg/L) of volatile compounds after six months of aging were reported in figure 27.

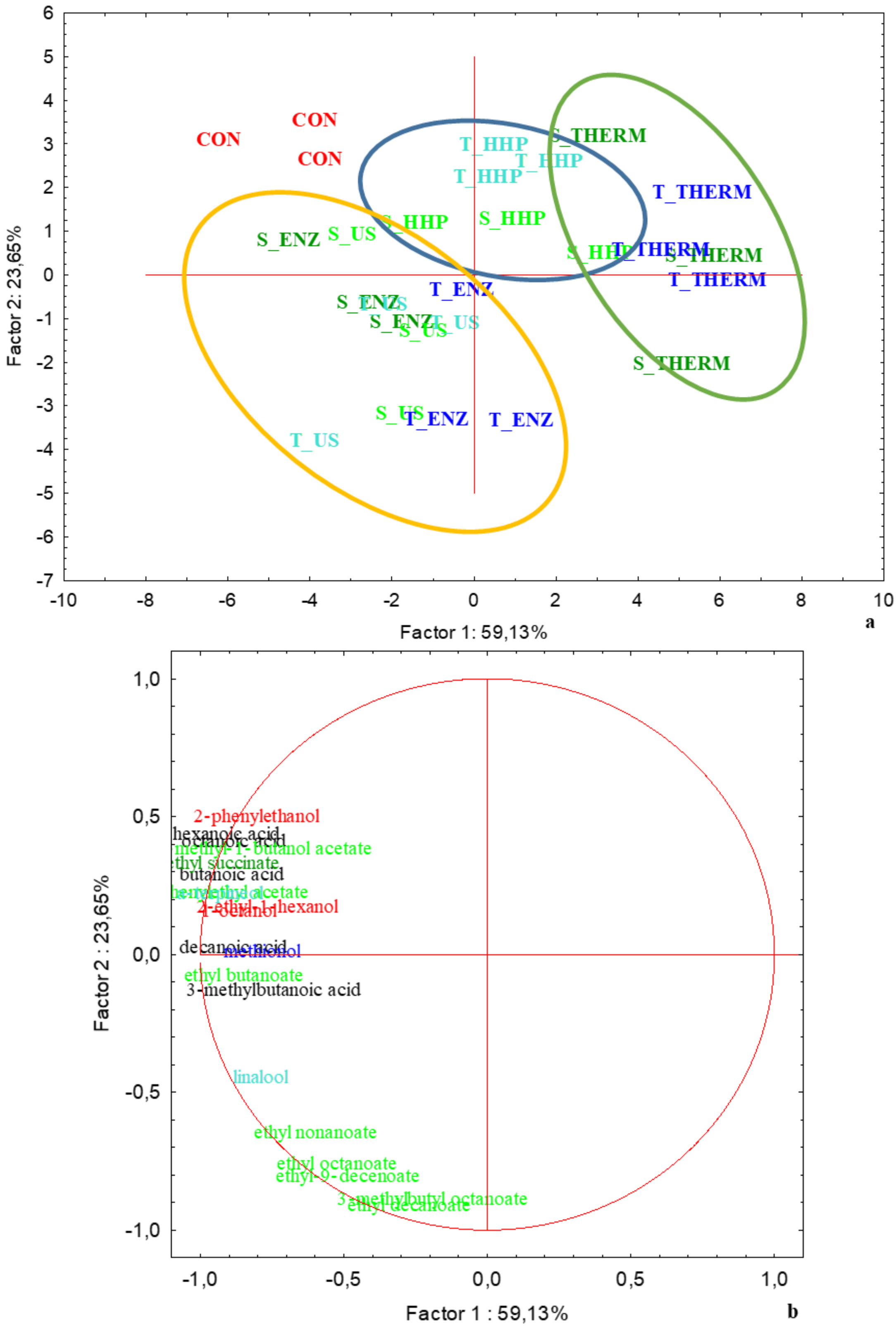


Figure 27. Results of the PCA carried out on concentration (µg/L) of volatile compounds detected in the headspace of wines after six months of aging. Projection of case (samples) (**a**) and variables (volatile compounds) (**b**) on the factor-plan were reported. Factor Loadings (FL) were calculated by Factorial Analysis, and the most relevant. variables were selected for marked FL > 0.7. S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; HHP: high hydrostatic pressure: US: ultrasound.

The influence of the different derivatives on wine volatile profile seemed to be mostly dependent on the manufacturing process previously performed for their production, rather than the starting yeast strains. Wines added with THERM products showed the poorest volatile profile, whereas wines added with US and ENZ derivatives were the most characterized in terms of aroma compounds, together with the control. Derivatives previously obtained by HHP partially impact on wine aroma - showing a profile intermediate between the first two groups - however quite similar to THERM. Tendentially, higher mean concentration of fatty acids and higher alcohols were detected in the control, whereas esters, some acids (*i.e.*, 3-methyl butanoic acid and decanoic acid) and linalool were the volatile compounds mainly characterizing wines treated with S_ ENZ, S_US, T_ENZ and T_US.

No correlation was found between the aroma compounds of the powders and of the wines, whereas correlations between some chemical parameters analyzed - *i.e.*, insoluble solids of powders, mannans and soluble colloids of wines - and the aroma compounds were found and reported in table 19; the aroma compounds considered were the most significant variables after factorial analysis and PCA (fig. 27). Negative correlation was found between insoluble solids and 3-methylbutyl octanoate (r = -0.78), whereas a positive correlation between the amounts of total soluble colloids detected into the wines and esters was observed: ethyl octanoate (r = 0.77), ethyl nonanoate (r = 0.80), ethyl decanoate (r = 0.89), 3-methylbutyl octanoate (r = 0.90) and ethyl-9-decenoate (r = 0.81).

Table 20. Correlation between chemical composition (insoluble solids in yeast derivatives; amounts of mannan and of soluble colloids in wines after six months) and the aroma compounds of wines after six months of aging. Marked correlation were significant at $p < 0.05$ (*) and at $p < 0.01$ (**).

	Insoluble solids (mg/g)	Mannans (mg/L)	Soluble colloids (mg/L)
butanoic acid	-0.20	-0.28	0.20
3-methylbutanoic acid	-0.36	0.21	0.56
hexanoic acid	-0.24	-0.26	0.24
octanoic acid	-0.07	-0.47	0.05
decanoic acid	-0.15	-0.19	0.28
2-ethyl-1-hexanol	-0.60	0.34	0.69
1-octanol	-0.39	0.03	0.41
2-phenylethanol	0.08	-0.63	-0.20
ethyl butanoate	-0.41	0.11	0.56
3-methyl-1-butanol acetate	-0.18	-0.32	0.11
ethyl hexanoate	0.27	-0.22	-0.57
hexyl acetate	-0.35	0.11	0.37
ethyl lactate	-0.25	0.34	0.21
ethyl octanoate	-0.58	0.41	**0.77***
ethyl nonanoate	-0.60	0.42	**0.80***
ethyl decanoate	-0.66	0.59	**0.89****
3-methylbutyl octanoate	**-0.78***	0.57	**0.90****
diethyl succinate	-0.21	-0.30	0.19
ethyl-9-decenoate	-0.63	0.46	**0.81***
2-phenylethyl acetate	-0.19	-0.33	0.18
methionol	-0.43	-0.04	0.47
linalool	-0.58	0.12	0.62
α-terpineol	-0.41	-0.02	0.46

These results agreed with those reported in literature; the addition of yeast derivatives may enhance the fruity and floral notes due to an increasing volatility of some esters when added at concentrations of about 200 mg/L [147], comparable to the dosage used in the present study (300 mg/L). Furthermore, polysaccharides - especially mannoproteins - may enhance the volatility of some aroma compounds, *i.e.*, alcohols and esters, also depending on the type of products and glycosylation degree of the polysaccharidic fractions [174,176]. This salting-out effect seems to occur during shorter contact time, whereas longer exposure of wines to yeast derivatives as well as the presence of insoluble fractions may determine the retention of wine aroma; moreover, a longer contact time may determine the release of some odor-active compounds from the powders, *i.e.*, pyrazines [157,177]. Nevertheless, retained volatile compounds may subsequentially be released into the wine during more prolonged aging time (up to six months), as previously observed by several authors [165,189].

These findings might support the results here obtained, explaining how wines added with derivatives obtained by THERM and HHP showed the poorest volatile profile, due to the higher mean content of insoluble solids in the respective derivatives (678 mg/g, 492 mg/g, 659 mg/g and 823 mg/g for S_THERM, S_HHP, T_THERM, T_HHP, respectively), together with the lower mean amounts of soluble colloids released into the wines after six months of aging.

No pyrazines were detected in the wines added with ENZ and US, even if the respective powders contained a certain amount of these odor-active compounds, similarly to the results obtained by Comuzzo et al. [94].

On the other hand, the greater concentration of total soluble colloids released into wines added with ENZ and US derivatives seemed to have promoted a retention of some volatile compounds after two months, especially for S_ENZ wines (as previously discussed); this was probably followed by a release of adsorbed aroma compounds when a more prolonged contact time was performed (six months), thus explaining the more intense volatile profile and the higher mean concentration of esters detected in such added wines, compared to the control.

5. Conclusion

T. delbreuckii showed a good aptitude to produce yeast derivatives, comparable to *S. cerevisiae*. After the addition of yeast derivatives, the chemical composition of wine changed also in relation to the contact time. As concerns wine evolution, independently on the starting composition of the respective derivatives, all the products offered a good protection against wine oxidation, in some cases comparable to sulfur dioxide, and the amounts of mannans and total soluble colloids at the end of aging was similar among all the samples. The addition of derivatives impacted on particles size and wine filterability; however, no differences were observed compared to the control. On the other hand, the manufacturing process and, consequentially, the initial composition of yeast derivatives seemed to mainly affect the volatile profile of the resulting wines. These products may improve wine aroma profile by increasing the volatility of compounds that generally confer floral and fruity notes - in the case of ENZ and US derivatives – and by reducing the release or by retaining some odor active compounds, *i.e.*, fatty acids, in the case THERM and HHP products. As previously discussed in the chapter 3 and by considering the effects on wine, it might be confirmed that emerging technologies might be a good alternative to the traditional methods for obtaining yeast autolysates or extracts by ultrasounds (instead of enzyme addition) and for producing cell wall preparations by high hydrostatic pressure (instead of thermal treatment). It would be interesting to elaborate on the addition of these products during fermentation with the purpose of enriching wine in soluble colloids and antioxidants, already during the first steps of winemaking process. In this regard, the use of derivatives specifically tailored for winemaking might allow to *i)* reduce the addition of sulfur dioxide, *ii)* improve the chemical stability of young wines or *iii)* effectively reduce the conventional period of aging on lees.

Chapter 5

Sequential inoculum of *Hanseniaspora* spp. and *Saccharomyces* spp. during fermentation of synthetic must: effect on chemical composition and volatile profile

1. Introduction

Due to the positive contribution on the overall wine quality, almost of all non-*Saccharomyces* strains have been well studied and used during winemaking process, also including *Hanseniaspora* spp. This genus represents a non-negligible part of natural microflora of grapes, and one of the most active strains during the early stages of fermentation [190]. Poorer growth kinetics and fermentative capacity, higher concentration of acetic acid and glycerol was reported compared to *Saccharomyces* spp., also depending on the strain [191,192]. On the other hand, sequential or mixed fermentation with *Saccharomyces* spp. allow to obtain wines with lower ethanol and acetic acid content [192] and an improved volatile profile and sensory perception [193,194], also related to grapevine variety and winemaking conditions [195].

The great ability of *Hanseniaspora* spp. to improve the aroma profile is mainly due to the highest production of acetate esters, such as 2-phenylethyl acetate, hexyl acetate and isoamyl acetate, with further increase when used in sequential fermentation, also depending on the strains [196] and nutrient sources [197]. Higher concentration of alcohols and lower production of sulfur compounds was also observed [196], together with an enhanced production of aldehydes (*e.g.*, benzaldehyde and trans-2-hexenal) and terpenes [31,194]. These peculiar features of *Hanseniaspora* spp. seem to be related to the presence of esterase and glucosidase enzymes [191], able to enhance the varietal characteristics of wines [194].

Besides the positive effect towards wine aroma profile, different *Hanseniaspora* spp. strains show a good aptitude to produce polysaccharides [198], with relevant amounts released both during single [4,13] and sequential fermentation [31]. However, during aging on lees, the amounts of polysaccharides released in model solution seems to be comparable to *Saccharomyces* spp. [29], or even much lower under real winemaking conditions [31].

Regarding to the role of this yeast towards wine oxidation, only few evidence is reported in literature, mainly concerning the increased production of acetaldehyde during sequential fermentation and the consequent formation of more stable pigments in red wines [199]. No evidence is reported about the release of antioxidant compounds and the potential contribution on protecting wine against oxidation, both during single and sequential fermentation.

2. Aim of the work

The aim of the work was to evaluate the aptitude of spontaneous *Hanseniaspora* spp. strain as co-starter during sequential fermentation in comparison with single fermentation (used as control), carried out by using commercial *Saccharomyces* spp. In this regard, strain H3 was selected by considering the notable release of soluble colloids and antioxidant compounds, as resulted from the first characterization (Chapter 1) and above all, after growth and enzyme-assisted lysis as reported in Chapter 2.

The experimental trial was performed in a synthetic must in order to assess the real contribution of the yeasts on the chemical composition, release of compounds of enological interest, and volatile profile of the resulted synthetic wines; for the first time, the release of antioxidant molecules - glutathione and cysteine - during sequential fermentation was assessed.

3. Materials and methods

3.1 Reagents

Sodium chloride for microbiology, bacteriological peptone, Malt Extract broth, WL Nutrient Agar and technical agar were purchased from Oxoid (Basingstoke, U.K.); glucose, fructose, sucrose, yeast extract, magnesium sulfate, ethanol (96 % v/v), mannan from *S. cerevisiae*, trifluoroacetic acid, riboflavin, L-glutathione reduced, L-cysteine, *p*-benzoquinone, 3-mercaptopropionic acid, and acetaldehyde (ACS reagent, ≥99.5%), glycerol, lactic, malic, succinic and acetic acid, and ethyl heptanoate were from Sigma Aldrich Italy (Milan, Italy). Methanol (HPLC grade) were purchased from VWR Chemicals (Milan, Italy). Tartaric and citric acid, boric acid, hydrochloric acid (37 % v/v), copper sulfate pentahydrate, sodium-potassium tartrate and sodium carbonate were purchased from Carlo Erba Reagents (Milan, Italy). MilliQ water was produced by MilliQ Advantage A10 apparatus (Merck Millipore, Billerica, MA, USA) and microfiltered at 0.22 μm before use. Commercial active dry yeast (ADY) *S. cerevisiae* was from Enologica Vason S.p.A. (San Pietro in Cariano, VR, Italy).

3.2 Yeasts strains and inoculum preparation

Spontaneous strain of *Hanseniaspora* spp. previously isolated from grape pomace and must (strain H3, Chapter 1) and commercial active dry yeast preparation (ADY) of *S. cerevisiae* were used as fermentation starters. For the former, the biomass production was carried out by using the best combination of nutrient sources and the most suitable growth conditions as reported in Chapter 2. Briefly, culture medium consisted 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 colony isolated on Malt Extract agar plates were grown overnight in 10 mL of the culture medium; yeast suspensions were then inoculated at 10 % (v/v), approximately corresponding to a concentration of 5.5 x 10^6 CFU/mL in 100 mL-sterile flasks containing 50 mL of the selected culture medium; after the first 24 hours of growth, the biomass was collected and fed with fresh culture medium until a final volume of 500 mL, then incubated for further 24 hours. The biomass production was carried out in an orbital shaker under constant agitation (150 rpm) at 30°C; the biomass was harvested by centrifugation (13000 rpm for 10 min at 4°C) and resuspended in the same volume of the synthetic must; the starting inoculum was approximately of 1 x 10^7 CFU/mL.

ADY of *S. cerevisiae* was rehydrated in 10 volumes of the synthetic must, inoculated at the same concentration (approximately 1 x 10^7 CFU/mL) at time 0 for single fermentation (used as control, hereafter referred to as S) and two days later than the initial inoculum of *Hanseniaspora* spp. for sequential fermentation (hereafter referred to as HS).

3.3 Alcoholic fermentation of synthetic grape must

To mimic the composition of grape must, a synthetic medium was prepared as described by Rossignol et al. [200], with slight modification: briefly, the sugars addition was 100 g/L of glucose and 100 g/L of fructose; malic acid (2.5 g/L), citric acid (0.7 g/L) and tartaric acid (2.5 g/L) were also added, and the pH was adjusted at 3.2 with KOH. Alcoholic fermentation was carried out in triplicate in 500 mL-glass bottles, previously sterilized and closed with hydrophobic cotton stoppers. In parallel, fermentations were also carried out in sterilized 9 L-stainless steel tanks, containing 7 L of synthetic must, hermetically closed and equipped with valve for releasing carbon dioxide; the latter was performed for obtaining both synthetic wine and fermentation lees for the following trials of aging (Chapter 6). After inoculating yeasts, prepared as described in the previous section, bottles and tanks were stored at temperature of 20°C±1 until the end of the fermentation process. Expect for cell viability, antioxidant compounds and aroma profile, wine samples were centrifuged at 3000 rpm for 15 min before the analyses.

3.4 Cell viability

Cell viability of both the strains was assessed before and after the inoculum, both in single and sequential fermentation; at the end of fermentation, the number of viable cells was evaluated both in wines and in lees. Cell viability was thus determined by plating 100 µL of each proper dilution on differential WL Nutrient Agar plates; the number of viable cells was counted after 48 and 72 hours of incubation at 30°C and expressed as logarithm of colony forming units per mL (log CFU/mL).

3.5 Monitoring of fermentation process by weighting and by residual sugars content

Fermentation kinetics was monitored by daily weighting the bottles due to the loss of carbon dioxide; fermentations were stopped when the daily weight loss was below 0.01 g/day, for at least three consecutive days.

For monitoring sugars consumption during fermentation, the content of residual sugars was also evaluated after two, seven and ten days of fermentation by Fehling method; briefly, a mixture of Fehling A (solution of $CuSO_4$, 69.278 g/L), Fehling B (solution of sodium-potassium tartrate, 346 g/L and sodium hydroxide, 100 g/L) and distilled water was freshly prepared with a ratio of 1:1:6. In a screw cap 10 mL-Pyrex tube, 5 mL of the mixture were added with 200 µL of wine and the samples were kept in oven at 100°C for 1 h; after cooling at room temperature, the absorbance at 694 nm was measured against water, by using a Spectrophotometer UV/VIS model V-530 (Jasco Inc, USA). The content of residual sugars was expressed in g/L in relation to a calibration curve made with glucose (0-25 g/L).

3.6 Basic enological parameters

Alcohol content (% v/v) was measured by Alcolyzer Plus Instrument (Antoon Paar, Graz, Austria), following the method suggested by the manufacturer; pH, total and free sulfur dioxide (in mg/L) were evaluated at the end of the alcoholic fermentation by using an automatic titrator (Flash Automatic Titrator, Steroglass, Italy).

3.7 Sugars and glycerol content and organic acids profile by HPLC-UV/IR

At the end of fermentation, 1 mL of wine sample was added to 9 mL of sulfuric acid (5mM) and collected at -20°C until the analysis. The amounts of residual sugars and glycerol, and organic acids profile were determined by HPLC-UV/IR as reported by Loira et al. [5]. Identification and quantitative analysis were performed by injecting solutions of external standards (glucose, fructose, glycerol, citric, tartaric, malic, lactic, succinic and acetic acid) at known concentrations, and the results were expressed in g/L.

3.8 Glutathione, cysteine and riboflavin by RP-HPLC

Glutathione content and cysteine were determined on wine samples as reported in Chapter 2, section 3.6, whereas riboflavin content was evaluated as reported in Chapter 2, section 3.7.

3.9 Total soluble colloids, particle size distribution and filtration assay

Total soluble colloids and mannans (SE-HPLC), particle size distribution (PSD) and filtration assay were determined as reported in Chapter 4, section 3.4.

3.10 Wine volatile profile

The volatile profile of wines after alcoholic fermentation were determined as previously described in Chapter 4, section 3.5.

3.11 Statistical analysis

Means and standard deviations, analysis of variance, factorial analysis and principal component analysis (PCA) were carried out as reported in Chapter 3, section 3.12.

4. Results and discussion

4.1 Monitoring of fermentation process and microbial count

The fermentation process was monitored by weighting loss due to the release of carbon dioxide; the alcoholic fermentation was almost completed after seven days, with a loss of weight lower than 0.01g/day; in order to be sure that all sugars were consumed, the samples were kept for further three days, then the concentration of residual sugars was evaluated by Fehling method, that confirmed the end of the fermentation process (glucose concentration < 1 g/L). At the end of the alcoholic fermentation, the number of viable cells detected in control wines (S) was 1.94 x 10^5 CFU/mL, whereas wines subjected to sequential fermentation (HS) showed a number of viable cells of 2.17 x 10^4 CFU/mL and 5.28 x 10^5 CFU/mL for *Hanseniaspora* spp. and *Saccharomyces* spp., respectively. Regarding fermentation lees, the number of viable cells of *Saccharomyces* spp. was approximately of 1 x 10^7 CFU/mL and 1 x 10^8 CFU/mL in S and HS respectively, whereas no viable cells (< 100 CFU/mL) were found for *Hanseniaspora* spp.

Different strains of *Hanseniaspora* spp. showed a good growth kinetics and fermentative capacity, good resistance at lower temperature and tolerance to sulfur dioxide; nevertheless, when the concentration of ethanol increased above 3%, a reduced growth was observed [192]. Furthermore, a reduction in cell viability after seven days of fermentation was also reported, faster than *Saccharomyces* spp., probably due to a higher lytic susceptibility of this strain [13]. This might explain the lowest cell viability in HS wines and the absence of viable cells in HS fermentation lees observed for *Hanseniaspora* spp.

4.2 Basic enological parameter, residual sugars, and organics acids

In table 20, the results about basic enological parameters (pH, total sulfur dioxide and alcohol), residual sugars and glycerol were reported.

Table 21. Basic enological parameters, residual sugars and glycerol determined in synthetic wines after alcoholic fermentation. Data were means and standard deviations of three replicates. Different letters within the same column marked significant differences, according to ANOVA and Tukey HSD test at $p < 0.05$. As concern sulfur dioxide, values in brackets were referred to the free form.

Fermentation	pH	Alcohol (% v/v)	Total SO_2 (mg/L)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Control (S)	2.88 ± 0.01 b	9.9 ± 0.0 b	14 (6) ± 2 a	0.12 ± 0.00 a	0.15 ± 0.01	2.11 ± 1.21
Sequential (HS)	2.80 ± 0.01 a	9.7 ± 0.0 a	22 (5) ± 1 b	0.13 ± 0.00 b	0.15 ± 0.01	3.15 ± 0.22

In both the conditions, the fermentation process was successfully completed, with few differences between control and sequential fermentation. Control wines showed the highest mean values in terms of pH (2.88) and alcohol content (9.9 % v/v) and lower concentration of residual glucose (0.12 g/L). Even if significant differences were observed, they may be considered negligible from the practical point of view: in fact, after sequential fermentation the pH, alcohol content and residual glucose of the resulting wines were similar to the control, with a mean value of 2.80, 9.7 % and 0.13 g/L, respectively. The highest mean value of total sulfur dioxide was found in HS wines, thus resulting significantly different from S, whereas the amount of sulfur dioxide in free form resulted practically the same (5 and 6 mg/L for HS and S, respectively). Regarding glycerol, even if no statistical differences were observed, the highest mean amount was detected in HS wines, with a concentration of 3.15 g/L, higher than S (2.11 g/L). Sequential fermentation did not modify the profile of organic acids that resulted the same as the control (data not shown); furthermore, for both the fermentations, the content of acetic acid was very low, of 0.23 g/L and 0.25 g/L detected in S and HS, respectively.

Sequential fermentation with *Hanseniaspora* spp. allowed to complete fermentation process and to obtain wines with chemical composition - in term of ethanol production and pH [31], total and volatile acidity and sulfur dioxide [195] comparable to the control. As concerns glycerol, *Hanseniaspora* spp. has showed a good ability to produce such compound under winemaking condition, also depending on the strains [62] and in some cases, in higher amounts compared to *Saccharomyces* spp. [192]. However, when sequential fermentations were performed, the production of glycerol seemed to be enhanced [201], with concentration almost twice higher compared to the control [192,194], thus confirming the results here obtained. In this regard, the higher concentration of such metabolite after sequential fermentation may positively contribute to improve wine sensory perception, since it is involved in mouthfeel, overall intensity flavor [202] and sweetness [203].

4.3 Release of glutathione and cysteine

The amount of antioxidant compounds, glutathione and cysteine (expressed in µmol/L), released after fermentation was reported in figure 28.

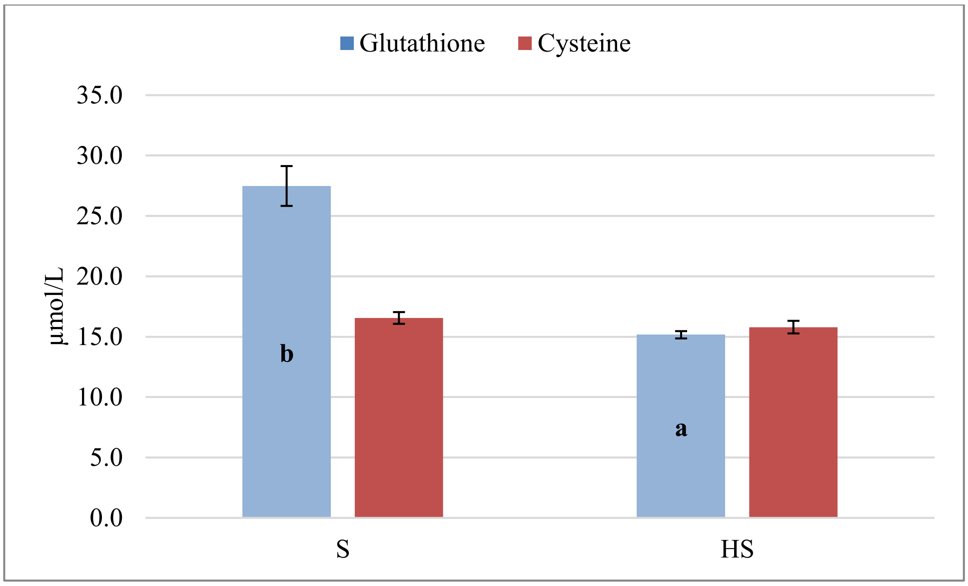


Figure 28. Amount of glutathione and cysteine (µmol/L) released after alcoholic fermentation, in control (S) and sequential fermentation (HS). Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$.

At the end of fermentation, the highest amount of glutathione was detected in control wine, with a content of 27.5 µmol/L, resulting significantly different from sequential fermentation (15.2 µmol/L). Regarding cysteine, no differences were found, and the amount detected was similar, ranging from 15.8 µmol/L (HS) to 16.6 µmol/L (S). These results were in contrast with those previously reported by Binati et al. [33] that observed how after sequential fermentation the amount of glutathione released into wine reached a final concentration up to 10 mg/L, also dependent on the strain used. Moreover, both the yeasts released lower amount of antioxidant molecules under winemaking conditions, conversely to what previously observed (Chapter 2, section 4.3.4).

Non-*Saccharomyces* yeasts are characterized by a different metabolism during winemaking conditions, with different consumption of sugars and amino acids that are dependent on the strain [7,62], type of amino nitrogen, temperature [6] and aeration [9]. Furthermore, Gobert et al. [6] also observed how the different utilization and consumption of nitrogen sources by some non-*Saccharomyces* strains conditioned the amount of amino acids available for *Saccharomyces* spp. sequentially inoculated, and then its metabolism. Similarly, under the winemaking condition here performed, the different composition of the fermentation medium and the different metabolic pathway might explain the lower amount of antioxidant compounds released after alcoholic fermentation, compared to the previous experiment performed (Chapter 2, section 4.3.4). Nevertheless, the results agree with those reported by Lemos Junior et al. [19] during alcoholic fermentation of synthetic must: by using *L. thermotolerans* and *S. bacillaris* strains as starters during single fermentation, the amounts of glutathione produced per mg of cell dry weight was comparable to those detected in *S. cerevisiae*, but a reduction in the accumulation of glutathione was observed when the two non-*Saccharomyces* strains were employed in sequential fermentation.

4.4 Release of riboflavin

The amount of riboflavin (expressed in µg/L) released after alcoholic fermentation was reported in figure 29.

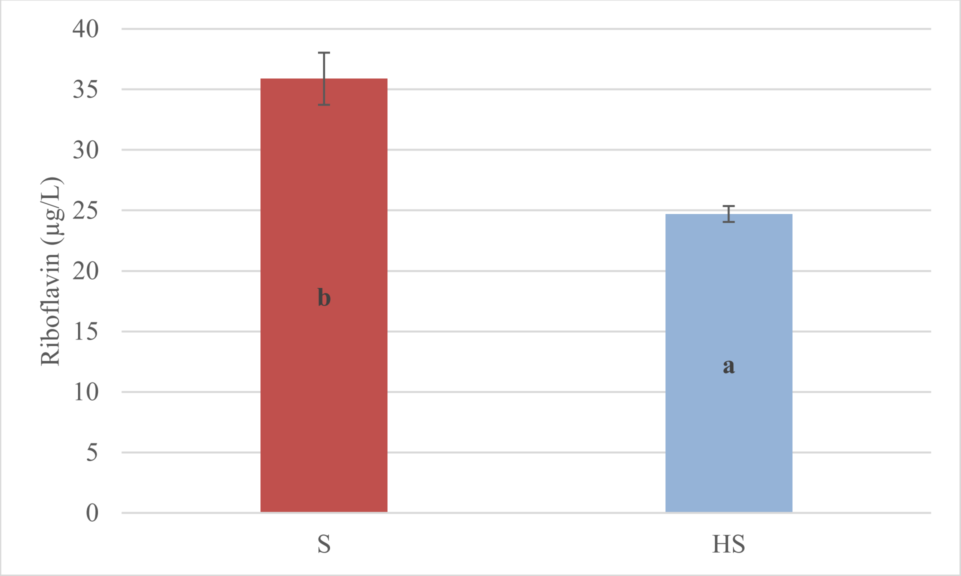


Figure 29. Amount of riboflavin (µg/L) released after alcoholic fermentation, in control (S) and sequential fermentation (HS). Data were means and standard deviations of three replicates. Different letters marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$.

S wines showed the highest content of riboflavin, with a mean value of 36 µg/L, thus resulting statistically different from HS. The amount of this compound increases during fermentation due to yeast metabolism, reaching a final concentration even higher than 100 µg/L in relation to the strains used [70]. Furthermore, as reported before, in the case of white wine and in presence of both riboflavin (at least 50 µg/L) and methionine (at least 1.5mg/L), the former may be involved in light-induced reaction thus determining the appearance of the so-called light-struck defect [74]. In the winemaking conditions here performed, the amount of riboflavin released into the wine might be considered negligible, since it resulted to be lower than the threshold level necessary to trigger the light-induced reactions. However, sequential fermentation would be a good strategy for reducing the amount of riboflavin produced, since it determined the lowest release into the resulting HS wines (25 µg/L).

4.5 Release of mannans and total soluble colloids, particle size distribution and filtration assay

In figure 30, the amount of mannans and total soluble colloids (expressed in mg/L) after alcoholic fermentation was reported.

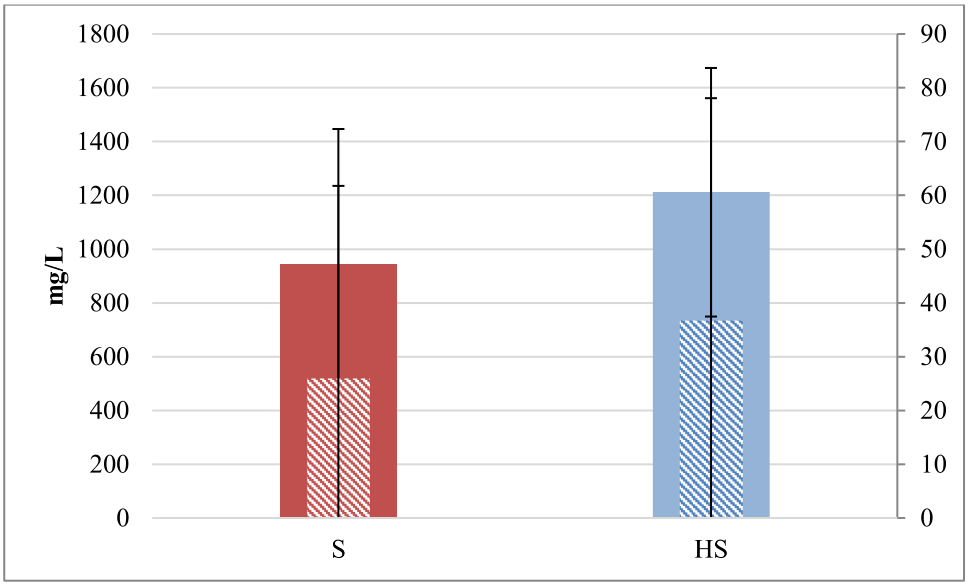


Figure 30. Amount of mannan and total soluble colloids (mg/L) released after alcoholic fermentation, in control (S) and sequential fermentation (HS). Data were means and standard deviations of three replicates. Filled bars: total soluble colloids; striped bars: mannans.

The amounts of both mannans and total soluble colloids released at the end of fermentation showed a similar trend. Even if no statistical differences were observed between the samples, higher mean content of mannans and soluble colloids were released during sequential fermentation, with a mean amount of 37 mg/L and 1211 mg/L respectively, compared to the control (26 mg/L and 944 mg/L for mannans and soluble colloids, respectively).

During single fermentation, the ability of *Hanseniaspora* spp. strains to produce polysaccharides was reported, with release comparable [4] or greater than *Saccharomyces* spp. [198]; furthermore, the sequential inoculum with *Hanseniaspora* spp. and *Saccharomyces* spp. also allowed to obtain wine richer in polysaccharides content at the end of alcoholic fermentation, at least in terms of mean values [31], similarly to the results obtained in the present study.

As concerns particle size distribution, the mean particle diameter of control wines was 1624 nm, resulting statistically different from HS wines (mean diameter of 807 nm), whereas no effect on wine filterability was observed (2.4 mL/min/cm^2 for S and 2.1 mL/min/cm^2 for HS).

4.6 Wine volatile profile

Twenty-five volatile compounds were tentatively identified and listed in Annex 9, whereas the results of the semi-quantitative analysis, with means and standard deviations and the results of the statistical analysis were reported in Annex 10.

The results of PCA carried out on the concentrations (in μg/L) of the volatile compounds detected in the headspace of wines after alcoholic fermentation were reported in figure 31 (a and b).

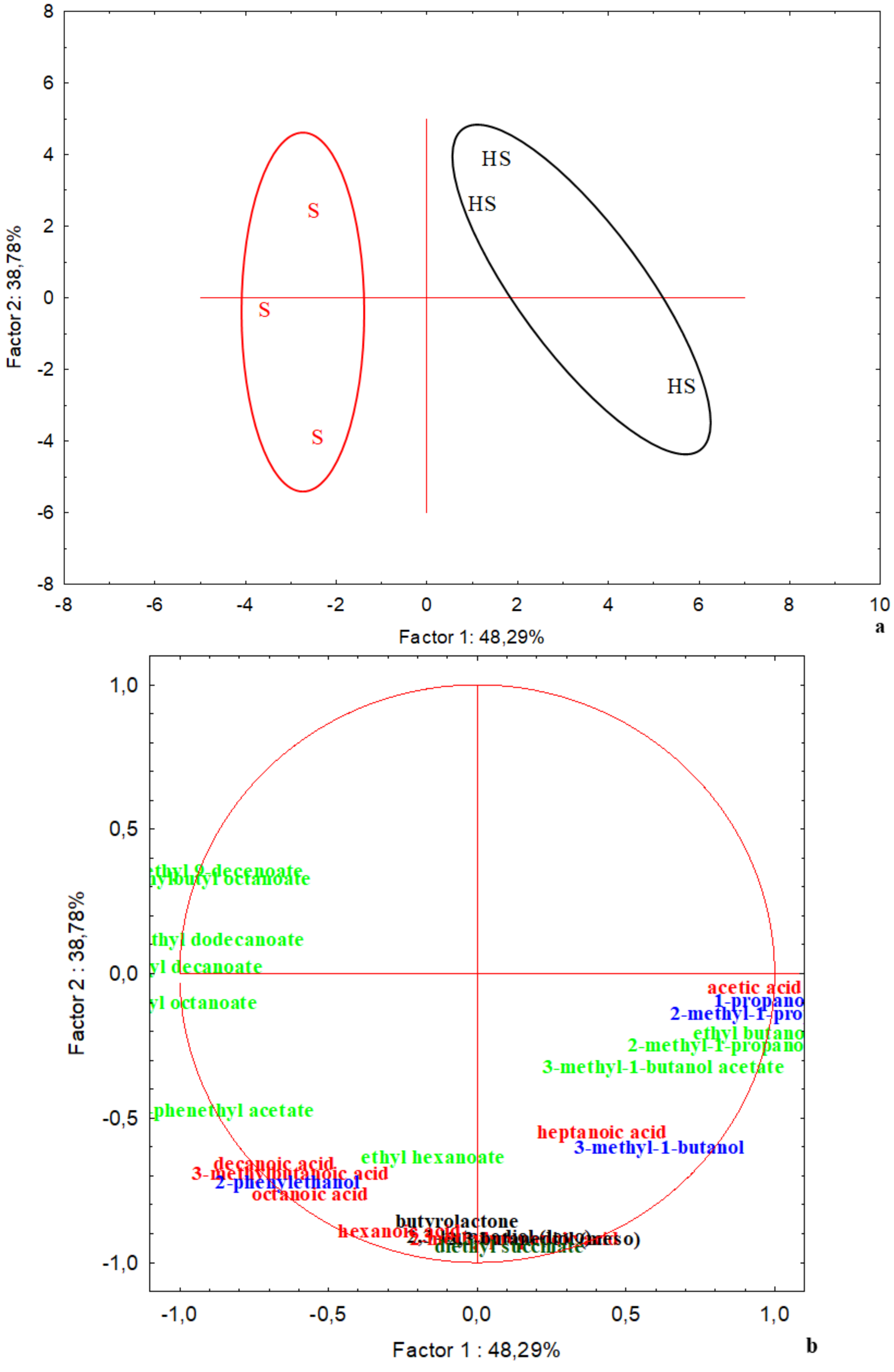


Figure 31. Results of PCA carried out on the concentrations (μg/L) of volatile compounds detected in the wines. Projection of case (samples) (**a**) and variables (volatile compounds) (**b**) on the factor-plan were reported. S: control; HS: sequential fermentation.

The two wine samples were well grouped, with S more characterized by esters and fatty acids, whereas alcohols, some acetate esters and acetic acid mostly characterized HS wines.

In particular, control wines (S) showed the highest mean concentration of ethyl octanoate, ethyl decanoate and 2-phenylethyl acetate, resulting significantly different from HS wines; the latter were instead characterized by tendential higher mean concentrations of 2-methyl-1-propanol acetate, ethyl butanoate and 3-methyl-1-butanol acetate (Annex 10).

It was reported the ability of *Hanseniaspora* spp. to produce higher amounts of esters compared to *Saccharomyces* spp. when separately inoculated, potentially conferring floral and fruity notes and improving wine aroma perception [29,190]. Higher concentration of ethyl esters (*i.e.*, ethyl hexanoate and ethyl octanoate) was found in wines obtained by single fermentation with *Saccharomyces* spp. whereas higher amount of acetate esters was detected in wines obtained after sequential fermentation [193], with differences also depending on grapevine variety [195]. These results confirmed the trend here observed as concern esters, except for 2-phenylethyl acetate that, conversely, mainly characterized S wines. However, in a study carried out by Moreira et al. [196], the authors observed how *Hanseniaspora* spp. strains have different ability to produce esters, with *H. uvarum* lower producer of 2-phenylethyl acetate compared to *H. guilliermondii*; the same authors also observed a further reduced production when sequentially inoculated with *S. cerevisiae*. This evidence, together with the fact that the winemaking conditions here adopted were different from those reported in literature (*i.e.*, fermentation medium, strains, time of inoculum) might justify the lowest amount of 2-phenylethyl acetate detected in HS compared to S.

Higher mean amounts of alcohols were also found in HS wines; on the other hand, 2-phenylethanol was mostly present in S wines, thus resulting statistically different from the former (Annex 10). The use of *Hanseniaspora* spp. in sequential fermentation allowed to obtain wines with higher content of alcohols [194,195]; however, the highest concentration of 2-phenylethanol was detected in Chardonnay wines obtained by single fermentation with commercial *S. cerevisiae* [193], also depending on the strain used [196], thus confirming the trend here observed.

As concern fatty acids, tendential lower concentration of such compounds was detected in HS wines; this could be a positive aspect since they may confer unpleasant odors to the wine, like rancid, oily and fatty notes [158]. On the other hand, the concentration of acetic acid was higher in wines obtained after sequential fermentation, with a mean content of 33.65 µg/L compared to control (9.35 µg/L), even if no significant difference was observed (Annex 10). Lower mean amounts of free fatty acids were found after performing sequential fermentation, compared to the control with only *Saccharomyces* spp. [193,195]; furthermore, higher production of acetic acid by *Hanseniaspora* spp. in comparison to *Saccharomyces* spp. was previously reported [191,192], thus confirming the trend here obtained.

Lastly, during both the fermentations performed minor compounds, *i.e.*, butyrolactone and 2-3-butanediol were also produced - without differences in terms of concentration - as previously observed by other authors [191,193].

5. Conclusion

Sequential fermentation with the selected *Hanseniaspora* spp. strain has not significantly modified the basic chemical composition, with tendential higher mean amount of glycerol compared to the control, potentially improving the mouthfeel and sweetness of the resulting wines. Sequential fermentation is confirmed to be a good strategy for enhancing the amount of mannans and total soluble colloids released, thus enriching wines in compounds positively involved in stability, aroma profile and sensory perception. The use of *Hanseniaspora* spp. as co-starter has also allowed to reduce the amount of riboflavin released, since it determined the lowest content into the resulting HS wines; nevertheless, the amount of glutathione was lower compared to *Saccharomyces* spp. alone. The volatile profile of wines obtained after sequential fermentation may be improved since they showed lower fatty acids content, together with increased concentration of some esters, *i.e.*, 2-methyl-1-propanol acetate, ethyl butanoate and 3-methyl-1-butanol acetate, potentially increasing the aroma complexity of the wines.

Chapter 6

Effect of lees from sequential fermentation (*Hanseniaspora* spp. and *Saccharomyces* spp.) and techniques for inducing autolysis on chemical composition and volatile profile of a synthetic and real wine during aging on lees

1. Introduction

Aging on lees is a common winemaking practice that aims to enrich wine with several compounds released from fermentation lees, *i.e.*, nitrogen compounds, polysaccharides, lipids and antioxidants as result of yeast autolysis [204]. During aging on lees, the release of mannoproteins may positively contribute on protein [30,166] and tartaric stabilization [167]. Interaction between polysaccharides and phenolic compounds may also affect wine color stability and astringency [168,169,205], as well as the interaction with wine aroma compounds [175] or the release of volatile compounds from lees [206] may improve the aroma complexity and sensory perception [164,189]. In this regard, non-*Saccharomyces* yeasts (NSY) may positively contribute to the overall wine quality and stability, since they showed a good ability to release highest amount of polysaccharides compared to *Saccharomyces* spp., both during fermentation [4,13] and wine aging on lees [12,14,189], possibly reducing the conventional period of aging. Interesting results were also obtained by different *Hanseniaspora* spp. strains, that showed a good aptitude to produce polysaccharides [198], both during single [4,13] and sequential fermentation [31]. However, during aging on lees, the amounts of polysaccharides released in model solution seemed to be comparable to *Saccharomyces* spp. [29], or even much lower under real winemaking conditions [31].

Another positive effect of aging on lees is related to the interaction of polysaccharides with phenolic fraction [166] and to the antioxidant properties of lees cell surface [16,151,207] that may reduce wine predisposition to browning and improve protection against oxidation. All these positive effects have been studied on *Saccharomyces* spp.; as concerns the impact of NSY towards wine oxidation, evidence is reported in literature about the increased production of pyruvic acid and acetaldehyde during fermentation and the subsequent formation of more stable pigments, mainly focused on red winemaking [3,15,199]. However, the increasing interest towards NSY have recently led some researchers to investigate their glutathione metabolism [18]; higher amount of reduced glutathione produced during single and sequential fermentation were also observed [19,33], highlighting once again the great potential of the use of NSY in winemaking. However, no evidence is till now carried out about the release of antioxidant compounds and the protection against wine oxidation, as well as the effect on protein and tartaric stability, during aging on lees obtained by sequential fermentation, especially as concerns *Hanseniaspora* spp.

The occurrence of yeast autolysis during aging on lees is a natural process that may last several months; in this regard, besides of the common addition of enzymes with β-glucanase activity, several practices are successfully employed for accelerating yeast autolysis. The amount of mannoproteins released during wine aging after processing fermentation lees by pulsed electric field have been reported [108,109]; ultrasounds treatment seems to enhance the release of polysaccharides and proteins [111], possibly reducing the conventional period of contact with lees [112,113]. Interesting results have been also obtained as concerns the application of high-pressure treatments for extracting cell wall polysaccharides [117], and for inactivating and inducing yeast autolysis for winemaking purposes [114–116].

2. Aim of the work

The aim of the work was to evaluate the aptitude of spontaneous *Hanseniaspora* spp. used as fermentation co-starter for improving wine aging on lees. In this regard, both fermentation lees obtained by single (S) and sequential fermentation (HS) and the respective synthetic wines as obtained in the previous chapter (Chapter 5) were used in the following experimental trial, consisting of two parts.

In the first part, the aim was to evaluate the contribution of lees from sequential fermentation to increase the amount of polysaccharides and, above all, antioxidant compounds in a synthetic wine, also in relation to the treatment performed for inducing autolysis. Lees from single and sequential fermentation were subjected to enzyme addition and high hydrostatic pressure, then added to a synthetic wine for the subsequent aging, together with untreated lees used as control. The chemical composition was evaluated in terms of antioxidant compounds (glutathione and cysteine), soluble colloids and riboflavin, together with the evaluation of volatile profile of the resulted aged wines; the results were reported and discussed in Results - Part I of the present chapter.

In the second part of the experimental plan, the aim was to evaluate - for the first time - the impact of lees from sequential fermentation on the chemical composition, volatile profile and sensory perception of a real wine, also in relation to the treatment performed for inducing autolysis. In this regard, both fermentation lees (S and HS) were subjected to the same treatments (enzyme addition and high hydrostatic pressure, untreated lees) then added to a white wine, together with untreated samples used as reference (without lees addition). The chemical evaluations concerned color evolution and browning potential, release of antioxidant compounds, soluble colloids and riboflavin, protein and tartaric stability, together with wine volatile profile and sensory analysis. The results regarding this part were reported and discussed in Results - Part II of the present chapter.

3. Materials and methods

3.1 Reagents

Ethanol (96 % v/v), mannan from *S. cerevisiae*, trifluoroacetic acid, riboflavin, L-glutathione reduced, L-cysteine, *p*-benzoquinone, 3-mercaptopropionic acid, and acetaldehyde (ACS reagent, ≥99.5%), (+)-catechin hydrate (≥ 98%, HPLC grade) and ethyl heptanoate were from Sigma Aldrich Italy (Milan, Italy). Methanol (HPLC grade) were purchased from VWR Chemicals (Milan, Italy). MilliQ water was produced by MilliQ Advantage A10 apparatus (Merck Millipore, Billerica, MA, USA) and microfiltered at 0.22 µm before use. Commercial enzyme preparation with β-glucanase activity and commercial active dry yeasts (ADY) *S. cerevisiae* were from Enologica Vason S.p.A. (San Pietro in Cariano, VR, Italy).

3.2 Fermentation lees and wines

As reported in Chapter 5, single (S) and sequential fermentation (HS) was also carried out in stainless steel tanks for obtaining both lees and synthetic wine to be used for the present trial of aging, whereas the white wine was a blend of Sauvignon Blanc and Pinot gris (vintage 2021). For the latter, the winemaking process was carried out in the experimental farm Azienda Agraria "A. Servadei" of the University of Udine (Italy); a small quantity of sulfur dioxide (30 mg/L of free sulfur dioxide) was added before starting the fermentation process and the removal of fermentation lees was carried out by decanting; no further treatment (*i.e.*, clarification, protein and tartaric stabilization) was performed. Moreover, before setting the trials, POM-test, total polyphenols index (TPI) and catechins content were determined, as better described below, in order to assess the oxidizability potential of the real wine; because of the occurrence of oxidation phenomena, catechins were not detected, then they were added up to a final concentration of 30 mg/L.

3.3 Treatments for inducing autolysis and aging on lees

The fermentation lees were harvested by decanting and divided in three aliquots for the following lytic treatments. For enzyme-assisted lysis (hereafter referred to as ENZ), a commercial preparation with β-glucanase activity was added at the maximum dose recommended by the supplier (4g/hL) whereas for high hydrostatic pressure (hereafter referred to as HHP), the treatment was performed as previously described in Chapter 3, section 3.3, by applying a pressure of 400 MPa. This latter was chosen as the most suitable treatment for preserving antioxidant compounds (Chapter 3, Part III); untreated lees were also used as control (hereafter referred to as CON). The lees thus obtained were added at 5% (v/v) in 500 mL and 750 mL-dark glass bottles previously filled with synthetic and white wine respectively, manually sealed with crown caps after nitrogen blowing; for aging, wine samples were stored at a temperature of 20°C±1 for three months, placed in horizontal position to allow a higher contact surface between lees and wine and mixed twice a week to simulate *bâtonnage* process. As concerns aging of synthetic wines, lees from S and HS were re-added in their respective wines after alcoholic fermentation and the experimental trial was performed in triplicates; as concerns aging of white wine, the trials were carried out in triplicate (two bottle were used for the chemical analyses and one bottle for sensory evaluation) and wine without lees addition was used as reference (hereafter referred to as RW). For the chemical evaluations, except for aroma profile, glutathione and catechins, wine samples were centrifuged at 3000 rpm for 15 min before the analyses.

3.4 Wine color and browning assay (POM-test), total polyphenols index (TPI) and catechins

Wine color (abs 420 nm and CieLab analysis), browning assay (POM-test) and total polyphenols index (TPI) was evaluated as reported in Chapter 4, section 3.3.

The amounts of catechins in white wine after aging was determined by RP-HPLC analysis, following the method described by Fracassetti et al. [60]. The analysis was performed by using a LC-2010 AHT liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an integrated autosampler and a UV–visible detector set at 280 nm; the separation column was a Zorbax Eclipse Plus C18, 5 μm, 150 x 4.6 mm (Agilent Technologies, Santa Clara, CA, USA), conditioned at 25°C. The eluents were water/trifluoroacetic acid (0.05% v/v) and methanol, the latter was increased from 10% to 35% in 18 minutes during the eluting gradient, at a flow rate of 1 mL/min; the injection volume was 20 μL. The concentrations were calculated in relation to a calibration curve prepared with standard solutions of catechin (0-30 mg/L) and expressed in mg/L.

3.5 Glutathione, cysteine and riboflavin by RP-HPLC

Glutathione content and cysteine were determined on wine samples as reported in Chapter 2, section 3.6, whereas riboflavin content was evaluated as reported in Chapter 2, section 3.7.

3.6 Total soluble colloids (SE-HPLC), particle size distribution (PSD) and filtration assay

Total soluble colloids and mannans, particle size distribution and filtration assay were determined as reported in Chapter 4, section 3.4. Regarding soluble colloids, the estimation of molecular weight was determined by comparing the retention time of detected peaks with those of external standard of PEG/PEO (InfinityLab EasiVial PEG/PEO, Agilent Technologies, Santa Clara, CA, USA) with known molecular weight (range 106 Da – 1.5 x 10^6 Da)

3.7 Protein and tartaric stability test

Protein stability of aged white wine was evaluated by using heat stability test, as reported by Comuzzo et al. [208]; briefly, wine samples were filtered on 0.45 µm pore size cellulose acetate membranes, heated at 80°C for 6 hours, then cooled at 0/+4°C for 16 hours. The turbidity measurements were carried out by using a AL250T-IR turbidimeter (Acqualytic, Dortmund, Germany) and the results were expressed as nephelometric turbidity unit (NTU). According to the test, wines were considered unstable if the differences in turbidity before and after heating (Δ_{TurbH}) was higher than 2.0 NTU.

Tartaric stability test was carried out by an external facility (Enologica Vason S.p.A., San Pietro in Cariano, VR, Italy) by mini-contact test and by cold test. The former is based on the measurement of the drop in electrical conductivity (expressed as ΔµS/cm) of chilled wine supersaturated with KHT; according to the test, the greater the drop in conductivity, the higher the tartaric instability of the wine. Cold test is a qualitative evaluation, based on the precipitation of potassium bitartrate after a period of cold storage (-4 °C for 6 days).

3.8 Wine volatile profile

The volatile profile of wines after aging on lees was determined as previously described in Chapter 4, section 3.5.

3.9 Sensory analysis

A blind sensory analysis was carried out on aged white wines by quantitative descriptive analysis (free-choice profile). The samples were labeled with three-digit numerical codes and submitted (according to a randomized service order) to the evaluation of a panel composed of twelve selected judges (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, giving them a score from 0 (low) to 10 (high), in relation to the intensity. Attributes were referred to: color (yellow hue and browning), smell (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 overall pleasantness. The sensory test was carried out in the sensory analysis laboratory (LABAS) of the University of Udine (Italy).

3.10 Statistical analysis

Means and standard deviations, analysis of variance, factorial analysis and principal component analysis (PCA) were carried out as reported in Chapter 3, section 3.12.

Two-ways ANOVA and Tukey HSD test was also carried out and the significance was fixed at $p < 0.05$. Relationships among parameters were investigated using Pearson correlation coefficient and the statistical significance was fixed at $p < 0.05$. ANOVA and least significant difference (LSD Fisher test, $p < 0.05$) was carried out for elaborating data concerned sensory analysis.

4. Results and discussion

***<u>Part I</u>* Chemical composition and volatile profile of synthetic wines after aging on lees**

In this part of the experimental trial, the aim was to assess the potential contribution of *Hanseniaspora* spp. used in sequential fermentation, on improving wine chemical composition and volatile profile during aging, also in relation to the treatment performed for inducing autolysis. Synthetic wines and lees obtained after fermentation in stainless steel tanks were used. Lees obtained by single (S) and sequential fermentation (HS) were subjected to enzyme addition (ENZ) and high hydrostatic pressure (HHP), together with untreated lees used as control (CON), then re-added to the respective synthetic wines (S and HS wines). The aging period lasted three months.

The results were first discussed in comparison to the initial composition of the wine, in order to evaluate the release of compounds of enological interest and the modification of aroma profile during aging, separately for S and HS.

Furthermore, the comparison between S and HS wines after aging were discussed, in order to better evaluate the effect of both the yeasts used in fermentation and the treatment performed for inducing autolysis on the chemical composition and volatile profile of the resulting wines. Means and standard deviations were calculated, and the results of one-way ANOVA were reported in table 21; in addition, the effect of lees typology and lees treatments was analyzed by two-ways ANOVA and the results were reported in table 21. All the chemical parameters evaluated and the volatile profile of wines after aging were discussed in detailed in their respective, following sections.

Table 22. Chemical composition of wines aged on S and HS lees. Data were means and standard deviations (SD) of three replicates and the results of one-way ANOVA were reported. The effect of lees typology (S and HS) and lees treatment (CON, ENZ, HHP) was analyzed by two-ways and data were the means calculated for group (lees typology and lees treatment). Different letters within the same row marked significant differences, according to ANOVA and Tukey HSD test at $p < 0.05$. S: S: single fermentation; HS: 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.

	one-way ANOVA						two-ways ANOVA						
							Lees Typology			Lees treatment			
	S_CON	S_ENZ	S_HHP	HS_CON	HS_ENZ	HS_HHP	S	HS	Sig.	CON	ENZ	HHP	Sig.
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean	Mean		Mean	Mean	Mean	
Cysteine (μmol/L)	22.40 ± 0.31 b	22.45 ± 0.13 b	15.74 ± 0.05 a	24.43 ± 0.76 b	23.78 ± 2.65 b	17.61 ± 0.09 a	20.20 a	21.94 b	**	23.41 b	23.11 b	16.67 a	***
Glutathione (μmol/L)	22.95 ± 0.23 d	21.36 ± 0.40 cd	17.59 ± 0.24 a	20.57 ± 0.74 bc	18.81 ± 1.84 ab	20.11 ± 0.14 bc	20.63	19.83	ns	21.76 b	20.09 a	18.85 a	***
Riboflavin (μg/L)	49 ± 3	50 ± 1	45 ± 1	48 ± 3	43 ± 8	53 ± 3	48	48	ns	49	47	49	ns
Total polysaccarides (mg/L)	3108 ± 181	3416 ± 1037	2395 ± 439	3747 ± 224	3764 ± 1147	2799 ± 72	2973	3437	ns	3428	3590	2597	ns
PSD (mean)	1624 ± 172 b	1649 ± 211 b	1734 ± 174 b	980 ± 19 a	918 ± 81 ab	840 ± 104 a	1669 b	913 a	***	1302	1284	1287	ns
Filtration flow (mL min^{-1} cm^{-2})	0.60 ± 0.04 ab	0.74 ± 0.02 c	0.60 ± 0.05 ab	0.51 ± 0.05 a	0.69 ± 0.05 bc	0.64 ± 0.03 bc	0.65	0.61	ns	0.56 a	0.71 c	0.62 b	***

ns: not significant; * $p < 0.01$ ** $p < 0.001$

4.1 Release of glutathione and cysteine

The amounts of glutathione and cysteine (µmol/L) released after aging in S and HS wines, in comparison to the initial concentration (at the end of alcoholic fermentation, T=0) were reported in figure 32 (a and b, respectively)

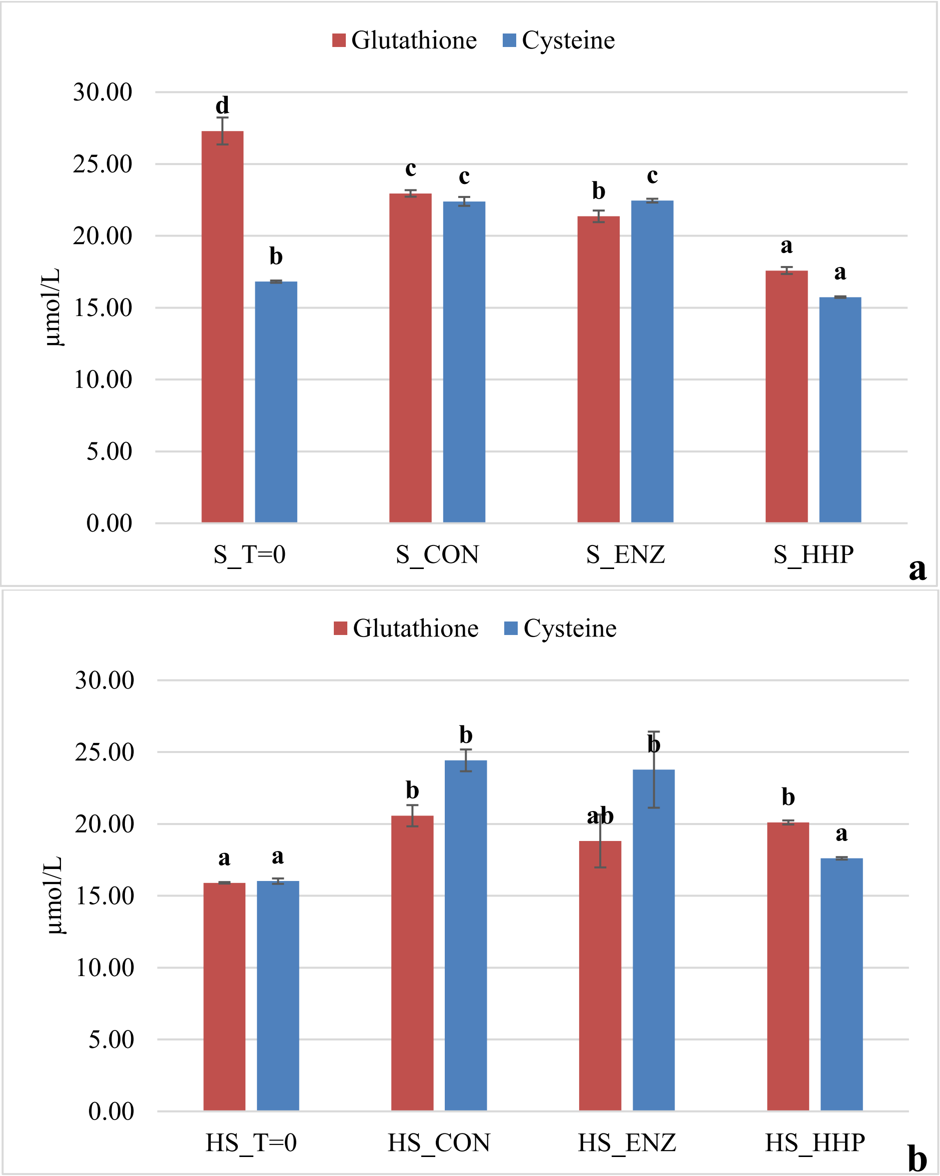


Figure 32. Differences between the initial and final concentrations of glutathione and cysteine (µmol/L) in S (**a**) and HS wines (**b**), in comparison to their respective starting wine (T=0). Data were means and standard deviation of three replicates. Different letters marked significant differences, according to ANOVA and Tukey HSD test at $p < 0.05$. S: single fermentation; HS: sequential fermentation; T=0; wines after alcoholic 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.

Starting from S, a decrease in the amount of glutathione during aging was observed; in fact, its starting concentration was about of 27.3 μmol/L, reaching the lowest mean value in S_HHP (15.74 μmol/L), followed by S_ENZ (21.36 μmol/L) and S_CON (22.95 μmol/L). On the other hand, the cysteine content slightly increased in S_CON and in S_ENZ compared to S_T=0 (+ 5.59 μmol/L and 5.65 μmol/L, respectively), whereas S_HHP showed a reduced amount (-1.07 μmol/L compared to T=0). Conversely, the content of glutathione tended to increase in HS wines during aging; the highest concentrations were detected in HS_CON and HS_HHP (20.57 μmol/L and 20.11 μmol/L, respectively), about 4 μmol/L more than HS_T=0 (15.9 μmol/L), whereas S_ENZ showed a mean content of 18.81 μmol/L; similar trend was also observed for cysteine, that tendentially increased in all the HS wines during aging. Even if after alcoholic fermentation the amount of these antioxidant molecules - especially glutathione - was higher in S wines, it is interesting to note, at least in term of mean values, how the use of lees obtained by sequential fermentation might increase the amount of glutathione released during the following period of aging, potentially improving the protection against oxidation in real winemaking condition.

By considering the effect of the factors (lees typology and treatment, table 21), the different lees used (S and HS) only affected the amount of cysteine, with highest values in HS, whereas the treatment was the most impacting factor on both the amounts of cysteine and glutathione, with HHP determining the lowest content. By considering all the samples, both lees treated by CON and ENZ showed the highest content of cysteine, thus resulting significantly different from S_HHP and HS_HHP (15.74 and 17.61 μmol/L, respectively); however, the highest concentrations of cysteine were detected in HS_CON and HS_ENZ (24.43 and 23.78 μmol/L, respectively), at least in terms of mean values. Regarding glutathione, the highest concentration was found in S_CON (22.95 μmol/L) that resulted significantly different from all the other samples, except from S_ENZ, whereas the lowest value was found in S_HHP (17.59 µmol/L), resulting significantly different from all the others, except from HS_ENZ. However, the amounts of glutathione detected in HS_CON and HS_HHP may not be considered negligible (about 20 µmol/L), even if they resulted statistically different from S_CON.

The non-negligible production of intracellular glutathione by non-*Saccharomyces* yeasts was observed [18], with amounts comparable or even higher than *Saccharomyces* spp. [19]. On the other hand, the higher activity of enzymes involved in the synthesis of glutathione showed by *Hanseniaspora* spp. strains [18,75] might have determined higher production of intracellular glutathione during fermentation, then released during aging when autolytic process occurred; this might explain the slight increase of glutathione observed in all HS wines during aging.

Regarding the treatment, the slow release of intracellular components occurred during natural autolysis (CON) might explain the high content of both cysteine and glutathione in wines added with untreated lees; on the other hand, HHP seemed to determine the lowest release of such antioxidant molecules. The main effect of high-pressure treatment seemed to be mostly related to cell wall breakage [144] and the inactivation of endogenous enzymes at high pressure might also occur [118]; this might explain the lower release of antioxidant compounds from lees treated by HHP compared to CON and also to ENZ. However, it should be considered that HHP allowed to obtain the highest insoluble fraction and the highest content of reducing proteins linked to cell walls, as observed for yeast derivatives production (Chapter 3, part III), thus potentially contributing to protect wine against oxidation under real winemaking condition. However, even if some statistical differences emerged, the final concentration of antioxidant compounds after HHP treatment was basically comparable to those detected in CON and ENZ, especially in the case of HS wines.

4.2 Release of riboflavin

The amount of riboflavin (µg/L) released after aging in both S and HS wines, in comparison to the initial concentration (at the end of alcoholic fermentation, T=0) was reported in figure 33.

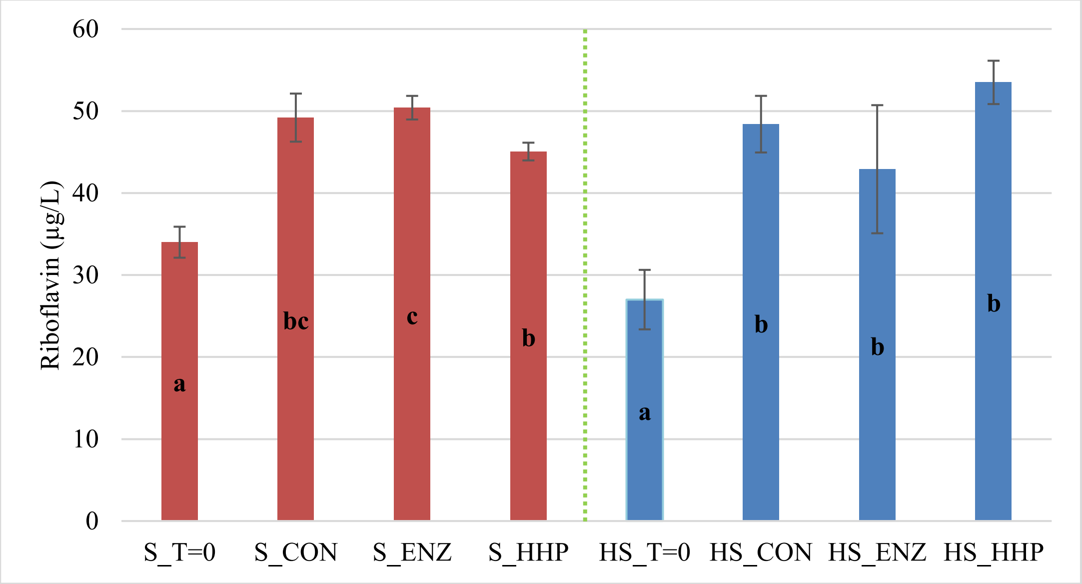


Figure 33. Differences between the initial and final concentrations of riboflavin (µg/L) in both S and HS wines, in comparison to their respective starting wine (S_T=0 and HS_T=0, respectively). Data were means and standard deviation of three replicates. Different letters marked significant differences, according to ANOVA and Tukey HSD test at $p < 0.05$. The statistical analysis was carried out separately for the two types of fermentation (S and HS). S: single fermentation; HS: sequential fermentation; T=0; wines after alcoholic 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.

During wine aging, as the result of yeast autolysis and the subsequent release of intracellular components, the amount of riboflavin tended to increase in both the wines, S and HS, both resulting significantly different compared to the respective starting wines (T=0). Furthermore, this increase seemed to be dependent neither on the lees typology nor the treatment performed. This was confirmed by looking at the results reported in table 21, in which no effect of the factors (lees and treatment) was observed, as well as no statistical differences emerged among the samples. In general, the concentration of such compound was quite similar among the samples, ranging from 43 µg/L in HS_ENZ to 53 (µg/L) in HS_HHP. The potential appearance of light-struck defect may occur since the concentration was closed to the threshold limit to trigger the redox reaction that involves riboflavin [74]; however, the sequential fermentation with this strain of *Hanseniaspora* spp. did not significantly affect the amount of riboflavin released during wine aging on lees.

4.3 Release of total soluble colloids, particle size distribution and filtration assay

The amount of total soluble colloids (mg/L) released after aging in both S and HS wines, in comparison to the initial concentration (at the end of alcoholic fermentation, T=0) was reported in figure 34.

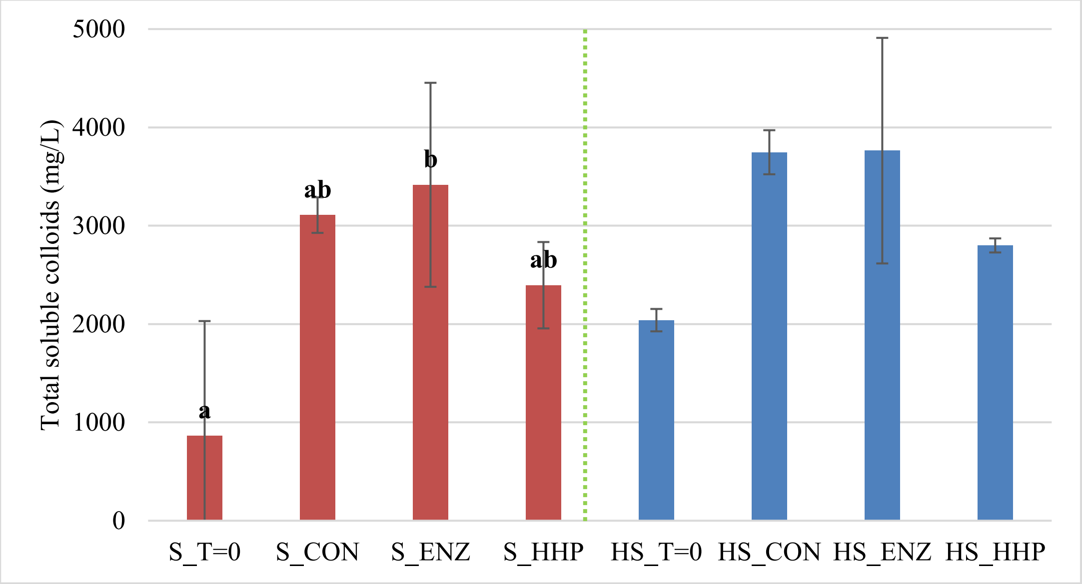


Figure 34. Differences between the initial and final concentrations of total soluble colloids (mg/L) in both S and HS wines, in comparison to their respective starting wine (S_T=0 and HS_T=0, respectively). Data were means and standard deviation of three replicates. Different letters marked significant differences, according to ANOVA and Tukey HSD test at $p < 0.05$. The statistical analysis was carried out separately for the two types of fermentation (S and HS). S: single fermentation; HS: sequential fermentation; T=0; wines after alcoholic 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.

A considerable release of soluble colloids was observed in all the wines after the period of aging. Starting from S, a significant increase was observed for S_ENZ - up to 3416 mg/L - compared to the starting concentration (862 mg/L); however, even if no significant differences emerged, the content of soluble colloids detected in wines added with untreated lees (3108 mg/L) and lees processed by HHP (2395 mg/L) was higher compared to the starting wine.

In the case of sequential fermentation, an increase in the amount of such compounds was also observed, even if no statistical differences between aged wines and wine after alcoholic fermentation was observed (*p* value = 0.052). However, the concentration of soluble colloids increased up to 3747 mg/L and 3764 mg/L in HS_CON and HS_ENZ, respectively; HHP determined the lowest release of such compounds during aging, with a mean value of 2799 mg/L, compared to the starting concentration (2040 mg/L). By considering the results reported in table 21, the release of such compounds was affected neither by the lees typology nor by the treatment, as well as no differences among the samples were observed. However, it would be said that after sequential fermentation and aging, HS wines were characterized by the highest concentration of soluble colloids compared to S wines, at least in terms of mean values, positively contributing to enrich wine in compounds involved in stability, and modulation of aroma profile and sensory perception. The results here obtained partially agreed with those reported in literature: by simulating aging on lees in model solution, the release of total polysaccharides by *Hanseniaspora* spp. was comparable to that detected for *Saccharomyces* spp. [29]. On the other hand, del Fresno et al. [31] observed that higher release of polysaccharides by *Hanseniaspora* spp. especially occurred at the end of sequential fermentation, whereas a reduced amount of such compounds was detected after wine aging on lees, compared to *Saccharomyces* spp.; the authors also found that polysaccharides released by *Hanseniapora* spp. mainly consisted of high molecular weight fraction, leading them to hypothesize a positive contribution on improving aroma profile and sensory perception.

Conversely, in the present study, an increase in total soluble colloids was observed after aging on HS lees, in some cases with mean concentrations higher than S*;* furthermore, high molecular weight fractions (higher than 500 kDa) were not detected, whereas an increase in lower molecular weight fractions (between 4 and 30 kDa) was observed after aging on lees. In this case, the lack of the peaks corresponding to soluble colloids, *i.e.*, mannans, higher than 500 kDa might be explained by the possible hydrolysis of higher molecular weight polysaccharides observed by other authors during aging [179].

In figure 35, the mean particles diameter (nm) in both S and HS aged wines, in comparison to the respective starting wines (at the end of alcoholic fermentation, T=0) was reported.

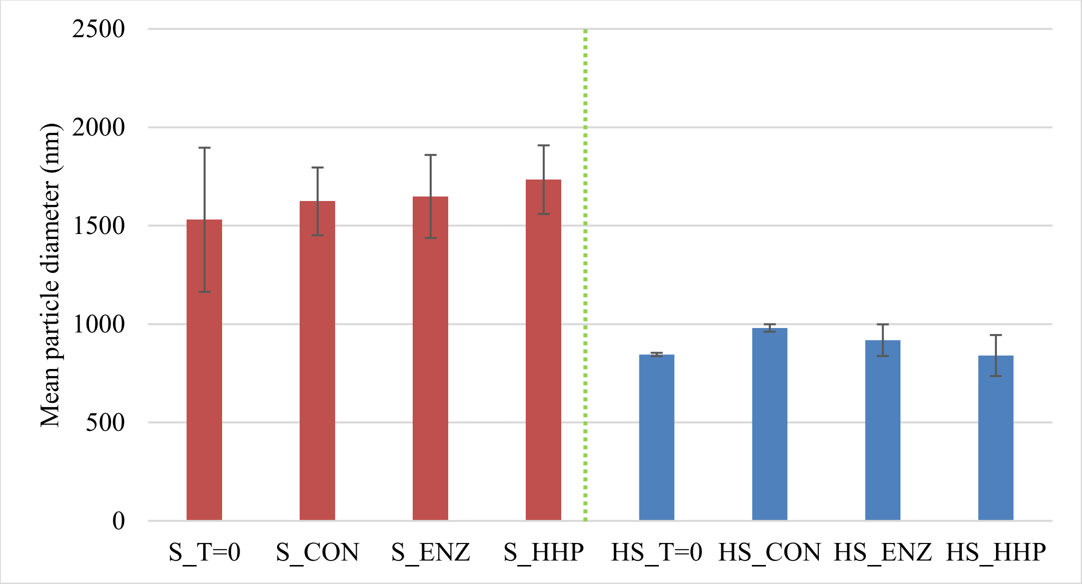


Figure 35. Mean particle diameter (nm) of wines after aging in comparison to the respective wine after alcoholic fermentation (T=0). Data were means and standard deviation of three replicates. S: single fermentation; HS: sequential fermentation; T=0; wines after alcoholic fermentation; CON: wines added with untreated lees; ENZ: wines added with lees treated by enzyme addition; HHP: wines added with lees treated by high hydrostatic pressure.

In both aged wines, no differences were observed in the mean particles size compared to the respective wines at the end of alcoholic fermentation, with mean values ranging from 1530 nm to 1734 nm for S_T=0 and S_HHP, respectively, and from 840 nm to 980 nm for HS_HHP and HS_ENZ, respectively. Looking at the results reported in table 21, lees typology (S or HS) affected the mean particles diameter, with the highest values detected in S compared to HS aged wines.

Lastly, as could be expected, the addition of lees determined a decrease in filtration flow rate when compared to their respective wines (data not shown). After fermentation, S wines showed a value of 2.60 mL/min/cm^2, whereas after aging a relevant (but not significant) decrease was observed, with values ranging from 0.60 mL/min/cm^2 (S_CON and S_HHP) to 0.74 mL/min/cm^2 (S_ENZ). Similarly, HS aged wines showed a mean value of 0.69, 0.64 and 0.51 mL/min/cm^2, all resulting statistically different from wine at the end of alcoholic fermentation

($2.48 mL/min/cm^2$). As reported in table 21, the treatment was the most impacting factor on filtration flow rate, with natural autolysis (CON) determining the highest reduction, followed by HHP and ENZ; the release of soluble colloids might have impacted on wine filterability, being potentially confirmed by the negative correlation found between the amount of total soluble colloids and filtration flow after aging ($r = - 0.66$). However, the addition of lees - both untreated and treated - determined a reduced filtration flow at the end of aging [187], thus confirming the results here obtained.

4.4 Wine volatile profile

Thirty-nine volatile compounds were tentatively identified in aged wines and listed in Annex 9. The results of the semi-quantitative analysis, with means and standard deviations, and the results of one-way ANOVA were reported in Annex 11; in addition, the effect of lees typology and lees treatments on volatile compounds was analyzed by two-ways ANOVA and the results were reported in Annex 11.

In order to understand the contribution of S and HS lees on wine volatile profile, the comparison between aroma profile of aged wines and the respective starting wine (at the end of alcoholic fermentation; T=0) was first discussed; the results of PCA carried out on the concentration (in μg/L) of volatile compounds detected in the headspace of wines were reported in figure 36 (**a** and **b**).

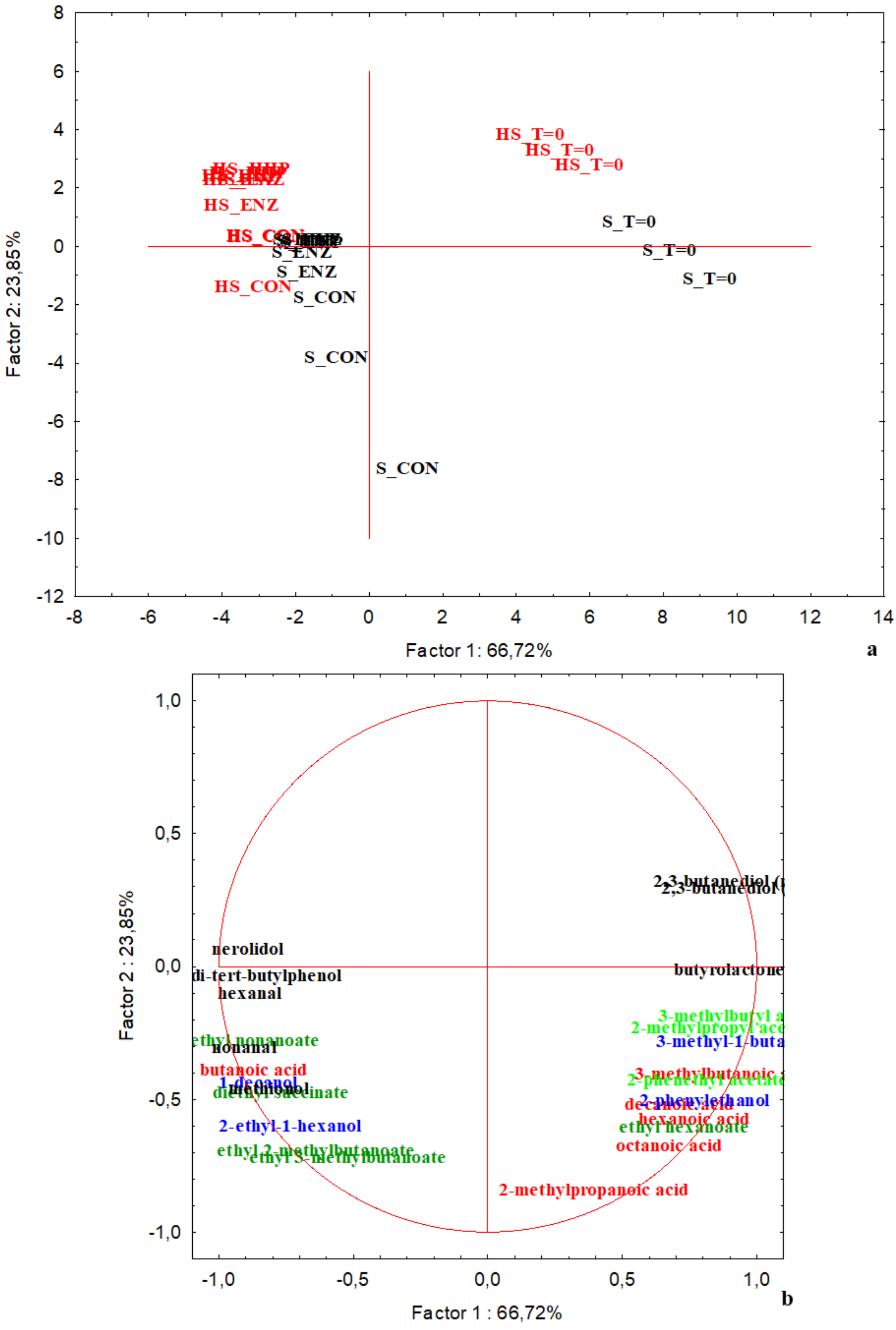


Figure 36 Results of PCA carried out on the concentrations (µg/L) of volatile compounds detected in aged synthetic wines in comparison to the respective starting wines. Projection of case (samples) (**a**) and variables (volatile compounds) (**b**) on the factor-plan were reported. Factor Loadings (FL) were calculated by Factorial Analysis, and the most relevant variables were selected for marked FL > 0.7. S: single fermentation; HS: sequential fermentation; T=0; wines after alcoholic 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.

As could be expected, the addition of lees impacted on the volatile profile after aging, both for S and HS wines, in comparison to their respective starting wines; a decrease in the amount of acetate esters and almost all the acids, 2-phenylethanol and 3-methyl-1-butanol was observed, whereas ethyl esters, 1-decanol and 2-ethyl-1-hexanol and other compounds (i.e., aldehydes, phenols, methionol and nerolidol) tended to increase after aging.

In order to understand if the release of soluble colloids during aging might have impacted on wine volatile profile, the correlations were calculated and reported in table 22. The volatile compounds considered were the most significant variables selected after calculating factor loadings, as reported in the previous PCA (figure 36).

Table 23. Correlation between soluble colloids and the aroma compounds of wines. Marked correlations were significant at $p < 0.01$ (*) and at $p < 0.001$ (**).

	Soluble colloids	
2-methylpropanoic acid	-0.18	
butanoic acid	0.73	**
3-methylbutanoic acid	-0.66	**
hexanoic acid	-0.63	**
octanoic acid	-0.55	*
decanoic acid	-0.62	*
2-methylpropyl acetate	-0.55	*
3-methylbutyl acetate	-0.71	**
2-phenethyl acetate	-0.71	**
ethyl 2-methylbutanoate	0.60	*
ethyl 3-methylbutanoate	0.53	*
ethyl hexanoate	-0.53	*
ethyl nonanoate	0.64	**
diethyl succinate	0.65	**
3-methyl-1-butanol	-0.62	*
2-ethyl-1-hexanol	0.64	**
1-decanol	0.71	**
2-phenylethanol	-0.67	**
butyrolactone	-0.62	*
2,3-butanediol (levo)	-0.74	**
2,3-butanediol (meso)	-0.71	**

Negative correlations were found between the concentrations of soluble colloids and all the acetate esters detected, almost all fatty acids, 2-phenylethanol and 3-methyl-1-butanol, butyrolactone and diols, being these latter not detected in all the aged wines; on the other hand, almost all ethyl esters (expect ethyl hexanoate), 2-ethyl-1-hexanol and 1-decanol were positively correlated with the amount of soluble colloids present into the wines, thus confirming what previously resulted by the PCA (figure 36).

Mannoproteins and polysaccharides released during aging were reported to interact with wine aroma compounds, thus modifying the volatile profile and the sensory perception [166,175]. Retention of isoamyl acetate and ethyl hexanoate in presence of polysaccharide-protein complexes was previously observed by Dufour et Bayonove [174], as well as the addition of yeast derivatives seemed to retain octanoic acid and 2-phenylethanol in model solution, also in relation to pH and presence of other volatile compounds [176]. This evidence supported the results obtained in the present study, thus justifying the tendential lower concentration of such compounds after aging. During wine aging on lees, tendential increase of ethyl esters and alcohols was reported, also depending on the type of strains used [189]; similarly, the addition of yeast derivatives and the consequent release of cell wall polysaccharides seemed to increase the volatility of the abovementioned compounds [165], thus further confirming the tendential higher concentration of such compounds in aged wines. As concern aldehydes, these compounds are produced by yeast during fermentation as intermediate of higher alcohols metabolism [158] or by oxidation phenomena [209], thus potentially explaining the presence of such compounds after aging. The trend observed for methionol was in line with those reported by Medina et al. [193], with the highest concentration detected in wines obtained by single fermentation with *Saccharomyces* spp. compared to sequential fermentation (*Hanseniaspora* spp. and *Saccharomyces* spp.).

By considering these results, wine volatile profile mainly changed in relation to the addition of lees, giving similar aroma profile at the end of aging. Thus, to better understand the impact of lees typology and treatment on the volatile profile, the comparison among the aged wines was reported in figure 37; as previously written, the results about the effect of the two factors (lees typology and lees treatments) on the concentrations of volatile compounds, and the statistical differences among the samples were reported in Annex 11.

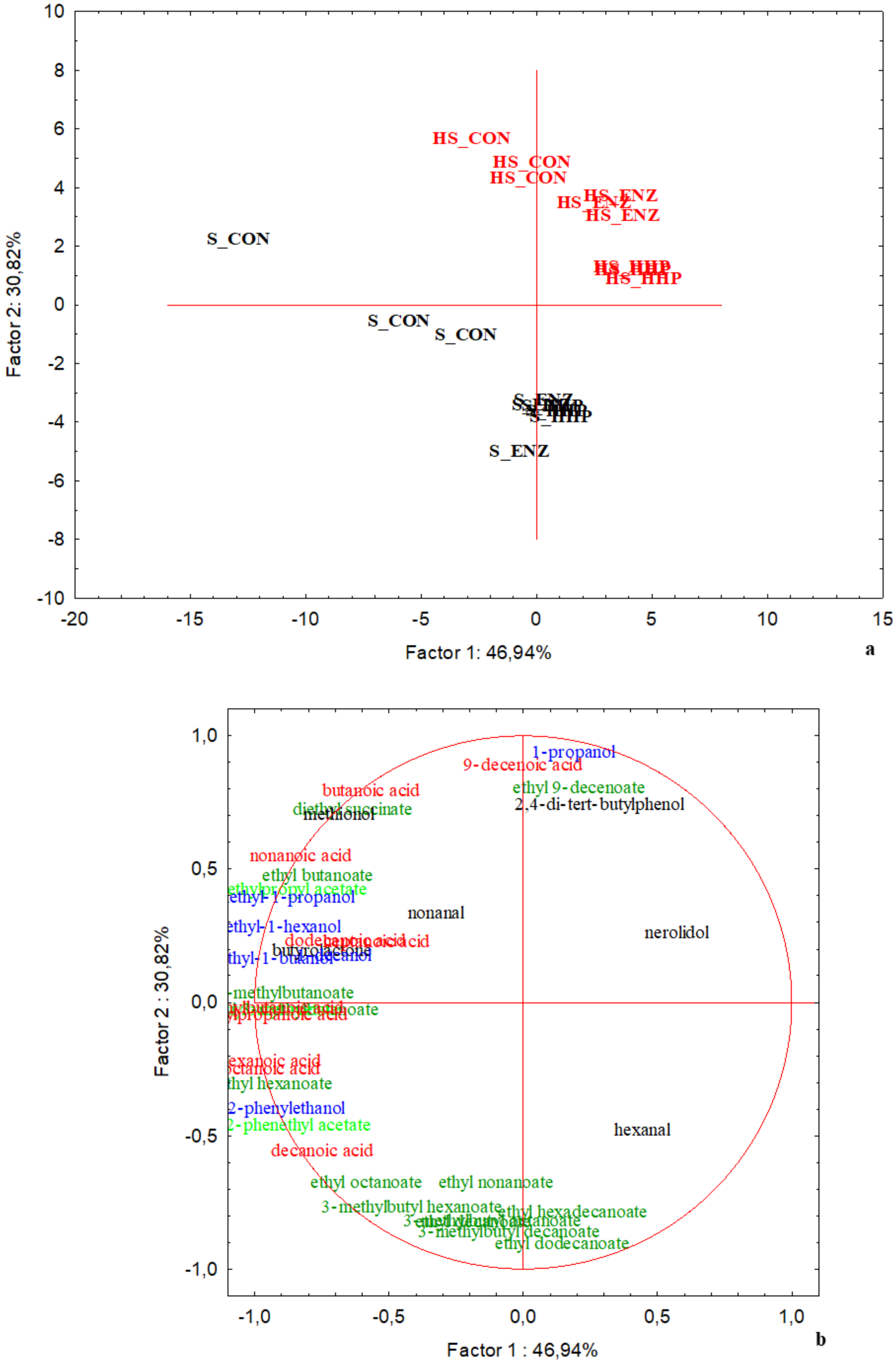


Figure 37. Results of PCA carried out on the chemical compounds detected in synthetic wines after three months of aging. Projection of case (samples) (**a**) and variables (volatile compounds) (**b**) on the factor-plan were reported. S: single fermentation; HS: sequential fermentation; CON: wines added with untreated lees; ENZ: wines added with lees treated by enzyme addition; HHP: wines added with lees treated by high hydrostatic pressure.

By looking at the results of PCA, it would be said that lees typology mostly affected the volatile profile of aged wines, with S wines the most characterized. However, also the treatment performed for inducing autolysis affected the aroma profile: wines aged on untreated lees were more characterized in term of alcohols, free fatty acids and esters (both ethyl and acetate esters); on the other hand, ENZ and HHP determined the less intense and complex volatile profile in the resulting wines, with non-negligible concentrations of ethyl esters; this trend was confirmed by looking at the results obtained by the statistical analysis (Annex 11). However, it is interesting to note how HS wines were characterized by higher mean concentration of nerolidol, also depending on the treatment performed (tendentially higher in ENZ and HHP compared to CON) and lower concentration of fatty acids compared to S wines.

Yeasts are able to produce some terpenic molecules [210], with further increase during aging [211]; moreover, the ability of *Hanseniaspora* spp. to increase the amounts of terpenes during sequential fermentation [31] together with lower release of fatty acids was also observed [193], thus explaining the trend here observed. Higher concentration of nerolidol and lower amount of fatty acids might positively impact on wine aroma profile since the former is described by floral to woody [212], whereas the latter by sour, pungent and cheese-like notes [147].

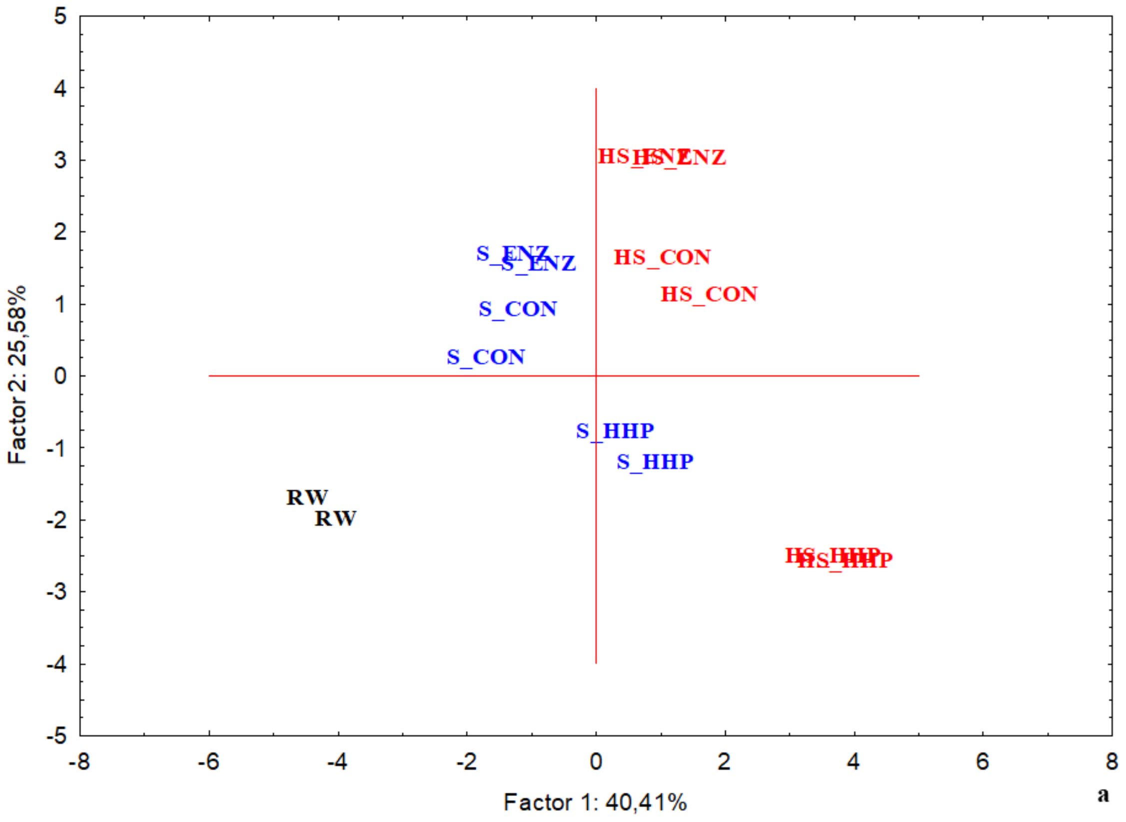
By considering the treatments, wine added with lees treated by HHP and ENZ showed the less complex volatile profile compared to CON; however, certain amounts of ethyl esters were detected. The use of both ENZ and HHP for inducing yeast autolysis resulted in relevant content of insoluble solids (Chapter 3, part III), as well as the addition of HHP derivatives determined low odor impact on the resulting added wine (Chapter 4), thus possibly explaining the trend here observed. However, the retention of compounds that may lead to the formation of *off-flavors*, *e.g.*, acids [147] and methionol [213] might be a positive aspect and, in this regard, HHP seemed to be interesting. By considering all these results, even if wines obtained after aging on HS lees showed the poorer volatile profile compared to S, the use of *Hanseniaspora* spp. as fermentation co-starters together with the application of HHP for processing lees might be a good strategy for improving the aroma profile and consequentially the sensory perception of the resulting wines, especially as concerning the reduction of fatty acids concentration, and the slight release of terpenic molecules.

Part II ***Chemical composition, volatile profile, and sensory analysis of white wines after aging on lees***

In this part of the experimental trial, the aim was to assess the potential contribution of lees from sequential fermentation on improving wine chemical composition and volatile profile during aging under real winemaking conditions, also in relation to the treatment performed for inducing autolysis. A white wine was added with lees obtained by single (S) and sequential fermentation (HS), previously subjected to enzyme addition (ENZ) and high hydrostatic pressure treatment (HHP), together with untreated lees (CON). Untreated wine (without lees addition) was also used as reference (hereafter referred to as RW, reference wine). The aging period lasted three months.

The chemical composition of aged wines was evaluated in terms of color evolution and browning potential, release of antioxidant compounds, protein and tartaric stabilization, together with the release of soluble colloids and mannans, wine volatile profile and sensory analysis.

The results of PCA carried out on all the chemical parameters evaluated were reported in figure 38, whereas means and standard deviations and the results of the statistical analysis were reported in table 23. The results about the chemical composition, volatile profile and sensory analysis of wines after aging were discussed in detail in their respective, following sections.



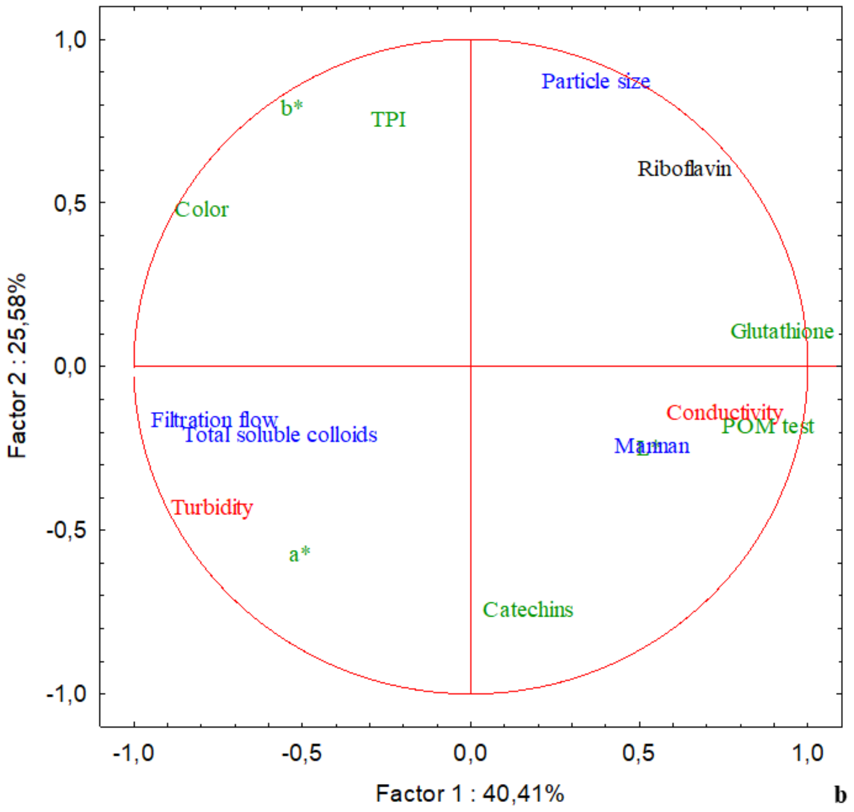


Figure 38. Results of PCA carried out on the chemical parameters evaluated in wines after three months of aging. Projection of case (samples) (**a**) and variables (volatile compounds) (**b**) on the factor-plan were reported. RW: reference wine (without lees addition); S: single fermentation; HS: sequential 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.

Table 24. Chemical composition of white wines after three months of aging on lees. Data were means and standard deviations of two replicates. Different letters within the same column marked significant differences, according to ANOVA and Tukey HSD test at $p < 0.05$. 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.

Sample code	Color (420 nm)	L*	a*	b*	POM test (%)	TPI (280 nm)	Catechins (mg/L)	Glutathione (μmol/L)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
RW	0.11 ± 0.00 d	97.68 ± 0.81	-0.56 ± 0.04 c	6.01 ± 0.19 bc	18 ± 2 a	7.0 ± 0.2 a	18.6 ± 0.0	11.8 ± 0.0 a
S_CON	0.09 ± 0.00 bc	98.07 ± 0.09	-0.64 ± 0.02 abc	5.90 ± 0.07 b	41 ± 4 b	9.1 ± 0.2 c	17.5 ± 0.0	14.2 ± 0.5 b
S_ENZ	0.10 ± 0.01 cd	97.55 ± 0.45	-0.64 ± 0.02 abc	5.91 ± 0.03 b	34 ± 11 ab	9.1 ± 0.2 c	17.6 ± 0.2	14.5 ± 0.0 b
S_HHP	0.08 ± 0.00 ab	97.84 ± 0.13	-0.60 ± 0.00 bc	5.09 ± 0.06 a	43 ± 1 b	8.3 ± 0.2 b	18.9 ± 0.9	15.4 ± 0.3 b
HS_CON	0.09 ± 0.00 bc	98.27 ± 0.04	-0.73 ± 0.02 a	5.99 ± 0.01 b	52 ± 2 bc	8.0 ± 0.1 b	17.9 ± 0.8	19.8 ± 0.5 c
HS_ENZ	0.10 ± 0.00 cd	97.71 ± 0.24	-0.67 ± 0.04 ab	6.34 ± 0.08 c	34 ± 8 ab	8.3 ± 0.0 b	17.4 ± 0.1	20.6 ± 0.1 c
HS_HHP	0.07 ± 0.00 a	98.41 ± 0.27	-0.63 ± 0.01 abc	4.81 ± 0.02 a	67 ± 5 c	6.4 ± 0.0 a	18.9 ± 1.0	23.4 ± 0.5 d

Sample code	Riboflavin (μg/L)	Mannan (mg/L)	Total soluble colloids (mg/L)	Mean particle diameter (nm)	Filtration flow (mL/min/cm^2)	Δ NTU	Mini-contact test ΔμS/cm
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
RW	67 ± 3 a	93 ± 11	3825 ± 673	514 ± 7 a	0.78 ± 0.02	79 ± 4	124 ± 0 abc
S_CON	81 ± 2 abc	123 ± 9	3768 ± 564	1096 ± 136 b	0.75 ± 0.03	68 ± 1	121 ± 1 ab
S_ENZ	83 ± 5 abc	138 ± 8	2953 ± 21	1464 ± 161 b	0.71 ± 0.04	65 ± 0	120 ± 2 a
S_HHP	76 ± 1 ab	143 ± 7	2512 ± 75	1047 ± 156 b	0.70 ± 0.03	62 ± 3	125 ± 0 bc
HS_CON	100 ± 8 c	99 ± 8	3058 ± 249	1203 ± 2 b	0.70 ± 0.00	65 ± 0	129 ± 2 cd
HS_ENZ	93 ± 8 bc	119 ± 48	2726 ± 81	2069 ± 107 c	0.69 ± 0.04	59 ± 0	132 ± 0 de
HS_HHP	86 ± 7 abc	169 ± 6	3030 ± 537	993 ± 213 ab	0.69 ± 0.03	63 ± 0	136 ± 1 e

4.1 Wine color evolution, POM-test and phenolic fraction

By considering the results of PCA (fig. 38), the addition of lees allowed a better wine color evolution in almost all the added wines compared to the reference. In general, all the wines added with HS lees and wines added with lees treated by HHP (both S and HS) showed tendential lower values in wine color (abs 420 nm), lower intensity in red and yellow components (a* and b*, respectively) and higher L* values (corresponding to lightness). This trend was also confirmed by looking at the results reported in table 23, with HS_HHP significantly different from RW as concern color (0.07 and 0.11, respectively) and b* value (4.81 and 6.01, respectively); the former also showed the highest mean value of L* (98.41), even if no statistical differences emerged.

Oxidizability potential, evaluated by browning test (POM-test) was also strongly affected by the addition of lees, with tendential higher mean values in all treated wines compared to the reference that, on the contrary, showed the lowest value (18%); also in this case, wines added with HS lees and added with lees treated by HHP (both for S and HS) tendentially gave the better results in term of protection against wine oxidation; the highest value of POM-test found in HS_HHP (67%), thus resulting significantly different from all the other samples, except for HS_CON (52 %) (table 23). Higher POM-test also corresponded to tendential lower TPI and tendential higher mean content of catechins (fig. 38); highest TPI was found in all the treated wines, that resulted statistically different from the reference (table 23), except for HS_HHP that, conversely, showed the lowest value (6.4). On the other hand, by considering the amount of catechins, the trend of such phenolic fraction was opposite compared to TPI, with tendential higher content in wines added with S_HHP and HS_HHP (18.9 mg/L) followed by RW (18.6 mg/L); the remaining samples showed lower mean values, ranging from 17.4 mg/L (HS_ENZ) to 17.9 mg/L (HS_CON); however no statistical differences were observed. In the case of RW, the lowest POM-test coupled with lower TPI value might be due to the occurrence of oxidation phenomena, thus also justifying the highest wine color intensity; similarly, in the case of wines added with S_ENZ and HS_ENZ oxidation might be occurred since lower POM-test (34 %) and catechins content (17.6 mg/L and 17.4 mg/L) was observed, together with higher intensity in wine color (table 23).

It is worth noting how, independently on the treatment performed, all wines added with HS lees showed the highest amount of glutathione, up to 23.4 μmol/L in HS_HHP, all resulted statistically different from RW and wines added with S lees. The highest release of glutathione in HS_HHP wines might have positively contributed to better preserve phenolics fractions, especially catechins content, thus resulting in lower browning, better color evolution and highest POM-test value.

The increase of glutathione previously observed in synthetic wines aged on HS lees (Part I, section 4.1 of the present Chapter) also occurred in this condition, thus confirming the previous hypothesis about the positive contribution of *Hanseniaspora* spp. towards protection against oxidation under real winemaking condition. Furthermore, the great ability of *Hanseniaspora* spp. to release glutathione after lysis treatments and to produce reducing proteins (Chapter 2, section 4.3.4) also supported the best results observed after the addition of HS lees.

On the other hand, a relevant impact of treatment performed was also observed, with HHP giving the best results towards wine color evolution and protection against oxidation. The chemical characterization of yeast derivatives obtained by different methods (Chapter 3, Part III) allowed to understand that HHP was the most suitable technology for preserving antioxidant compounds (both glutathione and reducing proteins linked to cell walls), thus explaining the highest content of glutathione and the best performance toward wine oxidation in wine added with lees treated by this technology, above all if combined with HS lees.

The protective effect of yeast lees towards wine oxidation was previously reported [16,152], depending on the amount of glutathione [155,171] and on compounds linked to cell wall fractions [32,151], thus explaining the lowest color intensity, highest POM-test and tendential higher content of catechins in HS_HHP wines.

On the other hand, such wines also showed the lowest TPI value. As previously reported (Chapter 3, Part III), HHP determined the highest median content of insoluble solids in the respective derivatives; similarly, potential higher presence of such fraction might be also occurred in HHP lees; this, together with potential adsorption of phenolic compounds on the insoluble fraction of yeast lees [153], might explain the lowest TPI value detected in such samples.

4.2 Release of riboflavin

The amount of riboflavin increased in all the wines after aging on lees (table 23), with the highest concentration detected in wines aged on HS, all resulting significantly different from RW; the maximum final concentration was found in HS_CON (100 µg/L), with an increase of 33 µg/L compared to RW (67 µg/L). The highest increase observed after aging and above all in HS lees, might be confirmed by the results previously obtained, concerning the greatest mean amount of riboflavin released by *Hanseniaspora* spp. compared to *Saccharomyces* spp. after lysis treatment (Chapter 2, section 4.3.3).

Possible occurrences of light-induced reactions and the appearance of light-struck defect might be enhanced after aging; however, it would be said that the contribution of lees might be considered negligible if compared to the initial concentration, since riboflavin was already present into the starting wine (RW), both deriving form grapes [149] and produced by yeast during alcoholic fermentation [70].

4.3 Release of mannans and soluble colloids, particle size distribution and filtration assay

The content of mannans and total soluble colloids followed a different trend. Starting from soluble colloids, no significant differences were observed among all the samples, with higher mean amounts detected in RW (3825 mg/L), followed by S_CON (3768 mg/L). The higher mean concentration detected in RW might be explained by the presence of polysaccharides deriving from grapes [179] and produced by yeast during alcoholic fermentation [168]. However, after aging on lees, the possible interaction between such compounds released by yeast lees and other wine colloidal substances might have determine the formation of higher aggregates that then precipitated [170], thus potentially explaining the tendential lower mean amount of such fraction in aged wined compared to RW.

By considering the amount of mannans, even if no significant differences were observed (*p* value = 0.06), the influence of wine aging on lees on the release of such polysaccharidic fraction was clear. Even if a certain amount of this fraction was already present into the RW (about 93 mg/L), for the reasons explained before as concern soluble colloids, the addition of lees determined an enhanced release of yeasts mannans, with the highest mean amount detected in HS_HHP (169 mg/L).

The positive effect of yeast lees addition on increasing the content of mannoproteins and total polysaccharides was known and seemed to occur during the first two months of aging [164,214], thus confirming the trend here observed.

The ability of *Hanseniaspora* spp. to produce relevant amounts of polysaccharides during single [4,13] and sequential fermentation [31] was reported, being also dependent on the strain [198]; furthermore, the good aptitude of this selected *Hanseniaspora* spp. strain to release highest amount of mannans, as previously assessed (Chapter 2, section 4.3.2), was also confirmed under winemaking condition, making it suitable for improving wine aging on lees. However, the release of such compounds was also influenced by the treatment performed for processing lees: an increase in polysaccharides after the addition of enzymes compared to untreated lees was previously observed by Cacciola et al. [111], thus confirming the tendential higher mean concentration of mannans detected in wines added with S_ENZ and HS_ENZ compared to the respective CON. Lastly, greater mean amounts were detected in wines added with HHP lees, both for S and HS, compared to enzyme addition; this trend was quite in line with what previously obtained concerning the amount of mannan released after wine aging on yeast derivatives (as discussed in Chapter 4, section 4.2), thus confirming the results here observed. Nevertheless, also in this case the best results were obtained by HS_HHP, that in fact determined the highest mean concentration of mannans in the resulting wines.

The addition of lees affected both mean particles size and filtration flow (table 23). Regarding the former, all the added wines significantly differed from RW (expect HS_HHP), as well as tendential reduction in filtration flow was observed in all the samples compared to RW, even if no statistical differences emerged. Similarly to what previously reported for wine aging on yeast derivatives (Chapter 4, section 4.3), the addition of lees and the subsequent release of soluble colloids and mannans might have influenced the colloidal state, consequentially impacting on particle size and wine filterability. In the same experimental trial abovementioned, HHP also determined the presence of particles with smaller size at the end of aging. This might further confirm the results here obtained, with wines added with HHP lees with mean particles size and filtration flow tendentially lower than the other samples.

4.4 Protein and tartaric stability

Starting from protein stability, the differences between wine turbidity before and after heating (expressed ad Δ NTU) was reported in table 23. According to the reference method used, wines were considered unstable if the differences were above 2 NTU. In general, all added wines showed a reduced Δ NTU - from 59 (in HS_ENZ) to 68 (in S_CON) - compared to RW (Δ NTU = 79) but they remained unstable also after treatments and no differences were observed from the statistical point of view.

As concern tartaric stability, the drop in conductivity was similar among the samples, with comparable values between RW and added wines. During wine aging, an improvement of protein and tartaric stability occurred [167]), due to the protective effect of mannoproteins [30], also depending on their composition and content of proteins linked to polysaccharide fraction [166]. However, in the present study the starting wine was highly unstable and the only release of mannans was probably not enough to obtain an evident stabilization.

4.5 Wine volatile profile

One hundred-eighteen volatile compounds were tentatively identified and listed in Annex 9. The results of the semi-quantitative analysis, with means and standard deviations and the results of the statistical analysis were reported in Annex 12.

To summarize, the total amount of aroma compounds – grouped for chemical class - was reported in table 24, with means and standard deviations and the results of the statistical analysis. Furthermore, the results of PCA carried out on the concentration (µg/L) of volatile compounds detected in the headspace of wines after three months of aging was reported in figure 39.

Table 25. Total amount of aroma compounds detected in the wines after three months of aging and grouped by chemical class. The concentrations were expressed in µg/L; data were means and standard deviation of two replicate. Different letters within the same row marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. 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	S_CON	S_ENZ	S_HHP	HS_CON	HS_ENZ	HS_HHP
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Acids	34.67 ± 7.20 a	67.52 ± 14.73 bc	80.82 ± 0.97 c	59.14 ± 2.38 abc	57.05 ± 1.68 abc	61.41 ± 4.69 bc	47.43 ± 2.01 ab
Acetate esters	113.29 ± 3.08 a	121.65 ± 4.96 ab	121.73 ± 3.20 ab	130.32 ± 2.29 b	130.01 ± 5.62 b	125.64 ± 3.74 ab	132.77 ± 0.70 b
Ethyl esters	543.01 ± 16.98 a	1209.76 ± 40.24 d	1193.93 ± 43.66 d	883.33 ± 41.54 bc	999.31 ± 18.60 c	981.00 ± 41.46 c	761.34 ± 7.50 b
Other esters	7.93 ± 0.75 a	12.51 ± 0.05 d	12.95 ± 0.54 d	9.68 ± 0.70 abc	11.97 ± 0.51 cd	11.33 ± 0.88 bcd	9.18 ± 0.21 ab
Alcohols	311.58 ± 8.03	329.52 ± 2.29	342.11 ± 8.11	325.05 ± 9.73	326.74 ± 20.36	318.43 ± 11.13	311.13 ± 0.03
Aldehydes	1.18 ± 1.02	0.52 ± 0.14	0.79 ± 0.46	0.65 ± 0.12	0.43 ± 0.06	0.53 ± 0.15	0.47 ± 0.10
Ketones	0.07 ± 0.01 a	0.10 ± 0.03 ab	0.12 ± 0.04 ab	0.16 ± 0.02 ab	0.22 ± 0.06 b	0.17 ± 0.05 ab	0.11 ± 0.01 ab
Norisoprenoids	4.98 ± 0.33 b	4.58 ± 0.03 ab	4.88 ± 0.04 ab	4.10 ± 0.27 ab	4.28 ± 0.39 ab	3.96 ± 0.36 ab	3.93 ± 0.07 a
Terpenes	7.95 ± 0.24	7.37 ± 0.03	7.77 ± 0.30	7.95 ± 0.76	7.93 ± 0.81	7.70 ± 0.35	8.07 ± 0.08
Phenols	24.16 ± 3.31 b	9.08 ± 1.95 a	10.96 ± 0.31 a	14.50 ± 1.92 a	10.37 ± 0.79 a	10.86 ± 0.92 a	14.03 ± 0.68 a
Sulfur compounds	0.73 ± 0.37	0.63 ± 0.16	0.79 ± 0.17	0.73 ± 0.04	0.63 ± 0.07	0.72 ± 0.02	0.61 ± 0.03

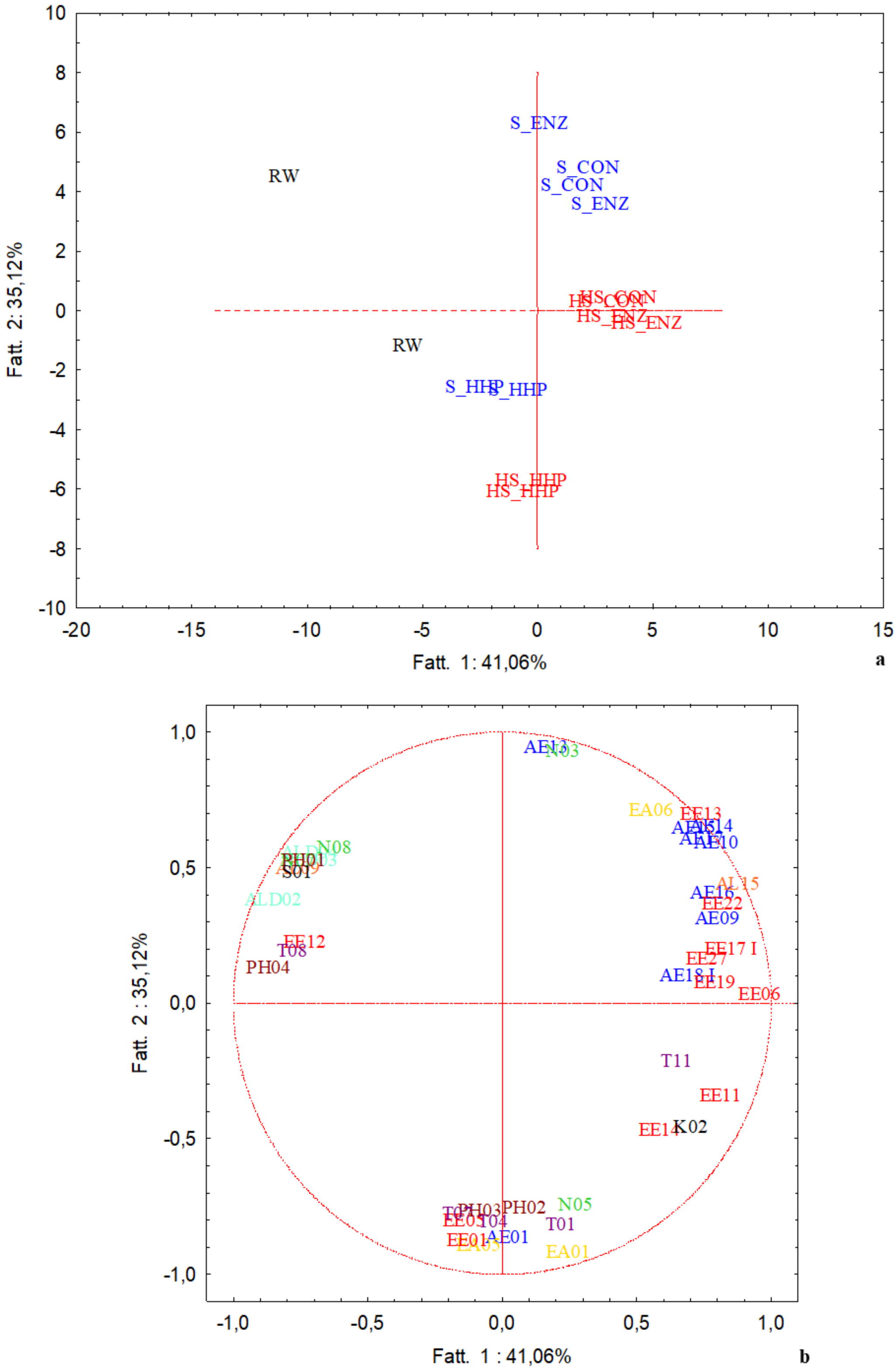


Figure 39. Results of PCA carried out on the concentration (µg/L) of volatile compounds detected in the headspace of wines after three months of aging. Projection of case (samples) (**a**) and variables (volatile compounds) (**b**) on the factor-plan were reported. Factor Loadings (FL) were calculated by Factorial Analysis, and the most relevant variables were selected for marked FL > 0.7. 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. EA: acetate esters (yellow); EE: ethyl esters (red); AE: other esters (blue); AL: alcohols (orange); ALD: aldehydes (light blue); K: ketones (black); N: norisoprenoids (light green); PH: phenols (brown); S: sulfur compounds (black); T: terpenes (violet).

As resulted by PCA, the addition of lees positively impacted on the volatile profile of aged wines, with treated samples as the most characterized. Wine added with CON and ENZ lees both from S and HS showed the tendential higher concentration of esters (both EE and AE), octyl acetate (EA06), 1-decanol (AL15), citronellol (T11) and vitispirane-like compound (N03). Higher concentration of acetate esters - ethyl acetate (EA01) and hexyl acetate (EA05) - 4-ethylguaiacol (PH02) and 4-ethylphenol (PH03), and several terpenes (β-myrcene, nerol oxide and linalool - in the PCAT01, T04 and T07 - respectively) mainly characterized S_HHP and HS_HHP; RW showed the poorest volatile profile, with tendential higher concentration of some phenols (PH01 and PH04), sulfur compounds (S1) and aldehydes (ALD), 2-butoxyethanol (AL09) and β-damascenone (N08). Yeast lees may release several aroma compounds, *i.e.*, esters and alcohols [206] as consequence of yeast metabolism [158]; during wine aging their concentration may be further increased with an improvement of wine aroma complexity and sensory perception [189], thus confirming the trend here observed.

However, even polysaccharides released by yeast lees during aging may be involved in the modulation of wine volatile profile. In this regard, the relationship between the content of mannans and soluble colloids and some of aroma compounds, as the most significant variables considered in the previous PCA, were calculated and reported in table 25.

Table 26. Correlation between the content of mannans and soluble colloids and the aroma compounds of wines. Marked correlation were significant at $p < 0.05$ (*) and at $p < 0.01$ (**).

Compound	Code	Mannans	Soluble colloids
3-methyl-1-butanol propanoate	AE01	0.65	-0.65
ethyl 3-methylbutyl succinate	AE18 I	0.13	-0.63
ethyl acetate	EA01	0.45	-0.57
hexyl acetate	EA05	0.48	-0.55
ethyl propanoate	EE01	0.80 *	-0.46
ethyl 3-methylbutanoate	EE05	0.78 *	-0.47
ethyl 6-heptenoate	EE12	-0.35	0.58
ethyl nonanoate	EE14	0.27	-0.71 *
2-furfural	ALD02	-0.50	0.53
dodecanal	ALD03	-0.45	0.49
tetradecanal	ALD04	-0.33	0.44
β-myrcene	T01	0.53	-0.52
nerol oxide	T04	0.94 **	-0.40
linalool	T07	0.67	-0.57
citronellol	T11	-0.04	-0.66
naphthalene	N07	-0.40	0.51
β-damascenone	N08	-0.39	0.66

Positive correlation between the mannans content with almost all the esters was found, possibly explaining the higher concentration of such compounds in wines added with lees; these latter in fact showed tendential higher mean concentration of mannans compared to RW. On the other hand, the negative correlation found between total soluble colloids and the same abovementioned volatile compounds might explain their lower mean concentration in RW that, in fact, showed the highest mean amount of total soluble colloids compared to the added wines. Previous findings suggested how wine volatile profile was modulated by the interaction between polysaccharides, especially mannoproteins, and wine aroma compounds [174,175]; these interaction may lead to both an increased volatility or retention phenomena, depending on pH, temperature and type of aroma compound [175,176], contact time [165,177] and glycosylation degree of the polysaccharidic fractions [174]. Similarly, it would be hypothesized that mannans have enhanced the volatility of some aroma compounds, thus determining the highest mean concentration of volatile compounds in wines aged on lees; on the other hand, soluble colloids seemed to have retained aroma compounds, thus possibly justifying the poorest volatile profile of RW.

By considering the total amount of aroma compounds, grouped for chemical class (table 24), these results confirmed the trend previously observed.

Increased concentrations in the total amount of ethyl esters, other esters (*i.e.*, methyl esters) and alcohols were detected in all added wines compared to RW, with tendential higher amounts in S wines, as previously observed by other authors [190,193]. Significant increase of acetate esters was instead detected in HS_HHP (132 μg/L), HS_CON and S_HHP (130 μg/L) that resulted statistically different from RW and the other added wines, potentially improving wine aroma profile by conferring fruity and floral notes. The ability of *Hanseniaspora* spp. to produce highest amounts of acetate esters during single [29,190] and sequential fermentation [193] was previously reported, also depending on the strains used [196] and grapevine variety [195]; this might confirm the trend here observed.

An increase in the total amount of fatty acids was observed compared to RW in all treated wines, with the highest mean concentration detected in S_ENZ, followed by S_CON, in terms of mean values. It is interesting to note how wines added with HS lees showed tendential lower mean concentration of such compounds compared to S wines subjected to the same treatment: the lowest total amount were detected in HS_HHP (47 μg/L) and HS_ CON (57 μg/L), lower than those detected in S_HHP (59 μg/L) and S_CON (67 μg/L), even if only in terms of mean values. This might positively impact on improving wine volatile profile and sensory perception, since such compounds are described with sour, pungent and cheese notes [147].

Aldehydes mostly characterized RW; no statistical differences were observed among the samples concerning the total amounts of such compounds, whereas lowest mean value of furfural was detected in HS_HHP, thus resulting significantly different from all the other samples, except for S_HHP (Annex 12). Aldehydes might be produced as intermediate of yeast metabolism [158] or by the occurrence of oxidation phenomena [209]; as previously observed concerning wine color evolution, RW in fact resulted the most oxidized sample, thus possibly explaining the tendential higher concentration of aldehydes formed by oxidation in such sample. Furthermore, aldehydes seemed to be negatively correlated with mannans (table 25); this polysaccharidic fraction might have retained such compounds, thus possibly explaining the lowest concentration found in added wines compared to RW.

Certain amounts of phenols were present in both RW and added wines, since they are produced during winemaking process [150]; HS_HHP and S_HHP were also characterized by the highest concentration of 4-ethylguaiacol (PH2) and 4-ethylphenol (PH04) (figure 39; for the concentration see Annex 12). However, by considering the total amount of such compounds (table 24), the highest concentration was found in RW, whereas a significant decrease was observed after aging, possibly due to the adsorption of these compounds on yeast lees surface. After the addition of lees and aging, the reduced concentrations observed for total phenols may positively improve the wine aroma profile, since such compounds are responsible of olfactory defects and described with woody, spicy or smoky to stable, horse notes [160].

Regarding terpenes, the total amount of such compounds was quite similar among all the samples and no differences were found in the total concentrations (table 24).

Among norisoprenoids, TDN, vitispirane and β-damascenone together with other compound that were tentatively identified as norisoprenoid-like molecules were detected in all the wines, with total highest concentration in RW. These compounds originate by thermal degradation of carotenoids present into the grapes, and their amount tend to increase during fermentation and aging both for enzymatic and chemical reactions [215,216]. These compounds may contribute to define wine varietal aroma, with positive notes, like floral or tropical fruits (β-damascenone) or negative notes like camphora (vitispirane) and kerosene (TDN) [150]. After aging, all these compounds showed a reduced concentration in treated wines, about half compared to RW, probably due to the possible adsorption on yeast lees or to possible interaction with mannans and soluble colloids, even if a poor correlation was found (table 25). However, since several of them are associated to *off-flavors*, the lower mean concentration in wines aged on lees may be positive from the aromatic point of view.

4.6 Sensory analysis

The results of sensory analysis carried out on white wines after three months of aging was reported in figure 40 (a and b): color (yellow hue and browning) and smell attributes were reported in figure 40 a, whereas taste and aftertaste attributes in figure 40 b. The sensory descriptors that resulted significantly different were listed in table 26.

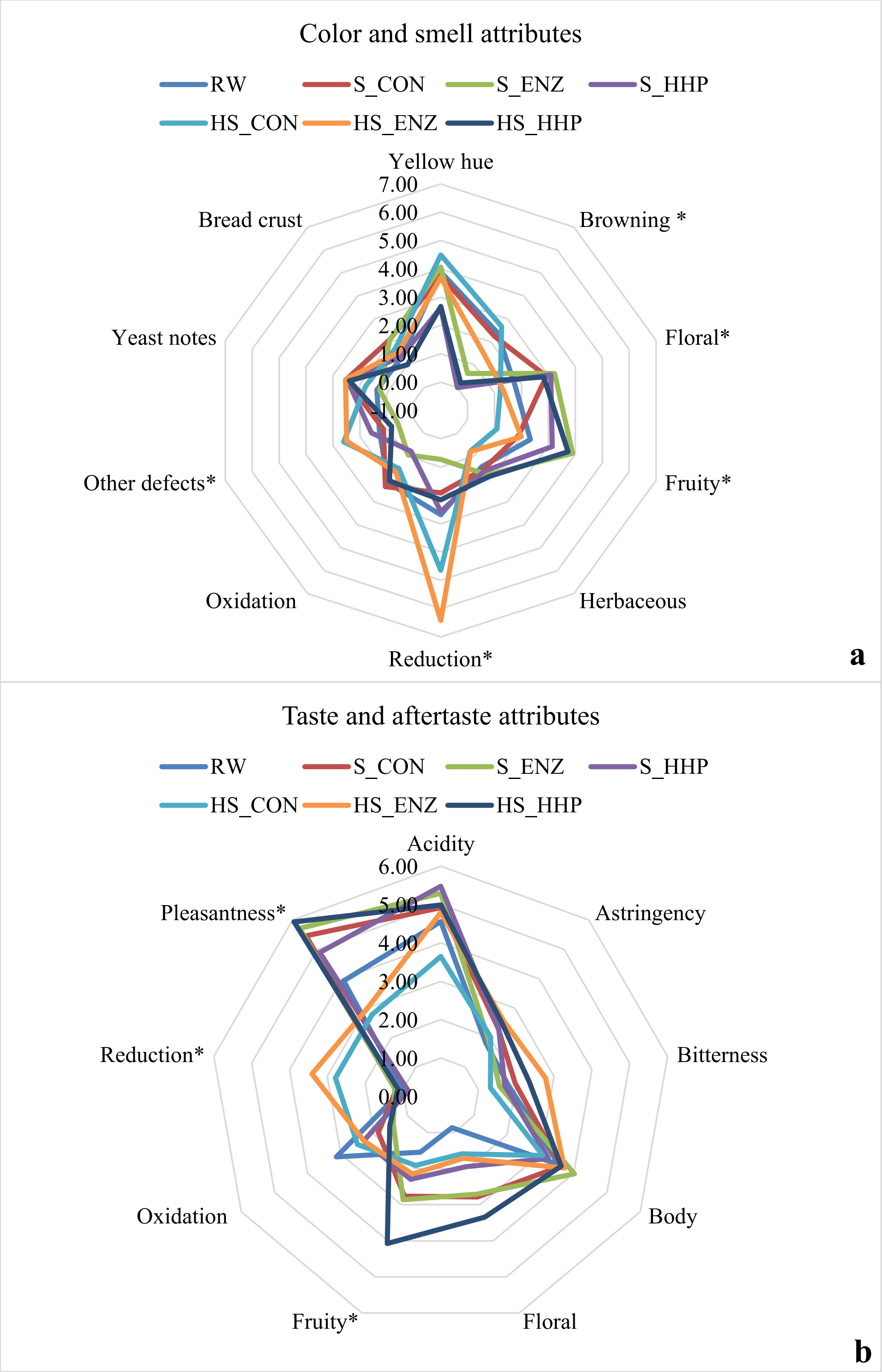


Figure 40. Mean scores of sensory profiles of wines after three months of aging. Color and smell attributes (**a**) and taste and aftertaste attributes (**b**). RW: reference wine, without lees addition; S: lees from single fermentation; HS: lees from sequential fermentation; CON: wines added with untreated lees; ENZ: wines added with lees treated by enzyme addition; HHP: wines added with lees treated by high hydrostatic pressure. *Significant different according to ANOVA and LSD Fisher test at $p < 0.05$.

Table 27. Mean scores of the descriptors resulted significantly different according to ANOVA and LSD Fisher test at $p < 0.05$. Data were the means of scores attributed by all the judges to the same wine for the same descriptor.

			Smell				After taste	
	Pleasantness	Browning	Floral	Fruity	Reduction	Other defects	Fruity	Reduction
RW	3.9 ab	2.4 c	1.8 a	2.3 ab	2.7 b	1.3 abc	1.5 a	1.0 a
S_CON	5.5 bc	2.2 c	2.9 b	1.9 ab	1.9 ab	1.1 ab	2.8 ab	1.2 a
S_ENZ	5.7 c	0.6 ab	3.2 b	3.9 c	0.7 a	0.6 a	2.9 ab	1.2 a
S_HHP	4.9 bc	0.0 a	3.1 b	3.1 bc	2.6 ab	1.6 abc	2.3 a	0.9 a
HS_CON	2.8 a	2.7 c	1.2 a	1.1 a	4.6 c	2.6 c	1.9 a	2.8 b
HS_ENZ	3.0 a	1.5 bc	1.3 a	2.0 ab	6.4 c	2.5 bc	2.2 a	3.4 b
HS_HHP	5.9 c	0.2 a	2.8 b	3.7 c	2.2 ab	0.8 a	4.1 b	1.1 a

After aging on lees, an improvement on aromatic intensity and complexity was observed in almost all the treated wines compared to RW, expect for HS_CON and HS_ENZ, that in fact showed the lowest scores in pleasantness. This improved wine sensory perception after aging was in line with that previously reported [189], thanks to the release of some volatile compounds from lees or to an enhanced volatility of aroma compounds due to interaction with mannoproteins [163,164,175].

Starting from color, the highest scores in terms of yellow hue and browning were attributed to RW, S_CON, S_ENZ and HS_ENZ whereas S_HHP and HS_HHP obtained to lowest ones. This was in line with what previously observed (section 4.1), with these latter samples with the lowest increase in wine color. S_HHP, S_ENZ and HS_HHP also showed the highest scores concerning smell attributes for floral and fruity notes and lowest values in reduction (table 26); HS_HHP presented also higher values in yeast and oxidation notes.

By considering taste and aftertaste, slight positive correlation was found between the total amounts of acetate esters and floral (r = 0.47) and fruity notes (r = 0.51), whereas positive correlation was found between aldehydes and oxidation notes (r = 0.51). The highest scores in terms of floral and fruity notes together with lower reduction and oxidation notes perception were attributed to HS_HHP that in fact showed the tendential higher mean concentration of acetate esters and some terpenes and the tendential lower mean concentration of aldehydes.

HS_HHP resulted the most preferred by the panelist group, with a final score in terms of pleasantness of 5.9 and sensory analysis confirmed what previously determined from the chemical point of view.

5. Conclusion

During aging on lees in model solution (synthetic wine), the content of both glutathione and cysteine tended to increase in all the wines aged on lees from sequential fermentation; interesting results were also obtained by processing lees by HHP, especially in the case of HS, thus potentially exhibiting non-negligible antioxidant properties under real winemaking conditions. The use of lees from sequential fermentation has not significantly affected the amount of riboflavin released during aging, and no effect was also observed in relation to the treatment performed; furthermore, the positive contribution of *Hanseniaspora* spp. on increasing the amount of soluble colloids was again observed and confirmed.

Modifications in wine aroma profile was observed in all the aged wines, both S and HS, in comparison with their respective starting wines. HS seemed to be characterized by the poorest volatile profile compared to the control at the end of aging period; nevertheless, the use of fermentation lees by sequential fermentation, also processing by HHP, might positively contribute to improve wine aroma profile, especially by considering the reduced mean concentration of fatty acids detected in such wines.

After the previous characterization in model wine, the overall interesting results obtained by HS_HHP lees was confirmed under real winemaking conditions. The best wine color evolution and highest protection against wine oxidation was observed, together with the highest content of glutathione released in such added wines. Highest concentration of mannans was also detected, whereas the amount of riboflavin released was comparable to the other samples. After aging, an improvement on intensity and complexity of wines were observed, with HS_HHP the most characterized in terms of acetate esters and some terpenes, together with lower amount of total fatty acids, aldehydes and norisoprenoids conferring negative notes (i.e., naphthalene and

TDN). Sensory analysis further confirmed the results obtained after chemical evaluation, being HS_HHP the most preferred wines by the panelist group.

This selected *Hanseniaspora* spp. strain has showed a great aptitude to be used as fermentation co-starter in sequential fermentation and for improving wine aging; the addition of the respective lees has determined an overall improvement of wine quality, giving the most interesting and promising results when in combination with HHP treatment.

Chapter 7

Potential human health benefits of yeast derivatives

1. Introduction

Yeast derivatives are characterized by a wide variety of macro and micronutrients - nitrogen compounds (amino acids and peptides), vitamins and minerals, nucleotides, polysaccharides, lipids and fatty acids - making them suitable for several technological applications.

Yeast extracts are first employed in food industry as additives in soups, snacks and sauces, thanks to the presence of nucleotides and aroma-active compounds that contribute to enhance the flavor of foods [89].

Furthermore, the presence of a non-negligible content of amino acids and peptides, polysaccharides, vitamins and minerals [82,84] makes them suitable as prebiotics [217–219] and as ingredient for functional foods preparation [220,221]. Interesting results have also been obtained by administering yeast derivatives as dietary supplements in animal feed, resulting in better growth performance and nutrients digestibility [222], improved intestinal development and better immune response against pathogenic microorganisms [223–225].

Another interesting application is related to the ability of yeast cell wall polysaccharides to bind several toxins produced by molds [226–228], thus potentially being employed as tool for decontaminating food products; furthermore, β-glucans and mannoproteins extracted from spent yeasts (as brewery and winemaking by-products) have also showed good oil and water-binding capacities [92,93,229], thus potentially being used as stabilizers for producing emulsions [230], dairy- and bakery-related products [220,231–233].

As concern the health benefits of yeast derivatives, several studies have demonstrated the positive effects of peptides extracted from spent yeasts towards inflammatory response [234], together with good anti-hypertensive and antioxidant activities, also evaluated *in vitro* gastrointestinal digestion [235].

Polysaccharides from yeasts - especially β-glucans - also demonstrated hypolipidemic effects, thus positively contributing on the reduction of blood cholesterol level when employed as dietary supplements *in vivo* assay [236].

The increasing attention of the consumers towards health have recently led several researchers to investigate the potential anti-diabetes effect of yeast polysaccharides, too. Some yeasts strains used as fermentation starters for producing dairy products [237], as well as yeast glucans administered as dietary supplement, have demonstrated a good hypoglycemic effect [236]; the latter seems to be related to the inhibition of the enzymes involved in the adsorption and regulation of glucose level in blood, *i.e.* α-glucosidase [237], or to the stimulation of immune response and inhibition of glucose transport in the gastrointestinal tract [238].

Polysaccharides from yeasts also have prebiotics effect, thus enhancing the activity and the metabolism of gut microbiota [218,219]. During digestion, these microorganisms produce several bioactive compounds - among them short chain-fatty acids - starting from both carbohydrates and proteins as precursors [239]; these compounds seem to exhibit antimicrobial activity [240], being also involved in inflammatory response regulation [241]. Similarly, yeasts derivatives contain certain amounts of short chain-fatty acids because of yeast metabolism [158], as well as polysaccharides and proteins as their precursors, possibly enhancing the positive effect linked to short chain-fatty acids when employed as ingredient for functional foods or as prebiotics.

2. Aim of the work

The aim of this work was to evaluate the potential health benefits of a yeast derivative (YD) to be employed as ingredient for functional foods preparation or as dietary supplement for human nutrition. Yeast derivative obtained by ultrasounds treatment was selected as the best compromise between nitrogen compounds (both amino acids and proteins) and polysaccharides content (Chapter 3, Part III); the potential human health benefits were evaluated in terms of antioxidant properties, hypolipidemic and hypoglycemic effects, and content of short chain-fatty acids.

3. Materials and methods

3.1 Reagents

Absolute ethanol, ascorbic acid, sodium phosphate, potassium ferricyanide, trichloroacetic acid, ferric chloride, cholesterol standard, sodium cholate, cholestyramine, sodium carbonate, sodium citrate, acetic acid (99% v/v), sulfuric acid (96% v/v), furfural, α-glucosidase and α-amylase, acarbose p-nitrophenyl-α-D-glucopyranoside, starch, dinitrosalicylic acid, sodium potassium tartrate tetrahydrate and 2,2-diphenyil-picrylhydrazyl ($DPPH^{\bullet}$) were purchased from Sigma Aldrich (St. Louis, MO, USA). MilliQ water was produced by MilliQ Advantage A10 apparatus (Merck Millipore, Billerica, MA, USA) and microfiltered at 0.22 µm before use. Commercial active dry yeasts (ADY) *S. cerevisiae* were from Enologica Vason S.p.A. (San Pietro in Cariano, VR, Italy).

3.2 Yeast derivative production

Commercial active dry yeast preparation of *S. cerevisiae* was used to produce YD after resuspension in water solution at a concentration of 10% (w/v) and the following US lysis treatment was performed as previously reported in Chapter 3, section 3.3. The YD powder was then subjected to the analyses described below.

3.3 Antioxidant properties

Radical scavenging activity was evaluated by DPPH assay as reported in Chapter 3, section 3.9; the results were expressed as percentage of absorbance decrease (OD 517 nm) observed in DPPH solution in presence of antioxidant molecules. Reducing power was evaluated by FRAP assay as reported by Dilna et al. [242]; the results were expressed as absorbance value (OD 700 nm). For both the assays, sample was employed at concentration of 10 mg/mL, with ascorbic acid used as positive control.

3.4 Cholesterol and sodium cholate binding capacity

Potential hypolipidemic effects were assessed by evaluating the ability of YD to bind cholesterol and sodium cholate. Regarding the former, the analysis was carried out as reported by Zhang et al. [243] and the residual content in the samples (expressed in mg/mL) were determined in relation to a calibration curve made with cholesterol standard at increasing concentrations (0-1.5 mg/mL). For sodium cholate binding capacity, the assay was performed as reported by Lin et al. [244], with slight modification. Briefly, 0.1g of YD powder was suspended in 100 mL of carbonate buffer (1 mmol/L of sodium cholate); after incubation, 0.5 mL of supernatant was added with 3mL of sulfuric acid (45% v/v) and 0.5 mL of furfural (0.3% v/v). The residual concentration in the samples (expressed as mmol/L) was evaluated by a calibration curve made with different amount of sodium cholate (0-1.5 mmol/L); cholestyramine was used as positive control.

3.5 α-glucosidase and α-amylase inhibition assays

The potential hypoglycemic effects of YD were assessed by performing both α-glucosidase and α-amylase inhibition assays, as reported by Xu et al. [245]. The inhibition effect was calculated as reported by the authors and expressed as inhibition rate (percentage).

3.6 Determination of short chain fatty acids content

The evaluation of short chain-fatty acids (SCFA) was performed by SPME-GC-MS. For sample preparation, 0.1 g of YD powder was resuspended in both 10 mL of citrate buffer at pH 2 and in 10 mL of carbonate buffer at pH 7, to simulate stomach and intestine conditions, respectively. The samples were pre-conditioned at 50°C for 15 minutes under agitation and the micro-extraction was carried out at the same temperature for 20 minutes. The operating conditions related to GC separation was the same reported in Chapter 3, section 3.11. The SCFA were tentatively identified by comparing their mass spectrum with those of external standard and 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; furthermore, the quantitative analysis was carried out by adding deuterated propionic acid as internal standard at a concentration of 0.5 mg/L.

3.7 Statistical analysis

The results obtained were means and standard deviations of three replicates. One-way ANOVA and Tukey HSD test were carried out for all the parameters evaluated and differences were considered significant at $p < 0.05$. All elaborations were carried out by the software Statistica for Windows Version 8.0 (StatSoft, Tulsa, OK, USA).

4. Results and discussion

4.1 Antioxidant properties

The antioxidant activity of the YD was evaluated in relation to ascorbic acid, widely employed as additive for preserving foods and beverages from oxidation; radical scavenging activity and reducing power were assessed by DPPH and FRAP assays respectively, and the results were reported in figure 41 (a and b, respectively).

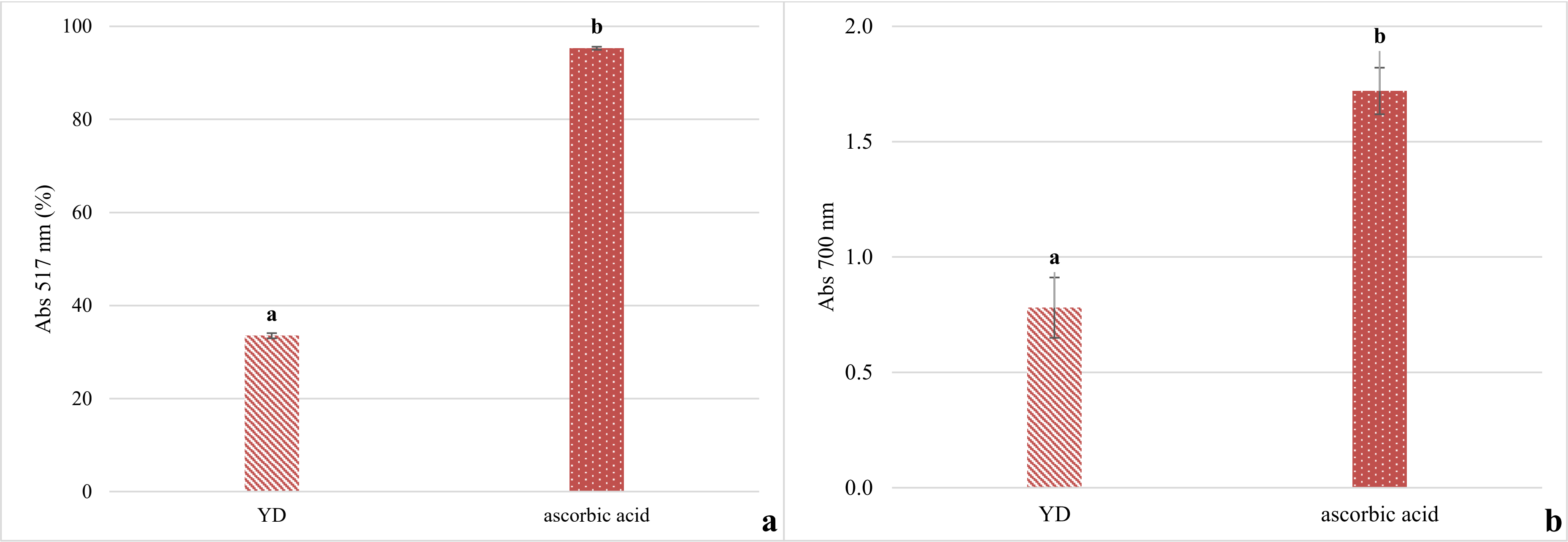


Figure 41. Radical scavenging activity evaluated by DPPH assay (**a**) (as percentage decrease of abs 517 nm, %) and reducing power evaluated by FRAP assay (**b**) (as abs 700nm). Data were means and standard deviation of three replicates. Different letters marked significant differences, according to ANOVA and Tukey HSD test at $p < 0.05$. YD: yeast derivative.

The radical scavenging activity was expressed as percentage of absorbance decrease (abs 517 nm) observed in DPPH solution in presence of antioxidant molecules. As expected, ascorbic acid showed a total radical scavenging capacity (95.3%) significantly different from YD, that conversely, showed a percentage decrease of 33.5%. Even if a significant difference resulted by the statistical analysis, the antioxidant activity exhibited by the sample was not negligible, representing about a third of the positive control. As concern FRAP assay, the highest the absorbance (OD at 700 nm) the highest the reducing power: the values of OD measured for the control (ascorbic acid) and the sample were 1.7 and 0.8, respectively, with significant differences between samples. Nevertheless, YD exhibited a good reducing power that corresponded to about 50% of the control, highlighting its potential antioxidant capacity.

Antioxidant properties of yeast extracts was previously reported [84], mainly due to the presence of small peptides with low molecular weight (< 3kDa) containing valine, tyrosine, or arginine in their sequence [154,246]; similarly, in the present study, the non-negligible antioxidant activity showed by YD might be related to the presence of such compounds as results of yeast autolysis and subsequent release of intracellular components.

4.2 Cholesterol and sodium cholate binding capacity

The ability of YD to interfere in the adsorption of cholesterol by direct interaction or by reducing the availability of bile salts (sodium cholate used as model) was evaluated and the results were reported in figure 42 (a and b, respectively).

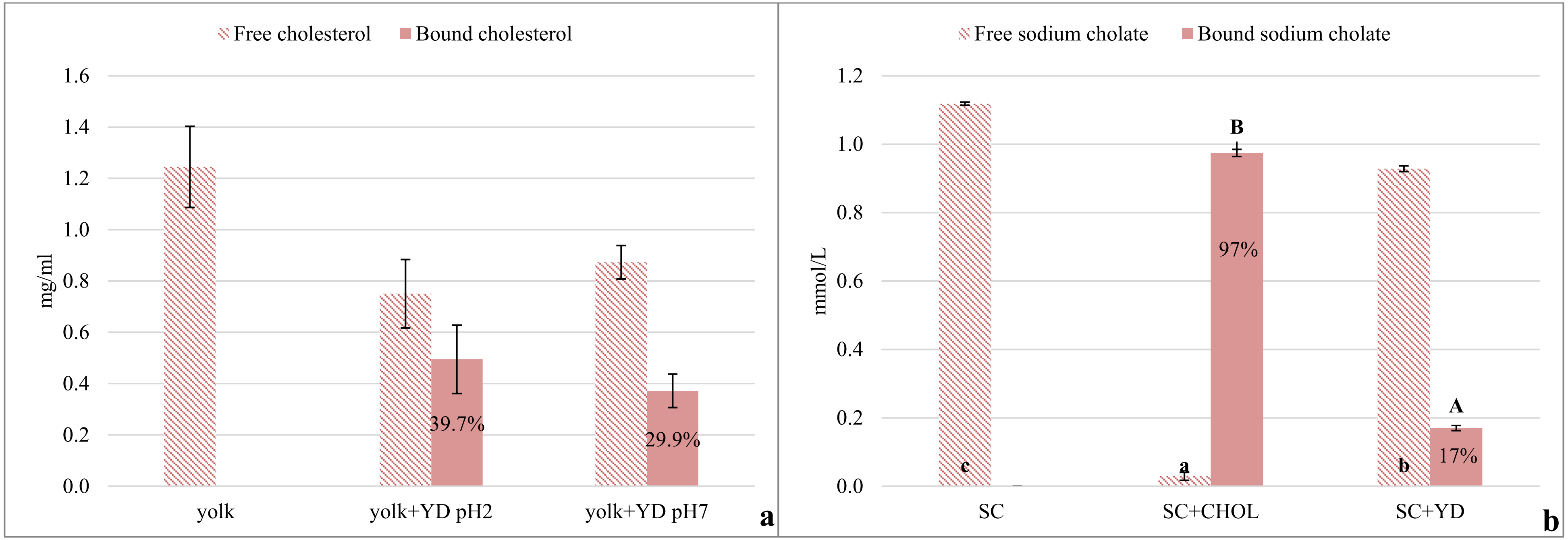


Figure 42. Cholesterol binding capacity expressed as mg/mL (**a**), and sodium cholate binding capacity expressed as mmol/L (**b**). Data were means and standard deviation of three replicates. Different letters marked significant differences among samples, according to ANOVA and Tukey HSD test at $p < 0.05$. Lowercase letters: significant differences between the starting solution of sodium cholate and solutions added with cholestyramine and yeast derivative. Uppercase letters: significant differences referred to bound sodium cholate. SC: starting solution of sodium cholate; SC + CHOL: sodium cholate solution added with cholestyramine; SC + YD: sodium cholate solution added with yeast derivative.

As concern the ability of YD to directly bind cholesterol (fig. 42 a), the starting concentration of the solution was 1.2 mg/mL; after the addition of YD and incubation, it was observed a good capacity to reduce total cholesterol content: in fact, the amount of bound form represented about 39.7 % and 29.9 % of the total starting concentration at pH 2 and pH 7, respectively. Under conditions simulating gastric environment (pH 2), the binding capacity was slightly higher compared to those simulating small intestine (pH 7); these results were in contrast with those reported in literature, in which the higher adsorption of cholesterol by dietary fibers was found in small intestine environment [243,244]. Furthermore, the chemical composition and structure of polysaccharides from different food matrices [247], as well as the solubility of dietary fibers influenced this property [248,249]. The different chemical composition, size and structure of polysaccharides from yeast cell walls and from vegetable cells might explain the different behavior towards cholesterol binding capacity observed in the present study. Nevertheless, exopolysaccharides produced by prebiotics have exhibited a lowering cholesterol activity *in vitro*, ranging from 30% to 60%, in relation to the strain, size and structure of polysaccharides [242,250]; this ability might be related to the surfactant property of polysaccharides that, consequentially, might lead to the formation of micelles, thus preventing cholesterol adsorption at gastro-intestinal level.

Regarding to sodium cholate (fig. 42 b), generally used as model system for bile salts, the binding capacity showed by YD was only of 17%, compared to cholestyramine that, on the contrary, showed the maximum adsorption capacity (97,4%). The latter has a high affinity with bile salt anions, including sodium cholate [251,252], thus reducing the adsorption of lipids in intestinal tract and, consequentially, controlling blood cholesterol level [253]. The capacity of dietary fibers from vegetable matrices, *e.g.*, apple, wheat or soybean was studied [243,244], whereas no scientific evidence was about the effect of polysaccharides produced by microorganisms towards binding capacity of bile salts. However, even if the ability to bind sodium cholate exhibited by YD was lower compared to cholestyramine, a certain hypolipidemic effect was observed.

4.3 α-glucosidase and α-amylase activity

The potential hypoglycemic effects of YD were evaluated by both α-glucosidase and α-amylase inhibition assays, with acarbose used as positive control.

As concern α-glucosidase, acarbose exhibited inhibition level of 39,4%, whereas YD seemed to enhance the activity of such enzyme. This result was not in line with those reported by Koh et al. [237] that observed how β-glucans from yeasts showed a good α-glucosidase inhibition rate.

Conversely, α-amylase was slightly inhibited by YD with a reduction of the activity of 25%, whereas acarbose did not show inhibitory effect. This inhibitory effect might be related to polysaccharidic fraction; exopolysaccharides produced by *L. plantarum* [242] and polysaccharides extracted from green tea [254] were reported to inhibit the activity of this enzyme, with inhibition rate also dependent on molecular size, sugar composition and concentration [245].

In the present study, the enhanced activity of α-glucosidase observed might be due to the presence of glucose in YD composition, resulting from the hydrolysis of cell wall polysaccharides induced by lysis treatment, as well as other yeast cell components might have acted as substrate for the enzyme activity. However, a certain inhibition rate was observed, at least as concern α-amylase. For the latter, further investigations are necessary for evaluating the inhibitory effect of YD in comparison to a positive control.

4.4 Short chain fatty acids (SCFA) content

A total of seven short chain-fatty acids (C2-C6) were tentatively identified in YD powder. The amount of each SCFA was detected in YD after resuspension in buffer solution at pH 2 and pH 7, for simulating stomach and intestine conditions, respectively; the results were reported in figure 43.

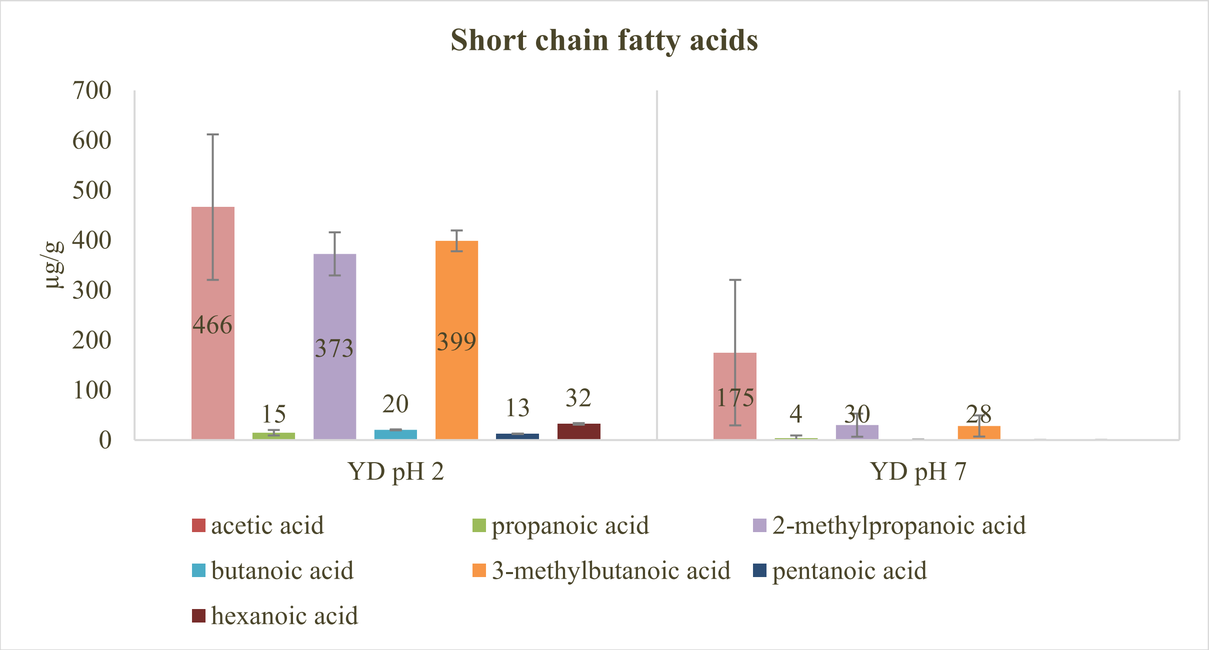


Figure 43. Short chain fatty acids detected in yeast derivative at pH 2 and pH 7. The results were expressed in μg per gram of yeast derivative. Data were means and standard deviation of three replicate. YD: yeast derivative.

Among the seven SCFA detected, the most representative was acetic acid, followed by isobutanoic and isovaleric acids, highly released from YD at both pH conditions. Except for acetic acid, with a content of 466 μg/g and 175 μg/g at pH 2 and pH 7 respectively, the other two abovementioned SCFA were released in gastric conditions in an amount 10 times higher compared to intestine-simulating condition (pH 7).

SCFA are produced by fermentative metabolism of yeasts [158] and represent the most abundant end products of bacteria in human intestine, as result of carbohydrates fermentation and amino acids metabolism [239]. The recent increasing interest on SCFA production by gut microbiota is mainly related to their positive effects on human health at gastro-intestinal level by modulating different processes, including immune response and inflammatory regulation [241]; furthermore, an antimicrobial activity against oral microorganisms was also observed [240]. Dietary intake of yeast derivative containing a good amount of SCFA might be helpful in enhancing these effects - especially in the first gastro-intestinal tract - due to the higher release of such compounds in acidic conditions. However, in a recent study carried out by Oba et al. [219], the authors observed how the addition of water soluble mannan from yeasts determined an increase in SCFA production by gut microbiota in *in vitro* human feces fermentation system. Considering above, the presence of both polysaccharides and nitrogen compounds (proteins and free amino acids) in yeast derivative might be used as substrate for fermentative metabolism by intestinal microbiota, possibly enhancing the production of SCFA.

5. Conclusion

Yeast derivative used in the present study has showed a non-negligible antioxidant property and a slight hypoglycemic effect due to the inhibition of α-amylase enzyme activity. Good ability to directly bind cholesterol was observed, up to 40 % and 30 % under simulating gastro-intestinal conditions; however, the contribution on binding bile salts (sodium cholate) with further reduction of lipids adsorption at intestinal level was also observed, even if in less intense manner. Lastly, YD was characterized by a certain amount of SCFA, thus potentially contributing to enhance the inflammatory response at least at the first gastrointestinal tract (stomach), condition that determined the highest release of such compounds.

Thanks to this bioactivity, YD obtained by ultrasounds treatment might be potentially employed as prebiotics and as dietary supplements for functional foods preparation or in pharmaceutical formulations.

Further investigations are needed in order to assess their impact on sensory attributes of supplemented foods, their sensory acceptability and their potential human health benefits also *in vivo* assays.

General conclusion

In light of the results obtained in the present research project, spontaneous non-*Saccharomyces* yeasts have showed a good aptitude to release antioxidant molecules and polysaccharides, highlighting their non-negligible contribution to enrich wine in substances involved in protection against oxidation, overall wine stability and quality.

The possibility to produce innovative yeast derivatives might be possible, by using non-*Saccharomyces* strains as starting material, as well as the chemical composition of the products might be managed by selecting appropriate yeast, for example by using high-polysaccharides and high-antioxidants producing strains. Moreover, the right management of growth conditions for biomass production as well as methods for inducing autolysis may also influence the release of compounds of enological interest, thus obtaining products with different chemical composition and specifically tailored for various applications in winemaking. In this regard, derivatives obtained from *Pichia* spp. or by applying ultrasounds may be useful for obtaining protein and yeast extracts, rich in soluble fraction, to be used as fermentation enhancers. The use of *Hanseniaspora* spp and *Torulaspora* spp. or the application of high-pressure treatment may be helpful for obtaining derivatives more characterized by polysaccharides and antioxidant molecules; their potential use both during fermentation and aging might potentially allow to reduce the amount of sulfur dioxide and improve the overall wine quality and stability, even for medium-low quality wines.

The use of non-*Saccharomyces* strains, as fermentation co-starters or for producing yeast derivatives, may potentially redefine and implement the current winemaking process. In this regard, the results of the present work underlined two possibilities: *i)* the use of selected strains as fermentation co-starters for subsequently modulating lees composition or *ii)* the use of selected non-*Saccharomyces* strains for producing yeast derivatives or synthetic fresh lees (also by applying appropriate emerging technologies), to be added to wines for managing their aging.

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Annexes

Annex 1. Output of the complete alignment of the sequenced strains. Source: NCBI, Blast.

C
Candida californica

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Query  3     GGA-TGCCTCAGTAGCGGGCGAGTGAAGCGGCAAGAGCTCAGATTTGAAATCGTGTTTTG   61
             ||| |||||||||||| |||||||||||||||||||||||||||||||||||||||||||
Sbjct  5      GGATTGCCTCAGTAGC-GGCGAGTGAAGCGGCAAGAGCTCAGATTTGAAATCGTGTTTTG   63

Query  62     GCACGAGTTGTAGAGTGTAGGTGGGAGTCTCTGCGGAGCACAGTGTCCAAGTCCCTTGGA   121
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  64    GCACGAGTTGTAGAGTGTAGGTGGGAGTCTCTGCGGAGCACAGTGTCCAAGTCCCTTGGA   123

Query  122    ACAGGGCGCCTGAGAGGGTGAGAGCCCCGTGGGGTGCTGTGCGAAGCTTTGAGGCCCTGC   181
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  124   ACAGGGCGCCTGAGAGGGTGAGAGCCCCGTGGGGTGCTGTGCGAAGCTTTGAGGCCCTGC   183

Query  182    TGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGCGGGTGGTAAATTCCATCTAAGGCT   241
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  184   TGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGCGGGTGGTAAATTCCATCTAAGGCT   243

Query  242    AAATATTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTT   301
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  244   AAATATTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAAAGCACTTT   303

Query  302    GAAAAGAGAGTGAAACAGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCCCGACATG   361
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  304   GAAAAGAGAGTGAAACAGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGCCCGACATG   363

Query  362    GGGAGTGCGCACCGCTGTCTCTTGTAGGCGGCGCTCTGGGCGCTCTCTGGGCCAGCATCG   421
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  364   GGGAGTGCGCACCGCTGTCTCTTGTAGGCGGCGCTCTGGGCGCTCTCTGGGCCAGCATCG   423

Query  422    GTTCCTGCTGCGAGAGAAAGGGTTCCGGAAAGTGGCTCTTCGGAGTGTTATAGCCGGGGC   481
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  424   GTTCCTGCTGCGAGAGAAAGGGTTCCGGAAAGTGGCTCTTCGGAGTGTTATAGCCGGGGC   483

Query  482    CAGATGTCGCGTGTGGGGACCGAGGACTGCGGCTTCTGTCTCGGATGCTGGCACAACGGC   541
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  484   CAGATGTCGCGTGTGGGGACCGAGGACTGCGGCTTCTGTCTCGGATGCTGGCACAACGGC   543

Query  542   GCAATACCGCCCGTCT  557
             ||||||||||||||||
Sbjct  544   GCAATACCGCCCGTCT  559
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H4

Hanseniaspora uvarum

```
Query  2    CCTTAGTAACGGGCGAGTGAAGCGGGTAAAAGCTCAAATTTGAAATCTGGTACTTTCAGT   61
            |||||||||| |||||||||||| ||||||||||||||||||||||||||||||||||||
Sbjct  11    CCTTAGTAAC-GGCGAGTGAAGC-GGTAAAAGCTCAAATTTGAAATCTGGTACTTTCAGT  68

Query  62    GCCCGAGTTGTAATTTGTAGAATTTGTCTTTGATTAGGTCCTTGTCTATGTTCCTTGGAA  121
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  69   GCCCGAGTTGTAATTTGTAGAATTTGTCTTTGATTAGGTCCTTGTCTATGTTCCTTGGAA   128

Query  122   CAGGACGTCATAGAGGGTGAGAATCCCGTTTGGCGAGGATACCTTTTCTCTGTAAGACTT  181
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  129  CAGGACGTCATAGAGGGTGAGAATCCCGTTTGGCGAGGATACCTTTTCTCTGTAAGACTT   188

Query  182   TTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAATTCCATCTAAA  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  189  TTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAATTCCATCTAAA   248

Query  242   GCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAAC  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  249  GCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAAGAAC   308

Query  302   TTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGAC  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  309  TTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATCAGAC   368

Query  362   ATGGTGTTTTTTGCATGCACTCGCCTCTCGTGGGCTTGGGCCTCTCAAAAATTTCACTGG  421
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  369  ATGGTGTTTTTTGCATGCACTCGCCTCTCGTGGGCTTGGGCCTCTCAAAAATTTCACTGG   428

Query  422   GCCAACATCAATTCTGGCAGCAGGATAAATCATTAAGAATGTAGCTACTTCGGTAGTGTT  481
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  429  GCCAACATCAATTCTGGCAGCAGGATAAATCATTAAGAATGTAGCTACTTCGGTAGTGTT   488

Query  482   ATAGCTTTTTGGAATACTGTTAGCCGGGATTGAGGACTGCGCTTCGGCAAGGATGTTGGC  541
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  489  ATAGCTTTTTGGAATACTGTTAGCCGGGATTGAGGACTGCGCTTCGGCAAGGATGTTGGC   548

Query  542  ATAATGGTTAAATGCCGCCCGTCTAA  567
            ||||||||||||||||||||||||||
Sbjct  549  ATAATGGTTAAATGCCGCCCGTCTAA  574
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H5

Hanseniaspora opuntiae

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Query  2    GGGA-T-CCTTAGTAACGGCGAGTGAAGCGGTAAAAGCTCAAATTTGAAATCTGGTACTT   59
            |||| | |||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  37    GGGATTACCTTAGTAACGGCGAGTGAAGCGGTAAAAGCTCAAATTTGAAATCTGGTACTT  96

Query  60    TCAGTGCCCGAGTTGTAATTTGTAGAATTTGTCTTTGATTAGGTCCTTGTCTATGTTCCT  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  97   TCAGTGCCCGAGTTGTAATTTGTAGAATTTGTCTTTGATTAGGTCCTTGTCTATGTTCCT   156

Query  120   TGGAACAGGACGTCATAGAGGGTGAGAATCCCGTTTGGCGAGGATACCTTTTCTCTGTAA  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  157  TGGAACAGGACGTCATAGAGGGTGAGAATCCCGTTTGGCGAGGATACCTTTTCTCTGTAA   216

Query  180   GACTTTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAATTCCAT  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  217  GACTTTTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAATTCCAT   276

Query  240   CTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAA  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  277  CTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAA   336

Query  300   AGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGAT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  337  AGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGAT   396

Query  360   CAGACATGGTGTTTTTTGCATGCACTCGCCTCTCGTGGGCTTGGGCCTCTCAAAAATTTC  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  397  CAGACATGGTGTTTTTTGCATGCACTCGCCTCTCGTGGGCTTGGGCCTCTCAAAAATTTC   456

Query  420   ACTGGGCCAACATCAATTCTGGCAGTAGGATAAATCATTAAGAATGTAGCTACCTCGGTA  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  457  ACTGGGCCAACATCAATTCTGGCAGTAGGATAAATCATTAAGAATGTAGCTACCTCGGTA   516

Query  480   GTGTTATAGCTTATTGGAATACTGCTAGCTGGGATTGAGGACTGCGCTTCGGCAAGGATG  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  517  GTGTTATAGCTTATTGGAATACTGCTAGCTGGGATTGAGGACTGCGCTTCGGCAAGGATG   576

Query  540  TTGGCATAATGGTTAAATGCCGCCCGTCTTGAACAACGGACCAAA  584
            |||||||||||||||||||||||||||||||||  ||||||||||
Sbjct  577  TTGGCATAATGGTTAAATGCCGCCCGTCTTGAAACACGGACCAAA  621
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M

Metschnikowia fructicola

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Query  14    GAGTGAAGCGGCAAAAGCTCAAATTTGAAATCCCCCGGGAATTGTAATTTGAAGAGATTT   73
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     GAGTGAAGCGGCAAAAGCTCAAATTTGAAATCCCCCGGGAATTGTAATTTGAAGAGATTT   60

Query  74    GGGTCCGGCCGGCAGGGGTTAAGTCCACTGGAAAGTGGCGCCACAGAGGGTGACAGCCCC   133
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    GGGTCCGGCCGGCAGGGGTTAAGTCCACTGGAAAGTGGCGCCACAGAGGGTGACAGCCCC   120

Query  134   GTGAACCCCTTTAACGCCTTCATCCCAGATCTCCAAGAGTCGAGTTGTTTGGGAATGCAG   193
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GTGAACCCCTTTAACGCCTTCATCCCAGATCTCCAAGAGTCGAGTTGTTTGGGAATGCAG   180

Query  194   CTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATACCGGCGAGAGACCGATAGCGAAC   253
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   CTCTAAGTGGGTGGTAAATTCCATCTAAAGCTAAATACCGGCGAGAGACCGATAGCGAAC   240

Query  254   AAGTACAGTGATGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAAAGTACGTGAAAT   313
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   AAGTACAGTGATGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAAAAGTACGTGAAAT   300

Query  314   TGTTGAAAGGGAAGGGCTTGCAAGCAGACACTTAACTGGGCCAGCATCGGGGCGGCGGGA   373
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   TGTTGAAAGGGAAGGGCTTGCAAGCAGACACTTAACTGGGCCAGCATCGGGGCGGCGGGA   360

Query  374   AACAAAACCACCGGGGAATGTACCTTTCGAGGATTATAACCCCGGTCTCTATTTCCTTGT   433
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   AACAAAACCACCGGGGAATGTACCTTTCGAGGATTATAACCCCGGTCTCTATTTCCTTGT   420

Query  434   TGCCCCGAGGCCTGCAATCTAAGGATGCTGGCGTAATGGTTGCAAGTCGCCCGTCT  489
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   TGCCCCGAGGCCTGCAATCTAAGGATGCTGGCGTAATGGTTGCAAGTCGCCCGTCT  476
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P2

Pichia kluyveri

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Query  1     CCAA-AGGGA-TGCCTCAGTAGCGGCGAGTGAAGCGGCAAGAGCTCAGATTTGAAATCTC   58
             |||| ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  28     CCAACAGGGATTGCCTCAGTAGCGGCGAGTGAAGCGGCAAGAGCTCAGATTTGAAATCTC  87

Query  59     ACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACGTGTGTGCAAGTC  118
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  88    ACCTAGTGTGCGAGTTGTAAATTGCAGGTTGGAGTCTCGGGTTAGACGTGTGTGCAAGTC   147

Query  119    CCTTGGAACAGGGTGCCACTGAGGGTGAGAGCCCCGTATCGTGCATGTCGACACCTGTGA  178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  148   CCTTGGAACAGGGTGCCACTGAGGGTGAGAGCCCCGTATCGTGCATGTCGACACCTGTGA   207

Query  179    GGCCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCAT  238
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  208   GGCCCTTCTGACGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCAT   267

Query  239    CTAAGGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAA  298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  268   CTAAGGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACTGTGAAGGAAAGATGAAA   327

Query  299    AGCACTTTGAAAAGAGAGTGAAACAGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGC  358
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  328   AGCACTTTGAAAAGAGAGTGAAACAGCACGTGAAATTGTTGAAAGGGAAGGGTATTGGGC   387

Query  359    TCGACATGGGATTTACGCATCGTTGCCTCTCGTGGGCGGCGCTCTGGGTTTTTCCTGGGC  418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  388   TCGACATGGGATTTACGCATCGTTGCCTCTCGTGGGCGGCGCTCTGGGTTTTTCCTGGGC   447

Query  419    CAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTCGGAGTGTTATA  478
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  448   CAGCATCGGTTTTCGTTGCAGGATAAGGACAATTGGAATGTGGCTCCTCGGAGTGTTATA   507

Query  479    GCCTTTTGTAGATGCTGCGTATGGGGACCGAGGGCTGCGGCGGACTCGTTTCGTCTCGGA  538
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  508   GCCTTTTGTAGATGCTGCGTATGGGGACCGAGGGCTGCGGCGGACTCGTTTCGTCTCGGA   567

Query  539   TGCTGGCACAACGGCGCAATACCGCCCGTCTTGAAAC  575
             |||||||||||||||||||||||||||||||||||||
Sbjct  568   TGCTGGCACAACGGCGCAATACCGCCCGTCTTGAAAC  604
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S2

Saccharomyces cerevisiae

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Query  6    GGGGATGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTGGTACCT  65
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8    GGGGATGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTGGTACCT  67

Query  66   TCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCC  125
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  68   TCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTATGTTCC  127

Query  126  TTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTA  185
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  128  TTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTCTTTGTA  187

Query  186  AAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCA  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  188  AAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAAATTCCA  247

Query  246  TCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAA  305
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  248  TCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAA  307

Query  306  AAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGA  365
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308  AAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGA  367

Query  366  TCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGGGTAGGGGAATCTCGCATTTCACTG  425
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  368  TCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGGGTAGGGGAATCTCGCATTTCACTG  427

Query  426  GGCCAGCATCAGTTTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGT  485
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428  GGCCAGCATCAGTTTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCGGTAAGT  487

Query  486  ATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAAGTCAAGGATG  545
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  488  ATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAAGTCAAGGATG  547

Query  546  CTGGCATAATGGTTATATGCCGCCCGTCTTGAAC  579
            ||||||||||||||||||||||||||||||||||
Sbjct  548  CTGGCATAATGGTTATATGCCGCCCGTCTTGAAC  581
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S5

Saccharomyces cerevisiae

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Query  1    CCAAAGGGGA-TGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTG   59
            |||| ||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  19   CCAACGGGGATTGCCTTAGTAACGGCGAGTGAAGCGGCAAAAGCTCAAATTTGAAATCTG   78

Query  60   GTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTA   119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  79   GTACCTTCGGTGCCCGAGTTGTAATTTGGAGAGGGCAACTTTGGGGCCGTTCCTTGTCTA   138

Query  120  TGTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTC   179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  139  TGTTCCTTGGAACAGGACGTCATAGAGGGTGAGAATCCCGTGTGGCGAGGAGTGCGGTTC   198

Query  180  TTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAA   239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  199  TTTGTAAAGTGCCTTCGAAGAGTCGAGTTGTTTGGGAATGCAGCTCTAAGTGGGTGGTAA   258

Query  240  ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAA   299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  259  ATTCCATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAA   318

Query  300  GATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGC   359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  319  GATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGC   378

Query  360  ATTTGATCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGGGTAGGGGAATCTCGCATT   419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  379  ATTTGATCAGACATGGTGTTTTGTGCCCTCTGCTCCTTGTGGGTAGGGGAATCTCGCATT   438

Query  420  TCACTGGGCCAGCATCAGTTTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCG   479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  439  TCACTGGGCCAGCATCAGTTTTGGTGGCAGGATAAATCCATAGGAATGTAGCTTGCCTCG   498

Query  480  GTAAGTATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAAGTCA   539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  499  GTAAGTATTATAGCCTGTGGGAATACTGCCAGCTGGGACTGAGGACTGCGACGTAAGTCA   558

Query  540  AGGATGCTGGCATAATGGTTATATGCCGCCCGTCTTGAAAC  580
            |||||||||||||||||||||||||||||||||||||||||
Sbjct  559  AGGATGCTGGCATAATGGTTATATGCCGCCCGTCTTGAAAC  599
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ST1
Starmerella bacillaris

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Query  1    CCAA-AGGGA-TGCCCTAGTAACGGCGAGTGAACAGGCAAGAGCTCAGATTTGAAAGGCA    58
             |||| ||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  16    CCAACAGGGATTGCCCTAGTAACGGCGAGTGAACAGGCAAGAGCTCAGATTTGAAAGGCA   75

Query  59    CTTTTGTGCCGTTGTATTCTGAAGTTAGGGTCCTGAGAAACGATGCTTAAGTCTTCTGGA   118
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  76   CTTTTGTGCCGTTGTATTCTGAAGTTAGGGTCCTGAGAAACGATGCTTAAGTCTTCTGGA    135

Query  119   AAGGAGCGCCATGGAGGGTGATAGCCCCGTCTAGCATTGACCTCATATAGGATCTTAACA   178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  136  AAGGAGCGCCATGGAGGGTGATAGCCCCGTCTAGCATTGACCTCATATAGGATCTTAACA    195

Query  179   TGGAGTCGAGTTGTTTGGGAATGCAGCTCAAATGGGTGGTATGCTCCATCTAAAGCTAAA   238
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  196  TGGAGTCGAGTTGTTTGGGAATGCAGCTCAAATGGGTGGTATGCTCCATCTAAAGCTAAA    255

Query  239   TATCTGCGAGAGACCGATAGTAAACAAGTACTGTGAGGGAAAGATGAAAAGAACTTTGAA   298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  256  TATCTGCGAGAGACCGATAGTAAACAAGTACTGTGAGGGAAAGATGAAAAGAACTTTGAA    315

Query  299   AAGAGAGTGAAAAAGTACGTGAAATTGTTGAAATGGAAGGGTAGGCCGCTAACCATGTAG   358
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  316  AAGAGAGTGAAAAAGTACGTGAAATTGTTGAAATGGAAGGGTAGGCCGCTAACCATGTAG    375

Query  359   AGCCGTGTTTGGGGGGAAGATAAATGCTGTAGAATGTAGCTCCTCGGAGTATTATAGATG   418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  376  AGCCGTGTTTGGGGGGAAGATAAATGCTGTAGAATGTAGCTCCTCGGAGTATTATAGATG    435

Query  419   CAGTTCATATTCCCACCCGAGCGCGAGGATCTCAGGTTCTACTAAATGGTGGTCTACCAC   478
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  436  CAGTTCATATTCCCACCCGAGCGCGAGGATCTCAGGTTCTACTAAATGGTGGTCTACCAC    495

Query  479  CCGTCTTGAACCACGGACCAA  499
             |||||||||||||||||||||
Sbjct  496  CCGTCTTGAACCACGGACCAA 516
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ST2

Starmerella bacillaris

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Query  1     CCAA-AGGGA-TGCCCTAGTAACGGCGAGTGAACAGGCAAGAGCTCAGATTTGAAAGGCA   58
             |||| ||||| |||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  29     CCAACAGGGATTGCCCTAGTAACGGCGAGTGAACAGGCAAGAGCTCAGATTTGAAAGGCA  88

Query  59     CTTTTGTGCCGTTGTATTCTGAAGTTAGGGTCCTGAGAAACGATGCTTAAGTCTTCTGGA  118
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  89    CTTTTGTGCCGTTGTATTCTGAAGTTAGGGTCCTGAGAAACGATGCTTAAGTCTTCTGGA   148

Query  119    AAGGAGCGCCATGGAGGGTGATAGCCCCGTCTAGCATTGACCTCATATAGGATCTTAACA  178
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  149   AAGGAGCGCCATGGAGGGTGATAGCCCCGTCTAGCATTGACCTCATATAGGATCTTAACA   208

Query  179    TGGAGTCGAGTTGTTTGGGAATGCAGCTCAAATGGGTGGTATGCTCCATCTAAAGCTAAA  238
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  209   TGGAGTCGAGTTGTTTGGGAATGCAGCTCAAATGGGTGGTATGCTCCATCTAAAGCTAAA   268

Query  239    TATCTGCGAGAGACCGATAGTAAACAAGTACTGTGAGGGAAAGATGAAAAGAACTTTGAA  298
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  269   TATCTGCGAGAGACCGATAGTAAACAAGTACTGTGAGGGAAAGATGAAAAGAACTTTGAA   328

Query  299    AAGAGAGTGAAAAAGTACGTGAAATTGTTGAAATGGAAGGGTAGGCCGCTAACCATGTAG  358
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  329   AAGAGAGTGAAAAAGTACGTGAAATTGTTGAAATGGAAGGGTAGGCCGCTAACCATGTAG   388

Query  359    AGCCGTGTTTGGGGGGAAGATAAATGCTGTAGAATGTAGCTCCTCGGAGTATTATAGATG  418
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  389   AGCCGTGTTTGGGGGGAAGATAAATGCTGTAGAATGTAGCTCCTCGGAGTATTATAGATG   448

Query  419    CAGTTCATATTCCCACCCGAGCGCGAGGATCTCAGGTTCTACTAAATGGTGGTCTACCAC  478
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  449   CAGTTCATATTCCCACCCGAGCGCGAGGATCTCAGGTTCTACTAAATGGTGGTCTACCAC   508

Query  479   CCGTCTTGAA-CAACCGGACC  498
             |||||||||| |||| |||||
Sbjct  509   CCGTCTTGAAACAAC-GGACC 528
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Annex 2. Effect of growth conditions on cell viability, biomass production and release of compounds of enological interest. Data were means and standard deviations (SD) of three replicates. Positive *and negative values indicated a release or a consumption respectively, in relation to the initial composition of culture medium*. The concentrations were expressed per liter and standardized to gram of biomass, except for amino acids. H: *Hanseniaspora* spp.; S: *Saccharomyces* spp.; - : growth without shaking to mimic anaerobic condition; + : growth with constant shaking (150 rpm) to mimic aerobic conditions.

Yeast	Temperature	Aeration	Sample code	Viable cells (log CFU/mL)	Biomass (g/L)	Amino acids (mg/L)	Mannans (mg/L)	Mannans (mg/g)	Riboflavin (µg/L)	Riboflavin (µg/g)	Glutathione (µmol/L)	Glutathione (µmol/g)
				Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Hanseniaspora spp.	30°C	-	H_30°C-	7.8 ± 0.1	1.6 ± 0.6	-417 ± 29	70 ± 58	49 ± 51	83 ± 10	58 ± 23	38.4 ± 0.6	26.2 ± 8.6
	22°C	-	H_22°C-	7.6 ± 0.1	1.6 ± 0.5	-518 ± 43	-15 ± 29	-9 ± 22	150 ± 11	105 ± 36	37.5 ± 0.3	25.8 ± 7.4
	30°C	+	H_30°C+	8.4 ± 0.0	3.3 ± 0.2	-343 ± 106	98 ± 15	30 ± 6	84 ± 12	26 ± 5	38.1 ± 0.4	11.5 ± 0.8
	22°C	+	H_22°C+	8.6 ± 0.1	3.2 ± 0.5	-678 ± 65	22 ± 10	7 ± 2	121 ± 7	39 ± 5	36.4 ± 0.5	11.7 ± 2.0
Saccharomyces spp.	30°C	-	S_30°C-	7.9 ± 0.1	9.2 ± 1.2	-397 ± 110	90 ± 5	10 ± 2	120 ± 7	13 ± 1	37.5 ± 0.2	4.1 ± 0.6
	22°C	-	S_22°C-	7.5 ± 0.7	10.3 ± 1.7	-610 ± 114	-30 ± 18	-3 ± 2	159 ± 26	15 ± 1	36.2 ± 0.3	3.6 ± 0.6
	30°C	+	S_30°C+	7.7 ± 0.1	5.2 ± 0.2	-323 ± 204	114 ± 24	22 ± 4	142 ± 3	27 ± 1	38.4 ± 0.8	7.4 ± 0.4
	22°C	+	S_22°C+	8.0 ± 0.1	3.9 ± 0.1	-579 ± 210	32 ± 13	8 ± 3	178 ± 13	45 ± 5	48.6 ± 1.5	12.4 ± 0.2

Annex 3. Chemical composition of yeast autolyzed suspensions after enzyme addition. Data were means and standard deviations (SD) of three replicates. The concentrations were expressed per liter and standardized to gram of biomass. H: *Hanseniaspora* spp.; S: *Saccharomyces* spp.; - : growth without shaking to mimic anaerobic condition; + : growth with constant shaking (150 rpm) to mimic aerobic conditions. RPC: reducing proteins containing cysteine.

Yeast	Temperature	Aeration	Sample code	Amino acids (mg/L)	Amino acids (mg/g)	Mannans (mg/L)	Mannans (mg/g)	Riboflavin (µg/L)	Riboflavin (µg/g)
				Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Hanseniaspora spp.	30°C	-	H_30°C -	49 ± 9	34 ± 14	316 ± 25	211 ± 57	59 ± 6	62 ± 22
	22°C	-	H_22°C -	14 ± 5	10 ± 5	212 ± 68	144 ± 63	50 ± 5	55 ± 19
	30°C	+	H_30°C +	165 ± 26	50 ± 11	1224 ± 45	370 ± 27	125 ± 7	59 ± 3
	22°C	+	H_22°C +	162 ± 60	54 ± 28	1165 ± 188	367 ± 13	157 ± 11	78 ± 14
Saccharomyces spp.	30°C	-	S_30°C -	95 ± 64	11 ± 7	194 ± 82	22 ± 10	49 ± 20	9 ± 4
	22°C	-	S_22°C -	46 ± 27	5 ± 4	125 ± 92	13 ± 12	24 ± 17	4 ± 3
	30°C	+	S_30°C +	496 ± 197	96 ± 40	360 ± 193	69 ± 39	176 ± 52	52 ± 16
	22°C	+	S_22°C +	573 ± 123	146 ± 32	660 ± 120	169 ± 35	166 ± 17	66 ± 6

Yeast	Temperature	Aeration	Sample code	Glutathione (µmol/L)	Glutathione (µmol/g)	Cysteine (µmol/L)	Cysteine (µmol/g)	RPC (µmol/L)	RPC (µmol/g)
				Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Hanseniaspora spp.	30°C	-	H_30°C -	51.8 ± 3.1	35.6 ± 12.9	38.2 ± 1.6	26.2 ± 9.1	nd[*]	nd
	22°C	-	H_22°C -	59.3 ± 11.8	42.0 ± 17.3	37.2 ± 3.6	26.0 ± 8.9	nd	nd
	30°C	+	H_30°C +	119.8 ± 8.0	36.3 ± 3.5	42.1 ± 3.2	12.7 ± 1.2	1636.8 ± 827.9	488.2 ± 227.8
	22°C	+	H_22°C +	166.9 ± 27.8	53.1 ± 9.4	62.9 ± 13.6	19.7 ± 1.4	1257.7 ± 148.3	398.0 ± 16.2
Saccharomyces spp.	30°C	-	S_30°C -	56.2 ± 18.0	6.3 ± 2.4	42.4 ± 10.6	4.7 ± 1.5	nd	nd
	22°C	-	S_22°C -	37.2 ± 10.3	3.8 ± 1.7	30.0 ± 1.8	3.0 ± 0.6	nd	nd
	30°C	+	S_30°C +	376.8 ± 96.7	72.1 ± 17.2	148.1 ± 31.3	28.4 ± 6.3	nd	nd
	22°C	+	S_22°C +	333.4 ± 20.1	85.2 ± 3.2	163.5 ± 5.3	41.8 ± 2.6	nd	nd

[*] nd: not detected.

Annex 4. Chemical composition of yeast derivatives powders. Data were means and standard deviations (SD) of three replicates. The concentrations were expressed per gram of powder. RSA: radical scavenging activity; RSA total fraction, determined on whole derivative; RSA soluble fraction, determined on the supernatant of derivative suspension. RPC: reducing proteins containing cysteine.

	Free amino acids (mg/g)	Soluble proteins (mg/g)	Mannan (mg/g)	Soluble colloids (mg/g)	Insoluble solids (mg/g)	Particle size (mean diameter nm)	Riboflavin (µg/g)	RSA total fraction	RSA soluble fraction	Cysteine (µmol/g)	Glutathione (µmol/g)	RPC (µmol/g)
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean + SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
S_ENZ	10 ± 1	58 ± 3	17 ± 4	111 ± 16	616 ± 49	1495 + 3	11 ± 1	29.4 ± 1.9	24.6 ± 2.4	1.6 ± 0.0	4.0 ± 0.4	66.5 ± 2.3
S_THERM	5 ± 0	62 ± 4	117 ± 10	187 ± 12	678 ± 7	657 + 1	7 ± 0	46.8 ± 6.7	28.5 ± 4.5	1.8 ± 0.1	5.0 ± 0.5	33.9 ± 2.0
S_US	32 ± 5	215 ± 37	116 ± 20	215 ± 25	289 ± 2	1184 + 4	18 ± 3	51.1 ± 7.4	41.6 ± 0.8	2.9 ± 0.4	3.6 ± 0.4	40.5 ± 5.5
S_HHP	9 ± 2	53 ± 13	11 ± 1	104 ± 7	492 ± 82	2068 + 1	19 ± 2	36.0 ± 4.0	28.2 ± 6.0	1.7 ± 0.0	9.0 ± 0.5	49.8 ± 2.4
T_ENZ	5 ± 4	71 ± 66	178 ± 9	327 ± 11	350 ± 9	662 + 4	14 ± 1	60.6 ± 7.1	41.1 ± 1.9	3.1 ± 0.2	7.2 ± 0.8	1.3 ± 0.5
T_THERM	3 ± 0	49 ± 4	62 ± 6	157 ± 11	659 ± 40	358 + 15	6 ± 0	51.9 ± 5.9	37.6 ± 4.6	1.7 ± 0.1	3.5 ± 0.3	nd*
T_US	4 ± 0	87 ± 17	84 ± 1	171 ± 7	619 ± 112	168 + 1	9 ± 1	36.1 ± 7.5	22.6 ± 4.2	2.5 ± 0.2	4.2 ± 0.7	nd*
T_HHP	5 ± 1	33 ± 8	21 ± 3	133 ± 5	823 ± 10	1276 + 4	13 ± 1	31.3 ± 3.4	18.7 ± 0.3	1.6 ± 0.0	3.3 ± 0.3	35.7 ± 3.7

*nd: not detected.

Annex 5. Volatile compounds tentatively identified by SPME-GC-MS in the headspace of the yeast derivative powders (YD), and in wines aged on yeast derivatives and control wines (W).

Compound	RI[a]	RI $_{(lit)}$[b]	IM[c]	Sample
ethyl butanoate	1040	1058	MS RI	W
2-methyl-1-propanol	1101	1'114	MS RI	W
3-methyl-1-butanol acetate	1117	1121	MS RI	W
2-methyl-1-propanol	1127	1114	MS RI	YD
3-methyl-1-butanol	1222	1211	MS RI S	YD W
ethyl hexanoate	1243	1230	MS RI	W
hexyl acetate	1298	1305	MS RI	W
2,5-dimethylpyrazine	1327	1316	MS RI	YD
2,6-dimethylpyrazine	1334	1319	MS RI	YD
ethyl lactate	1346	1340	MS RI	YD W
1-hexanol	1362	1359	MS RI S	YD W
cis-3-hexen-1-ol	1390	1386	MS RI	W
2,3,5-trimethylpyrazine	1409	1408	MS RI	YD
ethyl octanoate	1437	1441	MS RI S	YD W
2-ethyl-3,6-dimethylpyrazine	1452	1455	MS RI	YD
acetic acid	1453	1465	MS RI S	YD W
1-heptanol	1466	1461	MS RI	YD
2-ethyl-1-hexanol	1494	1484	MS RI S	W
ethyl nonanoate	1540	1541	MS RI	W
propanoic acid	1545	1540	MS RI S	YD
2,3-butanediol (levo)	1546	1526	MS RI	YD W
linalool	1552	1552	MS RI	W
1-octanol	1565	1565	MS RI	YD W
2-methylpropanoic acid	1573	1581	MS RI S	YD
2,3-butanediol (meso)	1583	1580	MS RI	YD W
1,2-propanediol	1595	1600	MS RI	YD W
γ-valerolactone	1611	1619	MS RI	YD
γ-butyrolactone	1621	1595	MS RI S	YD W
butanoic acid	1635	1628	MS RI S	YD W
ethyl decanoate	1641	1643	MS RI S	W
3-methylbutyl octanoate	1662	1658	MS RI	W
3-methylbutanoic acid	1675	1666	MS RI S	YD W
diethyl succinate	1678	1694	MS RI	W
ethyl-9-decenoate	1693	1694	MS RI	W
α-terpineol	1699	1692	MS RI	W
methionol	1720	1720	MS RI	YD W
pentanoic acid	1748	1744	MS RI	YD
1-decanol	1769	1769	MS RI	W
2-phenylethyl acetate	1814	1822	MS RI	W
hexanoic acid	1849	1857	MS RI S	YD W
ethyl 3-methylbutyl succinate	1903	1901	MS RI	W
2-phenylethanol	1913	1'922	MS RI S	YD W
2-ethylhexanoic acid	1955	1950	MS RI	YD

(continue)

Annex 5. *(continue)*

1-dodecanol	1966	1940	MS RI	W
diethyl malate	2044	2060	MS RI	W
ethyl tetradecanoate	2046	2043	MS RI	W
octanoic acid	2063	2070	MS RI S	YD W
decanoic acid	2273	2278	MS RI	YD W
ethyl hydrogen succinate	2372	2368	MS RI	W

[a] RI: calculated retention index

[b] RI (lit): retention index from literature, source NIST Chemistry WebBook [255]

[c] IM: identification method; MS: comparison of mass spectra with those reported in NIST 20 mass spectrum library; RI: comparison of retention index with those reported in literature; S: comparison of mass spectra and retention time with those of standard compounds.

Annex 6. Results of the semi-quantitative analysis carried out on the absolute area/1000 of volatile compounds detected in the headspace of yeast derivative powders. Data were means and standard deviations (SD) of three replicates. S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

	S_ENZ	S_THERM	S_US	S_HHP	T_ENZ	T_THERM	T_US	T_HHP
Compound	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
acetic acid	43218 ± 8095	945 ± 104	57344 ± 22384	6736 ± 7744	29812 ± 1323	570 ± 66	30453 ± 6223	3589 ± 638
propanoic acid	395 ± 146	0 ± 0	923 ± 480	215 ± 228	1078 ± 27	0 ± 0	1442 ± 65	90 ± 23
2-methylpropanoic acid	22729 ± 1877	1502 ± 196	55844 ± 7323	3393 ± 3545	67539 ± 1992	2303 ± 589	77494 ± 3521	2345 ± 412
butanoic acid	1086 ± 63	114 ± 10	2510 ± 253	363 ± 363	7507 ± 248	223 ± 33	8907 ± 466	398 ± 40
2-methylbutanoic acid	14327 ± 802	612 ± 127	32218 ± 3720	1208 ± 1224	38953 ± 838	907 ± 376	40822 ± 2950	1480 ± 216
pentanoic acid	nd*	nd	229 ± 245	118 ± 112	79 ± 24	nd	121 ± 17	nd
hexanoic acid	305 ± 26	nd	553 ± 68	99 ± 102	362 ± 52	nd	439 ± 29	nd
2-ethylhexanoic acid	302 ± 347	nd	163 ± 24	nd	nd	nd	nd	nd
octanoic acid	nd	nd	89 ± 10	178 ± 308	nd	nd	nd	nd
decanoic acid	nd	nd	nd	53 ± 92	nd	nd	nd	nd
2-methyl-1-propanol	55051 ± 21058	724 ± 222	43441 ± 14289	1184 ± 1146	30518 ± 13245	nd	22101 ± 8330	nd
3-methyl-1-butanol	181811 ± 21200	3733 ± 256	188879 ± 26851	7523 ± 8123	47489 ± 2711	1036 ± 513	26686 ± 1744	1465 ± 235
1-hexanol	966 ± 86	nd	1030 ± 105	75 ± 65	1053 ± 121	nd	1564 ± 199	nd
1-heptanol	712 ± 188	nd	1012 ± 385	nd	144 ± 23	nd	402 ± 43	nd
1-octanol	nd	nd	651 ± 199	nd	108 ± 4	nd	138 ± 42	nd
2-phenylethanol	5529 ± 757	60 ± 15	9379 ± 1193	141 ± 116	3925 ± 284	nd	1665 ± 319	330 ± 42
2,3-butanediol (levo)	22216 ± 466	135 ± 46	12470 ± 3706	319 ± 394	15034 ± 993	108 ± 40	2506 ± 380	437 ± 19
2,3-butanediol (meso)	1841 ± 62	nd	3284 ± 967	143 ± 248	2275 ± 147	nd	1212 ± 127	170 ± 143
1,2-propanediol	12223 ± 491	96 ± 8	3582 ± 191	619 ± 693	1469 ± 18	nd	430 ± 91	237 ± 27
ethyl lactate	nd	nd	774 ± 247	nd	1525 ± 806	nd	249 ± 83	nd
ethyl octanoate	152 ± 59	nd	129 ± 74	nd	62 ± 2	nd	nd	nd
γ-valerolactone	0 ± 0	nd	1199 ± 497	nd	517 ± 59	nd	441 ± 144	nd
γ-butyrolactone	1015 ± 29	51 ± 11	2470 ± 1111	64 ± 66	1326 ± 172	nd	1073 ± 217	76 ± 21

(continue)

Annex 6. *(continue)*

2,5-dimethylpyrazine	nd	nd	212 ± 78	nd	577 ± 32	nd	225 ± 68	nd
2,6-dimethylpyrazine	nd	nd	397 ± 86	nd	1016 ± 116	nd	1417 ± 231	nd
2, 3, 5-trimethylpyrazine	202 ± 68	nd	154 ± 18	nd	163 ± 19	nd	138 ± 23	nd
2-ethyl-3,6-dimethylpyrazine	479 ± 318	nd	108 ± 95	nd	995 ± 114	nd	217 ± 116	nd
methionol	nd	nd	455 ± 153	nd	290 ± 9	nd	67 ± 16	nd

*nd: not detected

Annex 7. Results of the semi-quantitative analysis carried out on the concentrations (µg/L) of volatile compounds detected in the headspace of wines after two months of aging. Data were means and standard deviations (SD) of three replicates. Different letters within the same row marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. CON: Control; S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

	CON	S_ENZ	S_THERM	S_US	S_HHP	T_ENZ	T_THERM	T_US	T_HHP
Compound	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
acetic acid	3.9 ± 0.2 ab	4.2 ± 0.8 abc	8.9 ± 2.9 abc	11.0 ± 1.8 abc	11.9 ± 4.5 c	3.5 ± 0.5 a	11.4 ± 5.6 bc	8.7 ± 1.9 abc	8.0 ± 0.7 abc
butanoic acid	1.0 ± 0.2	0.9 ± 0.1	1.6 ± 0.3	1.8 ± 0.7	1.6 ± 0.8	0.6 ± 0.1	2.4 ± 1.5	1.5 ± 0.3	0.7 ± 0.6
3-methylbutanoic acid	0.9 ± 0.0	0.9 ± 0.1	2.1 ± 0.5	2.9 ± 1.4	2.1 ± 0.9	0.9 ± 0.1	3.4 ± 1.9	15.3 ± 22.3	1.1 ± 1.0
hexanoic acid	32.8 ± 1.5 a	26.5 ± 6.8 a	72.2 ± 17.1 ab	97.2 ± 36.3 b	69.0 ± 14.5 ab	27.2 ± 6.1 a	96.4 ± 39.2 b	76.9 ± 10.8 ab	51.1 ± 8.1 ab
octanoic acid	90.5 ± 6.8 a	72.7 ± 17.7 a	187.2 ± 53.4 ab	257.6 ± 92.6 b	176.9 ± 9.5 ab	75.4 ± 26.9 a	239.0 ± 68.4 b	198.3 ± 44.6 ab	133.1 ± 34.5 ab
decanoic acid	6.2 ± 0.3 a	4.5 ± 1.2 a	16.0 ± 5.2 ab	22.5 ± 6.9 b	13.3 ± 2.8 ab	4.8 ± 2.2 a	21.5 ± 6.6 b	16.8 ± 6.1 ab	9.5 ± 5.6 ab
2-methyl-1-propanol	11.1 ± 7.9 b	nd nd	nd	nd	nd	nd	nd	nd	nd
3-methyl-1-butanol	362.6 ± 9.0	345.1 ± 55.9	484.0 ± 70.7	667.4 ± 307.1	489.9 ± 141.0	328.1 ± 12.1	691.4 ± 479.4	531.1 ± 126.8	396.0 ± 48.0
1-hexanol	15.9 ± 0.9 a	13.6 ± 2.3 a	33.4 ± 6.5 ab	39.9 ± 9.4 ab	33.5 ± 12.6 ab	11.5 ± 2.4 a	52.5 ± 34.0 b	33.3 ± 2.1 ab	23.8 ± 2.1 ab
cis-3-hexen-1-ol	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-ethyl-1-hexanol	8.7 ± 1.9 ab	6.3 ± 0.9 a	17.6 ± 0.6 ab	23.1 ± 10.0 ab	8.7 ± 4.2 ab	6.2 ± 0.5 a	24.3 ± 13.9 b	19.6 ± 4.8 ab	6.1 ± 0.8 a
1-octanol	1.2 ± 0.1 a	1.1 ± 0.3 a	3.0 ± 0.3 ab	3.5 ± 1.2 ab	2.9 ± 0.8 ab	1.0 ± 0.3 a	3.9 ± 2.1 b	3.0 ± 0.2 ab	1.9 ± 0.3 ab
1-decanol	0.6 ± 0.1	0.4 ± 0.1	1.6 ± 0.6	1.5 ± 0.6	1.4 ± 0.4	0.5 ± 0.1	1.6 ± 0.7	1.3 ± 0.4	0.7 ± 0.6
2-phenylethanol	33.0 ± 1.5 a	29.5 ± 4.8 a	68.2 ± 17.7 ab	93.9 ± 38.9 b	67.0 ± 9.8 ab	27.5 ± 6.2 a	91.9 ± 36.3 b	74.7 ± 17.0 ab	49.7 ± 8.1 ab
1-dodecanol	nd	nd	nd	nd	nd	nd	nd	nd	nd
2,3-butanediol (levo)	5.5 ± 0.1 ab	5.7 ± 1.3 abc	13.0 ± 5.4 ab	16.3 ± 4.0 b	16.2 ± 4.4 b	4.8 ± 0.6 a	16.7 ± 7.6 b	13.1 ± 2.6 ab	9.5 ± 2.9 ab
2,3-butanediol (meso)	1.6 ± 0.0 ab	1.6 ± 0.2 ab	3.7 ± 1.2 abc	4.6 ± 1.2 bc	4.0 ± 0.8 abc	1.4 ± 0.1 a	5.1 ± 2.4 c	3.8 ± 0.5 abc	2.3 ± 0.9 abc
1,2-propanediol	0.4 ± 0.0	0.4 ± 0.1	0.6 ± 0.2	0.7 ± 0.2	0.8 ± 0.1	0.3 ± 0.1	0.8 ± 0.4	0.5 ± 0.2	0.3 ± 0.3
ethyl butanoate	nd	nd	nd	nd	nd	nd	nd	nd	nd
3-methyl-1-butanol acetate	76.4 ± 5.3	71.5 ± 23.4	139.1 ± 38.0	128.1 ± 8.3	121.7 ± 15.4	69.4 ± 4.2	152.2 ± 78.8	103.9 ± 24.6	119.6 ± 19.7
ethyl hexanoate	211.9 ± 27.8 a	235.5 ± 31.7 a	481.8 ± 104.9 bcd	508.4 ± 21.6 cd	341.4 ± 48.2 abc	250.7 ± 19.8 ab	588.6 ± 200.0 d	474.7 ± 49.9 bcd	407.1 ± 37.4 abcd
hexyl acetate	14.2 ± 2.6	14.5 ± 2.2	24.2 ± 10.9	23.7 ± 9.3	171.4 ± 255.2	15.0 ± 2.2	32.0 ± 8.3	30.7 ± 3.0	23.4 ± 3.3
ethyl lactate	64.5 ± 3.7	66.8 ± 4.8	49.7 ± 5.1	58.0 ± 13.9	51.8 ± 18.8	56.1 ± 7.0	65.1 ± 31.2	60.0 ± 16.5	35.0 ± 16.7
ethyl octanoate	452.9 ± 62.0 abc	396.2 ± 69.2 a	613.9 ± 35.7 abc	662.2 ± 145.7 c	640.5 ± 121.0 bc	410.3 ± 16.4 ab	605.9 ± 45.3 abc	614.9 ± 80.0 abc	462.3 ± 99.9 abc
ethyl nonanoate	1.3 ± 0.5	1.6 ± 0.7	2.4 ± 1.1	2.3 ± 0.7	2.2 ± 1.1	1.1 ± 0.5	1.3 ± 1.1	2.4 ± 0.6	0.8 ± 0.7

(continue)

Annex 7. *(continue)*

ethyl decanoate	43.3 ± 6.3	32.6 ± 1.9	48.1 ± 17.3	73.2 ± 54.6	57.2 ± 26.0	30.7 ± 4.2	57.4 ± 14.4	61.0 ± 31.2	36.9 ± 14.7
3-methylbutyl octanoate	1.2 ± 0.2	1.0 ± 0.2	1.9 ± 1.4	3.8 ± 3.9	1.4 ± 0.7	1.0 ± 0.2	2.8 ± 1.1	3.0 ± 2.4	1.2 ± 0.1
diethyl succinate	14.2 ± 0.7 a	12.5 ± 2.6 a	34.8 ± 9.1 ab	46.4 ± 16.5 b	34.7 ± 6.4 ab	12.4 ± 2.7 a	47.1 ± 20.6 b	37.5 ± 6.1 ab	25.9 ± 3.7 ab
ethyl-9-decenoate	7.6 ± 0.9	5.1 ± 0.4	10.1 ± 3.3	12.7 ± 7.8	12.2 ± 7.2	5.7 ± 0.7	10.8 ± 2.4	11.1 ± 4.4	7.6 ± 3.2
2-phenylethyl acetate	3.5 ± 0.2 ab	2.9 ± 0.6 a	7.0 ± 0.6 ab	9.5 ± 5.0 b	6.9 ± 1.5 ab	2.9 ± 0.7 ab	9.5 ± 4.2 ab	7.4 ± 1.5 ab	4.9 ± 0.2 ab
ethyl 3-methylbutyl succinate	0.2 ± 0.0 a	0.2 ± 0.1 a	0.6 ± 0.0 a	0.7 ± 0.4 a	0.7 ± 0.1 a	27.9 ± 6.5 b	0.8 ± 0.2 a	0.7 ± 0.1	0.3 ± 0.3 a
diethyl malate	0.5 ± 0.1	0.6 ± 0.2	1.3 ± 0.8	1.6 ± 0.3	1.5 ± 0.7	0.3 ± 0.2	1.0 ± 0.6	1.2 ± 0.5	0.6 ± 0.7
ethyl tetradecanoate	nd	nd	nd	nd	nd	nd	nd	nd	nd
ethyl hydrogen succinate	0.5 ± 0.1	0.5 ± 0.1	4.4 ± 4.1	5.3 ± 1.4	4.0 ± 3.2	0.2 ± 0.2	3.5 ± 4.3	4.2 ± 3.5	1.9 ± 3.1
γ-butyrolactone	nd	nd	nd	nd	nd	nd	nd	nd	nd
methionol	0.3 ± 0.1	0.2 ± 0.1	0.6 ± 0.1	0.7 ± 0.3	0.5 ± 0.1	0.2 ± 0.1	0.8 ± 0.5	0.6 ± 0.0	0.3 ± 0.2
linalool	1.2 ± 0.0	1.0 ± 0.2	1.5 ± 0.4	2.3 ± 1.8	2.2 ± 1.0	1.0 ± 0.2	1.8 ± 0.8	1.7 ± 1.0	1.5 ± 0.1
α-terpineol	0.8 ± 0.0 ab	0.6 ± 0.1 a	2.0 ± 0.4 abc	2.6 ± 0.9 bc	1.9 ± 0.6 abc	0.7 ± 0.1 a	2.9 ± 1.5 c	2.2 ± 0.2 abc	1.3 ± 0.2 abc

[*] nd: not detected

Annex 8. Results of the semi-quantitative analysis carried out on the concentrations (µg/L) of volatile compounds detected in the headspace of wines after six months of aging. Data were means and standard deviations (SD) of three replicates. Different letters within the same row marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. CON: Control; S: *S. cerevisiae*; T: *T. delbrueckii*; ENZ: enzyme addition; THERM: thermal inactivation; US: ultrasounds; HHP: high hydrostatic pressure.

	CON	S_ENZ	S_THERM	S_US	S_HHP	T_ENZ	T_THERM	T_US	T_HHP
Compound	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
acetic acid	9.9 ± 1.0	9.8 ± 0.9	9.6 ± 0.5	9.5 ± 0.9	9.3 ± 0.6	9.5 ± 0.5	11.6 ± 1.6	10.2 ± 0.8	10.1 ± 0.8
butanoic acid	1.4 ± 0.1 c	1.2 ± 0.0 bc	0.9 ± 0.1 a	1.2 ± 0.0 bc	1.1 ± 0.1 ab	1.1 ± 0.1 ab	1.0 ± 0.1 ab	1.1 ± 0.1 ab	1.1 ± 0.1 ab
3-methylbutanoic acid	1.3 ± 0.1	1.3 ± 0.1	1.0 ± 0.0	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.0	1.3 ± 0.3	1.1 ± 0.1
hexanoic acid	47.5 ± 1.7 c	39.4 ± 4.0 bc	28.7 ± 5.2 a	37.2 ± 3.6 ab	35.0 ± 3.6 ab	34.2 ± 2.6 ab	28.4 ± 0.4 a	37.1 ± 2.0 ab	35.8 ± 1.9 ab
octanoic acid	107.0 ± 6.9 c	96.4 ± 9.0 bc	57.1 ± 15.5 a	90.0 ± 7.8 bc	78.5 ± 11.6 ab	75.1 ± 8.5 ab	60.9 ± 3.2 a	88.4 ± 7.4 bc	93.3 ± 4.0 bc
decanoic acid	9.0 ± 1.0 d	8.5 ± 0.5 cd	3.1 ± 0.6 a	7.8 ± 0.2 cd	4.8 ± 0.8 ab	5.1 ± 0.9 ab	4.2 ± 0.4 ab	8.3 ± 1.3 cd	6.4 ± 1.2 bc
2-methyl-1-propanol	6.1 ± 1.9	4.4 ± 1.8	6.6 ± 0.7	5.7 ± 2.2	5.2 ± 2.4	5.9 ± 1.7	5.0 ± 1.3	5.2 ± 1.5	3.4 ± 0.7
3-methyl-1-butanol	367.3 ± 50.6 b	320.4 ± 63.7 b	238.7 ± 139.9 ab	305.4 ± 49.1 b	74.1 ± 13.0 a	311.7 ± 72.4 b	184.0 ± 25.0 ab	357.2 ± 52.8 bc	210.3 ± 11.7 ab
1-hexanol	37.8 ± 26.5	18.4 ± 1.5	16.8 ± 1.0	17.6 ± 1.7	18.6 ± 1.6	18.7 ± 0.7	16.4 ± 0.5	18.5 ± 1.5	18.2 ± 0.6
cis-3-hexen-1-ol	0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.0	0.8 ± 0.2
2-ethyl-1-hexanol	33.5 ± 4.6 e	22.9 ± 3.7 d	15.1 ± 1.0 abc	22.2 ± 3.2 cd	14.4 ± 0.9 ab	21.3 ± 0.7 bcd	15.2 ± 2.0 abc	20.2 ± 1.1 abcd	13.9 ± 1.5 a
1-octanol	1.9 ± 0.1 d	1.7 ± 0.1 cd	1.2 ± 0.2 ab	1.4 ± 0.2 bc	1.5 ± 0.1 bcd	1.5 ± 0.0 bcd	0.9 ± 0.2 a	1.5 ± 0.1 bcd	1.3 ± 0.2 abc
1-decanol	nd	nd	nd	nd	nd	nd	nd	nd	nd
2-phenylethanol	41.1 ± 3.8 c	40.1 ± 4.5 c	26.2 ± 5.1 a	36.0 ± 2.8 abc	32.7 ± 5.6 abc	28.4 ± 2.9 ab	26.6 ± 2.0 a	33.0 ± 1.1 abc	38.2 ± 1.3 bc
1-dodecanol	0.8 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.8 ± 0.5	0.5 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
2,3-butanediol (levo)	13.2 ± 1.1 b	11.0 ± 1.2 ab	10.0 ± 1.2 a	10.7 ± 0.3 ab	11.3 ± 1.4 ab	10.4 ± 1.3 ab	10.7 ± 0.6 ab	10.7 ± 0.5 ab	11.0 ± 0.8 ab
2,3-butanediol (meso)	4.5 ± 0.4 b	4.0 ± 0.2 ab	3.2 ± 0.3 a	3.9 ± 0.3 ab	3.7 ± 0.5 ab	3.5 ± 0.2 ab	3.9 ± 0.4 ab	4.2 ± 0.9 ab	3.7 ± 0.2 ab
1,2-propanediol	0.7 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.6 ± 0.3	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	0.6 ± 0.1
ethyl butanoate	10.3 ± 0.9 b	9.5 ± 1.1 ab	7.5 ± 0.7 ab	10.0 ± 0.4 b	8.7 ± 1.3 ab	8.4 ± 0.8 ab	6.9 ± 1.8 a	10.3 ± 0.9 ab	8.1 ± 1.2 ab
3-methyl-1-butanol acetate	49.1 ± 4.4 b	45.1 ± 4.1 ab	40.5 ± 1.9 ab	44.0 ± 2.8 ab	45.9 ± 4.3 ab	43.3 ± 3.1 ab	38.3 ± 1.5 a	44.6 ± 3.3 ab	44.9 ± 1.1 ab
ethyl hexanoate	171.8 ± 66.3 ab	143.4 ± 48.0 ab	199.1 ± 175.1 ab	111.4 ± 22.5 a	360.2 ± 26.0 b	141.4 ± 103.6 ab	257.7 ± 18.0 ab	74.5 ± 20.3 a	237.7 ± 71.0 ab
hexyl acetate	12.6 ± 1.9 b	13.8 ± 0.8 ab	11.2 ± 1.5 ab	11.2 ± 0.8 ab	11.1 ± 1.3 ab	13.6 ± 1.2 b	9.6 ± 0.4 a	11.5 ± 1.0 ab	10.8 ± 0.3 ab
ethyl lactate	47.5 ± 4.0 b	37.4 ± 3.8 ab	33.9 ± 1.2 a	38.1 ± 5.0 ab	34.5 ± 7.0 a	36.3 ± 2.6 ab	41.3 ± 3.6 ab	35.3 ± 3.0 ab	33.9 ± 6.4 a
ethyl octanoate	535.2 ± 69.4 ab	693.4 ± 14.1 c	533.6 ± 103.9 ab	667.7 ± 61.9 bc	567.8 ± 43.7 abc	683.1 ± 28.0 bc	472.4 ± 37.0 a	718.2 ± 52.6 c	489.5 ± 24.6 a
ethyl nonanoate	2.7 ± 0.0 abc	3.4 ± 0.1 bc	1.2 ± 0.3 ab	4.1 ± 1.2 c	0.7 ± 0.2 a	3.7 ± 0.6 c	1.3 ± 0.1 ab	4.0 ± 1.8 c	0.8 ± 0.3 a

(continue)

Annex 8. *(continue)*

ethyl decanoate	35.1 ± 3.8 ab	57.4 ± 5.1 abc	40.8 ± 17.3 abc	62.3 ± 15.5 abc	40.8 ± 1.8 abc	63.3 ± 13.0 bc	39.2 ± 9.2 abc	67.1 ± 16.1 c	30.5 ± 4.9 a
3-methylbutyl octanoate	1.2 ± 0.1 a	2.1 ± 0.1 ab	1.4 ± 0.9 a	2.4 ± 0.6 ab	1.7 ± 0.1 ab	2.9 ± 0.8 b	1.6 ± 0.3 ab	2.4 ± 0.4 ab	1.3 ± 0.2 a
diethyl succinate	25.2 ± 1.3 d	23.6 ± 2.2 cd	16.3 ± 3.1 ab	21.7 ± 1.8 cd	20.0 ± 2.4 abcd	19.5 ± 1.5 abc	15.5 ± 0.6 a	21.2 ± 1.0 bcd	20.8 ± 0.9 bcd
ethyl-9-decenoate	7.0 ± 1.0 abc	10.5 ± 1.4 bc	6.3 ± 2.3 ab	10.0 ± 1.7 bc	7.3 ± 0.4 abc	11.0 ± 2.4 c	5.3 ± 1.2 a	11.1 ± 1.6 c	5.3 ± 0.4 a
2-phenylethyl acetate	2.6 ± 0.2 c	2.5 ± 0.1 bd	1.6 ± 0.4 a	2.4 ± 0.2 bc	2.2 ± 0.2 bc	2.0 ± 0.2 ab	1.5 ± 0.1 a	2.3 ± 0.2 bc	2.3 ± 0.1 bc
ethyl 3-methylbutyl succinate	nd	nd	nd	nd	nd	nd	nd	nd	nd
diethyl malate	0.9 ± 0.2 abc	1.2 ± 0.2 bc	0.9 ± 0.1 abc	0.5 ± 0.0 a	0.8 ± 0.3 abc	0.6 ± 0.3 a	0.8 ± 0.2 abc	0.7 ± 0.1 ab	1.4 ± 0.3 c
ethyl tetradecanoate	0.7 ± 0.2 ab	0.5 ± 0.1 ab	0.8 ± 0.1 ab	0.5 ± 0.0 a	0.7 ± 0.2 ab	0.5 ± 0.0 a	0.8 ± 0.2 b	0.7 ± 0.1 ab	0.5 ± 0.0 a
ethyl hydrogen succinate	2.2 ± 0.6 bc	3.1 ± 0.2 bc	0.4 ± 0.1 a	2.5 ± 0.2 bc	2.3 ± 0.5 bc	2.0 ± 0.5 b	2.3 ± 0.7 bc	2.2 ± 0.6 bc	3.5 ± 0.6 c
γ-butyrolactone	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.0	0.7 ± 0.1
methionol	0.3 ± 0.0 bc	0.3 ± 0.0 bc	0.2 ± 0.1 b	0.3 ± 0.0 c	0.2 ± 0.1 bc	0.2 ± 0.0 bc	0.0 ± 0.0 a	0.3 ± 0.1 c	0.2 ± 0.1 bc
linalool	0.7 ± 0.1 bc	0.8 ± 0.1 c	0.6 ± 0.1 ab	0.8 ± 0.0 c	0.7 ± 0.1 bc	0.8 ± 0.1 c	0.5 ± 0.0 a	0.8 ± 0.1 c	0.7 ± 0.1 abc
α-terpineol	1.2 ± 0.2 c	0.9 ± 0.1 bc	0.5 ± 0.2 a	0.8 ± 0.1 b	0.7 ± 0.1 ab	0.8 ± 0.1 ab	0.5 ± 0.0 a	0.9 ± 0.1 b	0.7 ± 0.1 ab

[*]nd: not detected

Annex 9. Volatile compounds tentatively identified by SPME-GC-MS in the headspace of synthetic wines after alcoholic fermentation (Sf), synthetic wines after aging (Sa) and white wines after aging (RW).

	Compound	RI^{a}	RI $_{(lit)}{}^{b}$	IM^{c}	Sample
1	ethyl acetate	895	890	MS RI	W
2	ethyl propanoate	967	966	MS RI	W
3	ethyl 2-methylpropanoate	975	975	MS RI	W
4	2-methyl-1-propanol acetate	1019	1018	MS RI	Sf Sa W
5	ethyl butanoate	1044	1044	MS RI	Sf Sa W
6	1-propanol	1047	1025	MS RI	Sf Sa W
7	ethyl 2-methylbutanoate	1060	1063	MS RI	Sa W
8	ethyl 3-methylbutanoate	1074	1075	MS RI	Sa W
9	hexanal	1088	1083	MS RI	Sa W
10	2-methyl-1-propanol	1098	1094	MS RI	Sf Sa W
11	3-methyl-1-butanol acetate	1128	1126	MS RI	Sf Sa W
12	2-methyl-1-butanol acetate	1132	1134	MS RI	W
13	β-myrcene	1168	1164	MS RI	W
14	3-methyl-1-butanol propanoate	1194	1184	MS RI	W
15	3-methylbutyl 2-methylpropanoate	1197	1190	MS RI	W
16	d-limonene	1202	1202	MS RI	W
17	3-methyl-1-butanol	1213	1211	MS RI S	Sf Sa W
18	ethyl hexanoate	1241	1241	MS RI	Sf Sa W
19	3-methylbutyl butanoate	1271	1270	MS RI	W
20	hexyl acetate	1278	1276	MS RI	W
21	methyl heptanoate	1292	1290	MS RI	W
22	acetoin	1294	1287	MS RI	W
23	ethyl 3-hexenoate	1306	1303	MS RI	W
24	ethyl 3-hexenoate (isomer)	1309	1290	MS RI	W
25	4-methyl-1-pentanol	1309	1290	MS RI	W
26	propyl hexanoate	1316	1318	MS RI	W
27	linalool like	1325	1312	MS RI	W
28	3-methyl-1-pentanol	1328		MS	W
29	ethyl nonanoate	1330	1334	MS RI	Sa W
30	ethyl lactate	1350	1352	MS RI	W
31	1-hexanol	1356	1356	MS RI S	W
32	2-methylpropyl hexanoate	1359	1351	MS RI	W
33	*trans*-3-hexen-1-ol	1367	1366	MS RI	W
34	*cis*-3-hexen-1-ol	1387	1386	MS RI	W
35	nonanal	1389	1396	MS RI	Sa W
36	methyl octanoate	1395	1390	MS RI	W
37	ethyl 5-heptenoate	1398		MS	W
38	2-butoxyethanol	1406	1391	MS RI	W
39	ethyl 6-heptenoate	1411		MS	W

(continue)

Annex 9. *(continue)*

40	*cis*-2-hexen-1-ol	1417	1420	MS RI	W
41	ethyl octanoate	1446	1431	MS RI S	Sf Sa W
42	acetic acid	1458	1447	MS RI S	Sf W
43	3-methylbutyl hexanoate	1465	1453	MS RI	W
44	2-furfural	1476	1468	MS RI	W
45	octyl acetate	1478	1476	MS RI	W
46	nerol oxide	1480	1471	MS RI	W
47	2-ethyl-1-hexanol	1490	1484	MS RI S	Sa W
48	geranylethyl ether	1514	1506	MS RI	W
49	geranylethyl ether (isomer)	1514		MS	W
50	2-nonanol	1518	1508	MS RI	W
51	propyl octanoate	1524	1526	MS RI	W
52	2,3-butanediol (levo)	1539	1530	MS RI	Sf
53	linalool	1548	1552	MS RI	W
54	2-methylpropyl octanoate	1557	1550	MS RI	W
55	1-octanol	1558	1564	MS RI	W
56	2-methylpropanoic acid	1563	1574	MS RI S	Sf Sa W
57	3-methylbutyl heptanoate	1563	1552	MS RI	Sa W
58	TDN	1570	1567	MS RI	W
59	2,3-butanediol (meso)	1575	1567	MS RI	Sf
60	3-methylbutyl lactate	1575	1572	MS RI	W
61	methyl decanoate	1599	1592	MS RI	W
62	butyrolactone	1609	1595	MS RI S	Sf Sa
63	*trans*-γ-caryophyllene	1611	1613	MS RI	W
64	ethyl furoate	1632	1611	MS RI	W
65	butanoic acid	1633	1628	MS RI S	Sa W
66	ethyl decanoate	1650	1634	MS RI S	Sf Sa W
67	1-nonanol	1661	1666	MS RI	W
68	3-methylbutyl octanoate	1665	1658	MS RI	Sf Sa W
69	citronellol acetate	1672	1655	MS RI	W
70	3-methylbutanoic acid	1674	1666	MS RI S	Sf Sa W
71	2-methyl butanoic acid	1675	1674	MS RI	W
72	diethyl succinate	1681	1679	MS RI	Sf Sa W
73	decyl acetate	1684	1679	MS RI	W
74	ethyl 9-decenoate	1695	1694	MS RI	Sf Sa W
75	α-terpineol	1704	1692	MS RI	W
76	norisoprenoid-like (m/z 163)	1707		MS	W
77	dodecanal	1718	1723	MS RI	W
78	methionol	1724	1720	MS RI	Sa W
79	vitispirane-like (m/z 192)	1727		MS	W
80	norisoprenoid-like (m/z 163)	1738		MS	W
81	ethyl undecanoate	1745	1737	MS RI	W

(continue)

Annex 9. *(continue)*

82	norisoprenoid-like (m/z 138)	1752		MS	W
83	2-methylpropyl decanoate	1759	1750	MS RI	W
84	1-decanol	1762	1769	MS RI	Sa W
85	TDN	1762	1724	MS RI	W
86	naphthalene	1763	1762	MS RI	W
87	citronellol	1766	1755	MS RI	W
88	diethyl glutarate	1784	1774	MS RI	W
89	ethyl phenylacetate	1796	1793	MS RI	W
90	methyl dodecanoate	1806	1802	MS RI	W
91	2-phenethyl acetate	1828	1814	MS RI	Sf Sa W
92	β-damascenone	1835	1801	MS RI	W
93	hexanoic acid	1839	1849	MS RI S	Sf Sa
94	ethyl dodecanoate	1849	1833	MS RI	Sf Sa W
95	3-methylbutyl decanoate	1866	1851	MS RI	Sa W
96	phenylmethanol	1886	1886	MS RI	W
97	ethyl 3-hydroxyoctanoate	1894	1884	MS RI	W
98	ethyl 3-methylbutyl succinate	1905	1901	MS RI	W
99	2-phenylethanol	1922	1904	MS RI S	Sf Sa W
100	tetradecanal	1929	1924	MS RI	W
101	ethyl tridecanoate	1948	1943	MS RI	W
102	heptanoic acid	1954	1960	MS RI	Sf Sa W
103	1-dodecanol	1965	1940	MS RI	W
104	2-hexenoic acid	1970	1971	MS RI	W
105	benzothiazole	1978	2000	MS RI	W
106	phenol	2013	2008	MS RI	W
107	*trans*-nerolidol	2038	2036	MS RI	Sa W
108	4-ethylguaiacol	2040	2036	MS RI	W
109	diethyl malate	2047	2060	MS RI	W
110	ethyl tetradecanoate	2051	2043	MS RI	W
111	octanoic acid	2059	2070	MS RI S	Sf Sa W
112	ethyl 3-hydroxydodecanoate	2103		MS	W
113	nonanoic acid	2160	2169	MS RI	Sa W
114	4-ethylphenol	2175	2183	MS RI	W
115	2-methyl-4-hydroxyacetophenone	2208		MS	W
116	ethyl hexadecanoate	2256	2243	MS RI	Sa W
117	decanoic acid	2271	2278	MS RI	Sf Sa W
118	ethyl 9-hexadecenoate	2283	2283	MS RI	W
119	2,4-di-tert-butylphenol	2307	2321	MS RI	Sa W
120	9-decenoic acid	2332	2348	MS RI	Sa W
121	undecanoic acid	2376	2369	MS RI	W
122	ethyl hydrogen succinate	2381	2395	MS RI	W
123	dodecanoic acid	2480	2502	MS RI	Sa W

[a] RI: calculated retention index

[b] RI (lit): retention index from literature, source NIST Chemistry WebBook (webbook.nist.gov) [255]

[c] IM: identification method; MS: comparison of mass spectra with those reported in NIST 20 mass spectrum libraries; RI: comparison of order of elution with those reported in literature; S: comparison of mass spectra and retention time with those of standard compounds.

Annex 10. Results of semi-quantitative analysis carried out on the concentrations (µg/L) of volatile compounds detected in the headspace of wines after alcoholic fermentation. Data were means and standard deviations (SD) of three replicates. Different letters within the same row marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. S: control; HS: sequential fermentation.

	S				HS			
	Mean	±	SD		Mean	±	SD	
Esters								
2-methyl-1-propanol acetate	3.22	±	0.28		5.00	±	1.70	
3-methyl-1-butanol acetate	134.90	±	32.56		150.95	±	44.01	
2-phenethyl acetate	45.17	±	6.11	b	16.56	±	1.87	a
ethyl butanoate	2.66	±	0.31		4.28	±	1.10	
ethyl hexanoate	120.69	±	2.90		107.68	±	15.76	
ethyl octanoate	736.77	±	40.42	b	362.17	±	74.83	a
ethyl decanoate	344.61	±	45.53	b	112.14	±	54.71	a
3-methylbutyl octanoate	3.46	±	0.98		1.58	±	1.39	
diethyl succinate	0.49	±	0.10		0.45	±	0.12	
ethyl 9-decenoate	18.06	±	4.88		9.35	±	4.36	
ethyl dodecanoate	24.60	±	9.09		9.33	±	3.09	
Alcohols								
1-propanol	1.82	±	0.21	a	5.64	±	2.35	b
2-methyl-1-propanol	24.49	±	2.98		48.69	±	19.18	
3-methyl-1-butanol	294.22	±	15.19		320.04	±	115.75	
2-phenylethanol	113.21	±	31.47	b	58.47	±	12.53	a
Acids								
acetic acid	9.35	±	4.98		33.65	±	15.19*	
2-methylpropanoic acid	2.46	±	0.37		2.19	±	0.68	
3-methylbutanoic acid	2.57	±	0.38		1.90	±	0.29	
hexanoic acid	13.19	±	5.00		9.60	±	2.67	
heptanoic acid	0.27	±	0.23		0.38	±	0.10	
octanoic acid	68.92	±	19.56		42.97	±	8.10	
decanoic acid	46.52	±	15.52		22.21	±	4.48	
Minor compounds								
2,3-butanediol (levo)	5.91	±	1.81		5.33	±	1.26	
2,3-butanediol (meso)	2.35	±	0.68		2.14	±	0.94	
γ-butyrolactone	0.65	±	0.24		0.56	±	0.09	

*p value = 0.057

Annex 11. Results of semi-quantitative analysis carried out on the concentrations (μg/L) of volatile compounds detected in the headspace of synthetic wines aged on S and HS lees. Data were means and standard deviations (SD) of three replicates and the results of one-way ANOVA were reported. The effect of lees typology (S and HS) and lees treatment (CON, ENZ, HHP) was analyzed by two-ways ANOVA and data were the means calculated for group (lees typology and lees treatment). Different letters within the same row marked significant differences, according to ANOVA and Tukey HSD test at $p < 0.05$. S: single fermentation; HS: 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.

	one-way ANOVA						two-ways ANOVA						
	Differences among the samples						Lees typology			Lees treatment			
	S_CON	S_ENZ	S_HHP	HS_CON	HS_ENZ	HS_HHP	S	HS		CON	ENZ	HHP	
Esters	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean	Mean	Sig.	Mean	Mean	Mean	Sig.
2-methylpropyl acetate	2.26 ± 0.55 c	1.44 ± 0.06 ab	1.18 ± 0.04 a	1.87 ± 0.11 bc	1.45 ± 0.04 ab	1.14 ± 0.04 a	1.63	1.49	ns	2.06 b	1.45 a	1.16 a	***
3-methylbutyl acetate	87.93 ± 18.27 b	66.98 ± 1.36 ab	61.00 ± 1.12 a	64.05 ± 6.74 a	55.24 ± 3.54 a	45.73 ± 1.08 a	71.97 b	55.01 a	***	75.99 b	61.11 a	53.37 a	**
2-phenethyl acetate	21.97 ± 7.43 c	14.69 ± 1.73 bc	16.65 ± 0.49 c	7.83 ± 1.24 ab	5.64 ± 1.01 a	6.20 ± 0.56 ab	17.77 b	6.55 a	***	14.90	10.17	11.42	ns
ethyl butanoate	3.63 ± 1.29	2.75 ± 0.14	2.71 ± 0.08	3.31 ± 0.21	3.01 ± 0.19	2.78 ± 0.14	3.03	3.03	ns	3.47	2.88	2.74	ns
ethyl hexanoate	137.60 ± 27.34 d	108.08 ± 1.81 cd	98.09 ± 1.08 bc	84.28 ± 5.31 abc	72.12 ± 3.42 ab	64.75 ± 1.44 a	114.59 b	73.72 a	***	110.94 b	90.10 a	81.42 a	**
ethyl octanoate	576.04 ± 25.98 c	607.63 ± 29.68 c	479.87 ± 4.75 b	317.60 ± 45.46 a	335.26 ± 19.90 a	275.89 ± 8.41 a	554.51 b	309.58 a	***	446.82 b	471.45 b	377.88 a	***
ethyl nonanoate	0.63 ± 0.04 ab	0.81 ± 0.05 b	0.82 ± 0.02 b	0.66 ± 0.12 ab	0.51 ± 0.07 a	0.64 ± 0.07 ab	0.75 b	0.60 a	***	0.65	0.66	0.73	ns
ethyl decanoate	184.42 ± 60.56 b	280.68 ± 27.58 c	184.83 ± 1.17 b	75.41 ± 16.81 a	106.68 ± 13.47 ab	101.16 ± 9.19 a	216.64 b	94.42 a	***	129.92 a	193.68 b	143.00 a	**
ethyl dodecanoate	11.37 ± 5.47 a	21.15 ± 5.21 b	22.22 ± 0.25 b	5.25 ± 1.16 a	7.65 ± 2.64 a	13.39 ± 1.78 ab	18.25 b	8.77 a	***	8.31 a	14.40 b	17.81 b	**
ethyl 2-methylbutanoate	0.46 ± 0.11 c	0.33 ± 0.02 bc	0.27 ± 0.01 ab	0.34 ± 0.08 bc	0.22 ± 0.02 ab	0.15 ± 0.02 a	0.36 b	0.24 a	**	0.40 b	0.27 a	0.21 a	***
ethyl 3-methylbutanoate	0.74 ± 0.19 b	0.31 ± 0.26 a	0.43 ± 0.00 ab	0.37 ± 0.04 ab	0.28 ± 0.01 a	0.22 ± 0.01 a	0.49 b	0.29 a	**	0.56 b	0.30 a	0.32 a	**
3-methylbutyl hexanoate	1.14 ± 0.16 b	1.46 ± 0.09 c	1.11 ± 0.00 b	0.60 ± 0.11 a	0.58 ± 0.04 a	0.46 ± 0.05 a	1.24 b	0.55 a	***	0.87 a	1.02 b	0.79 a	**
3-methylbutyl octanoate	1.47 ± 0.74 ab	2.50 ± 0.34 c	1.66 ± 0.02 bc	0.59 ± 0.12 a	0.91 ± 0.11 ab	0.69 ± 0.07 a	1.88 b	0.73 a	***	1.03 a	1.70 b	1.17 a	*
diethyl succinate	3.55 ± 1.33	2.23 ± 0.17	2.21 ± 0.01	3.85 ± 0.61	2.81 ± 0.43	2.72 ± 0.08	2.66	3.13	ns	3.70 b	2.52 a	2.47 a	**
ethyl 9-decenoate	3.31 ± 0.67 b	4.51 ± 0.31 b	1.01 ± 0.05 a	12.54 ± 4.20 a	16.45 ± 2.42 a	4.32 ± 0.37 a	2.94 a	11.10 b	***	7.92 b	10.48 b	2.67 a	***
3-methylbutyl decanoate	0.50 ± 0.25 ab	1.11 ± 0.26 c	0.75 ± 0.02 bc	0.17 ± 0.06 a	0.29 ± 0.14 a	0.30 ± 0.04 a	0.79 b	0.25 a	***	0.34 a	0.70 b	0.52 ab	**
ethyl hexadecanoate	0.24 ± 0.10 ab	0.98 ± 0.47 c	0.73 ± 0.02 bc	0.11 ± 0.03 a	0.25 ± 0.18 ab	0.49 ± 0.11 abc	0.65 b	0.28 a	**	0.17 a	0.61 b	0.61 b	**

(continue)

Annex 11. *(continue)*

Alcohols													
1-propanol	1.99 ± 0.10 b	1.91 ± 0.07 b	1.38 ± 0.04 a	2.90 ± 0.08 d	2.99 ± 0.15 d	2.28 ± 0.12 c	1.76 a	2.72 b	***	2.45 b	2.45 b	1.83 a	***
2-methyl-1-propanol	23.43 ± 5.83 c	14.94 ± 0.37 ab	12.84 ± 0.13 ab	19.28 ± 1.11 bc	14.81 ± 0.89 ab	11.98 ± 0.72 a	17.07	15.36	ns	21.36 b	14.88 a	12.41 a	***
3-methyl-1-butanol	201.62 ± 49.86 b	132.38 ± 2.35 a	115.88 ± 2.69 a	147.66 ± 8.89 ab	111.82 ± 7.96 a	94.20 ± 1.35 a	149.96 b	117.89 a	**	174.64 b	122.10 a	105.04 a	***
2-ethyl-1-hexanol	11.61 ± 3.11 b	7.19 ± 0.50 a	6.19 ± 0.02 a	8.64 ± 0.51 ab	6.47 ± 0.44 a	6.28 ± 0.56 a	8.33	7.13	ns	10.12 b	6.83 a	6.24 a	***
1-decanol	1.77 ± 0.20 b	1.46 ± 0.16 ab	1.33 ± 0.05 ab	1.61 ± 0.31 ab	1.42 ± 0.20 ab	1.10 ± 0.05 a	1.52	1.38	ns	1.69 b	1.44 ab	1.21 a	**
2-phenylethanol	86.06 ± 22.19 d	61.92 ± 5.48 bcd	65.73 ± 0.58 cd	44.87 ± 6.70 abc	32.69 ± 5.68 a	34.76 ± 1.93 ab	71.24 b	37.44 a	***	65.47 b	47.30 a	50.24 ab	*
Acids													
2-methylpropanoic acid	2.84 ± 0.79 c	1.83 ± 0.02 b	1.28 ± 0.04 ab	1.69 ± 0.21 ab	0.87 ± 0.12 a	0.83 ± 0.04 a	1.98 b	1.13 a	***	2.26 b	1.35 a	1.06 a	***
butanoic acid	0.50 ± 0.05 bc	0.34 ± 0.02 a	0.31 ± 0.01 a	0.51 ± 0.08 c	0.43 ± 0.03 abc	0.38 ± 0.03 ab	0.38 a	0.44 b	*	0.50 b	0.38 a	0.35 a	***
3-methylbutanoic acid	1.65 ± 0.51 b	0.92 ± 0.01 a	0.99 ± 0.03 a	0.95 ± 0.07 a	0.73 ± 0.07 a	0.63 ± 0.07 a	1.19 b	0.77 a	**	1.30 b	0.83 a	0.81 a	**
hexanoic acid	14.73 ± 4.90 b	9.05 ± 1.12 ab	10.02 ± 0.35 ab	7.71 ± 1.09 a	5.48 ± 0.98 a	5.46 ± 0.07 a	11.27 b	6.22 a	***	11.22 b	7.27 a	7.74 a	*
heptanoic acid	0.39 ± 0.14 b	0.16 ± 0.05 a	0.32 ± 0.01 ab	0.33 ± 0.03 ab	0.15 ± 0.06 a	0.35 ± 0.04 b	0.29	0.28	ns	0.36 b	0.16 a	0.33 b	***
octanoic acid	72.62 ± 23.74 b	47.26 ± 5.33 ab	48.87 ± 1.24 ab	37.73 ± 4.79 a	27.66 ± 4.50 a	27.63 ± 1.78 a	56.25 b	31.01 a	***	55.18 b	37.46 a	38.25 a	*
nonanoic acid	0.40 ± 0.22 b	0.17 ± 0.01 ab	0.11 ± 0.01 a	0.39 ± 0.07 b	0.16 ± 0.01 ab	0.14 ± 0.01 a	0.23	0.23	ns	0.40 b	0.16 a	0.12 a	***
decanoic acid	38.24 ± 8.45 b	30.69 ± 4.30 b	29.38 ± 1.92 b	16.91 ± 3.87 a	13.79 ± 3.66 a	16.00 ± 1.18 a	32.77 b	15.56 a	***	27.57	22.24	22.69	ns
9-decenoic acid	0.12 ± 0.07 a	0.10 ± 0.04 a	0.00 ± 0.00 a	0.58 ± 0.21 b	0.44 ± 0.07 b	0.12 ± 0.01 a	0.07 a	0.38 b	***	0.35 b	0.27 b	0.06 a	***
dodecanoic acid	1.59 ± 1.14	0.89 ± 0.14	0.93 ± 0.03	1.73 ± 1.28	0.42 ± 0.11	0.66 ± 0.09	1.14	0.94	ns	1.66	0.66	0.79	ns
Aldehydes													
hexanal	0.43 ± 0.02 ab	0.54 ± 0.04 b	0.61 ± 0.08 ab	0.38 ± 0.14 a	0.51 ± 0.08 ab	0.59 ± 0.06 ab	0.53	0.50	ns	0.41 a	0.53 ab	0.60 b	**
nonanal	0.25 ± 0.04	0.22 ± 0.01	0.20 ± 0.01	0.24 ± 0.03	0.23 ± 0.05	0.20 ± 0.01	0.22	0.22	ns	0.24	0.23	0.20	ns

(continue)

Annex 11. *(continue)*

Other compounds													
butyrolactone	0.40 ± 0.03 b	0.29 ± 0.02 a	0.24 ± 0.03 a	0.33 ± 0.06 ab	0.27 ± 0.05 a	0.22 ± 0.04 a	0.31	0.27	ns	0.37 b	0.28 a	0.23 a	***
2,4-di-tert-butylphenol	0.89 ± 0.33 ab	0.91 ± 0.13 ab	0.72 ± 0.07 a	1.47 ± 0.47 b	1.57 ± 0.19 b	1.10 ± 0.15 ab	0.84 a	1.38 b	***	1.18	1.24	0.91	ns
methionol	1.42 ± 0.25 b	0.90 ± 0.02 a	0.82 ± 0.02 a	1.52 ± 0.05 b	1.00 ± 0.10 a	0.97 ± 0.06 a	1.05	1.16	ns	1.47 b	0.95 a	0.89 a	***
nerolidol	0.39 ± 0.20	0.58 ± 0.08	0.42 ± 0.02	0.49 ± 0.19	0.77 ± 0.11	0.65 ± 0.16	0.46 a	0.64 b	*	0.44 a	0.68 b	0.54 ab	*

ns: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Annex 12. Results of semi-quantitative analysis carried out on the concentrations (µg/L) of volatile compounds detected in the headspace of white wines after three months of aging. Data were means and standard deviations of two replicates. Different letters within the same row marked significant differences among the samples, according to ANOVA and Tukey HSD test at $p < 0.05$. 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	S_CON	S_ENZ	S_HHP	HS_CON	HS_ENZ	HS_HHP
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
acetic acid	AC01	11.66 ± 0.91	10.30 ± 1.05	12.78 ± 1.65	12.91 ± 1.34	13.23 ± 2.45	11.53 ± 0.19	12.08 ± 0.19
2-methyl propanoic acid	AC02	0.28 ± 0.03	0.29 ± 0.05	0.33 ± 0.06	0.40 ± 0.06	0.40 ± 0.06	0.33 ± 0.03	0.34 ± 0.02
butanoic acid	AC03	0.35 ± 0.16	0.26 ± 0.05	0.33 ± 0.01	0.32 ± 0.02	0.30 ± 0.05	0.27 ± 0.01	0.27 ± 0.00
3-methyl butanoic acid	AC04	0.41 ± 0.04	0.44 ± 0.06	0.53 ± 0.06	0.53 ± 0.07	0.55 ± 0.10	0.51 ± 0.04	0.49 ± 0.00
2-methyl butanoic acid	AC05	0.55 ± 0.06	0.56 ± 0.07	0.67 ± 0.10	0.69 ± 0.07	0.72 ± 0.14	0.66 ± 0.02	0.63 ± 0.00
heptanoic acid	AC06	0.46 ± 0.26	0.32 ± 0.08	0.36 ± 0.14	0.35 ± 0.04	0.32 ± 0.02	0.35 ± 0.07	0.30 ± 0.02
2-hexenoic acid	AC07	0.15 ± 0.04	0.13 ± 0.03	0.18 ± 0.03	0.15 ± 0.01	0.13 ± 0.00	0.18 ± 0.02	0.17 ± 0.02
octanoic acid	AC08	16.81 ± 3.63 a	24.10 ± 5.74 ab	28.84 ± 0.95 b	22.52 ± 0.28 ab	22.66 ± 0.17 ab	26.00 ± 1.90 ab	19.90 ± 1.09 ab
nonanoic acid	AC09	0.52 ± 0.53	0.25 ± 0.11	0.45 ± 0.20	0.37 ± 0.15	0.22 ± 0.02	0.36 ± 0.03	0.26 ± 0.03
decanoic acid	AC10	2.48 ± 0.90 a	28.77 ± 6.82 cd	33.81 ± 1.32 d	19.18 ± 1.02 bc	17.17 ± 1.25 b	19.69 ± 2.23 bc	11.76 ± 0.62 ab
9-decenoic acid	AC11	0.24 ± 0.07	0.24 ± 0.08	0.30 ± 0.00	0.19 ± 0.00	0.28 ± 0.04	0.35 ± 0.08	0.25 ± 0.01
undecanoic acid	AC12	0.22 ± 0.24	0.09 ± 0.04	0.18 ± 0.13	0.10 ± 0.03	0.03 ± 0.01	0.05 ± 0.00	0.04 ± 0.01
dodecanoic acid	AC13	0.54 ± 0.34 a	1.77 ± 0.55 bc	2.06 ± 0.10 c	1.43 ± 0.31 abc	1.04 ± 0.02 abc	1.13 ± 0.13 abc	0.95 ± 0.04 ab
Sum of acids		34.67 ± 7.20 a	67.52 ± 14.73 bc	80.82 ± 0.97 c	59.14 ± 2.38 abc	57.05 ± 1.68 abc	61.41 ± 4.69 bc	47.43 ± 2.01 ab
ethyl acetate	EA01	95.44 ± 2.99	99.17 ± 4.82	98.30 ± 2.76	102.86 ± 1.32	104.94 ± 4.22	100.12 ± 2.53	104.87 ± 0.37
2-methylpropyl acetate	EA02	0.27 ± 0.03	0.28 ± 0.11	0.34 ± 0.04	0.32 ± 0.07	0.28 ± 0.04	0.36 ± 0.03	0.29 ± 0.03
3-methyl-1-butanol acetate	EA03	11.18 ± 0.09 a	13.90 ± 0.34 b	14.05 ± 0.18 b	18.47 ± 0.24 d	17.03 ± 0.38 c	17.52 ± 0.48 cd	19.76 ± 0.23 e
2-methyl-1-butanol acetate	EA04	0.37 ± 0.01 a	0.43 ± 0.02 a	0.59 ± 0.04 ab	0.76 ± 0.18 ab	0.52 ± 0.22 ab	0.55 ± 0.01 ab	1.03 ± 0.22 b
hexyl acetate	EA05	1.45 ± 0.08	1.15 ± 0.14	1.21 ± 0.06	1.79 ± 0.13	1.51 ± 0.16	1.62 ± 0.23	1.95 ± 0.02

(continue)

Annex 12. *(continue)*

octyl acetate	EA06	0.01 ± 0.00 a	0.19 ± 0.02 c	0.20 ± 0.01 c	0.08 ± 0.01 b	0.09 ± 0.00 b	0.09 ± 0.01 b	0.04 ± 0.00 a
decyl acetate	EA07	0.00 ± 0.00 a	0.12 ± 0.03 bc	0.16 ± 0.03 c	0.07 ± 0.01 ab	0.10 ± 0.00 bc	0.09 ± 0.02 bc	0.06 ± 0.03 ab
2-phenylethyl acetate	EA08	4.58 ± 0.11 a	6.41 ± 0.25 bc	6.87 ± 0.29 c	5.95 ± 0.35 abc	5.53 ± 0.60 abc	5.28 ± 0.49 ab	4.78 ± 0.10 a
Sum of acetate esters		113.29 ± 3.08 a	121.65 ± 4.96 ab	121.73 ± 3.20 ab	130.32 ± 2.29 c	130.01 ± 5.62 c	125.64 ± 3.74 ab	132.77 ± 0.70 c
ethyl propanoate	EE01	2.40 ± 0.08 a	2.43 ± 0.11 a	2.41 ± 0.02 a	2.67 ± 0.01 b	2.42 ± 0.04 a	2.41 ± 0.07 a	2.71 ± 0.03 b
ethyl 2-methylpropanoate	EE02	1.36 ± 0.19	1.30 ± 0.01	1.27 ± 0.00	1.29 ± 0.11	1.23 ± 0.01	1.19 ± 0.13	1.36 ± 0.03
ethyl butanoate	EE03	3.18 ± 0.25	3.75 ± 0.02	3.79 ± 0.00	3.78 ± 0.30	3.73 ± 0.21	3.44 ± 0.34	3.71 ± 0.00
ethyl 2-methylbutanoate	EE04	0.74 ± 0.01 a	0.77 ± 0.02 a	0.74 ± 0.01 a	0.95 ± 0.04 b	0.77 ± 0.01 a	0.76 ± 0.03 a	0.88 ± 0.08 ab
ethyl 3-methylbutanoate	EE05	0.97 ± 0.03 a	1.00 ± 0.01 a	0.96 ± 0.00 a	1.10 ± 0.00 b	0.97 ± 0.01 a	0.99 ± 0.05 a	1.10 ± 0.00 b
ethyl hexanoate	EE06	64.89 ± 0.17	85.62 ± 4.42	81.97 ± 0.72	75.39 ± 0.12	87.93 ± 1.59	86.55 ± 3.14	75.86 ± 0.05
ethyl 3-hexenoate	EE07	0.05 ± 0.02	0.05 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.05 ± 0.02	0.05 ± 0.01	0.04 ± 0.00
ethyl 3-hexenoate (isomer)	EE08	0.07 ± 0.00	0.07 ± 0.02	0.07 ± 0.00	0.05 ± 0.03	0.08 ± 0.00	0.03 ± 0.00	0.05 ± 0.02
ethyl lactate	EE10	7.39 ± 0.66	7.21 ± 0.39	7.91 ± 1.04	8.19 ± 0.70	8.73 ± 1.67	7.10 ± 0.05	6.99 ± 0.07
ethyl 5-heptenoate	EE11	0.17 ± 0.03 a	0.23 ± 0.01 ab	0.21 ± 0.03 ab	0.19 ± 0.01 ab	0.25 ± 0.02 ab	0.27 ± 0.00 b	0.24 ± 0.00 ab
ethyl 6-heptenoate	EE12	0.40 ± 0.35	0.15 ± 0.03	0.17 ± 0.03	0.16 ± 0.02	0.14 ± 0.02	0.15 ± 0.02	0.21 ± 0.10
ethyl octanoate	EE13	340.17 ± 7.89 a	448.44 ± 19.31 c	439.14 ± 11.97 bc	360.77 ± 3.71 a	410.68 ± 9.13 bc	403.39 ± 10.48 b	338.96 ± 0.81 a
ethyl nonanoate	EE14	0.80 ± 0.02 a	1.12 ± 0.11 ab	1.14 ± 0.11 ab	1.40 ± 0.13 b	1.62 ± 0.26 b	1.39 ± 0.02 b	1.39 ± 0.13 b
ethyl decanoate	EE15	54.98 ± 3.41	504.87 ± 20.58	500.10 ± 29.49	315.11 ± 23.44	340.35 ± 0.53	337.45 ± 15.44	223.40 ± 3.39
ethyl furoate	EE16	0.55 ± 0.02	0.55 ± 0.02	0.56 ± 0.03	0.54 ± 0.04	0.58 ± 0.05	0.53 ± 0.03	0.53 ± 0.00
diethyl succinate	EE17 I	20.34 ± 1.75 a	29.00 ± 4.47 abc	32.03 ± 1.05 bc	25.74 ± 1.14 ab	35.36 ± 2.96 c	35.12 ± 1.40 c	24.78 ± 0.41 ab
ethyl 9-decenoate	EE18	25.69 ± 1.34 c	23.53 ± 0.35 bc	23.92 ± 0.34 bc	16.54 ± 1.69 a	26.90 ± 0.98 c	25.62 ± 2.11 c	20.07 ± 0.46 ab
ethyl undecanoate	EE19	0.18 ± 0.02 a	0.37 ± 0.01 ab	0.37 ± 0.09 ab	0.24 ± 0.03 ab	0.48 ± 0.08 ab	0.56 ± 0.19 b	0.31 ± 0.06 ab
diethyl glutarate	EE20	0.11 ± 0.02	0.10 ± 0.00	0.12 ± 0.01	0.11 ± 0.00	0.12 ± 0.01	0.13 ± 0.00	0.14 ± 0.00
ethyl phenylacetate	EE21	0.93 ± 0.02	0.99 ± 0.07	1.08 ± 0.07	0.98 ± 0.00	1.07 ± 0.16	1.02 ± 0.05	0.96 ± 0.02

(continue)

Annex 12. *(continue)*

ethyl dodecanoate	EE22	13.82 ± 0.07 a	93.65 ± 0.40 d	90.62 ± 3.63 cd	64.52 ± 10.98 b	71.50 ± 2.31 bc	68.21 ± 6.94 b	53.37 ± 2.95 b
ethyl 3-hydroxyoctanoate	EE23	0.04 ± 0.00	0.04 ± 0.02	0.06 ± 0.01	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01
ethyl tridecanoate	EE24	0.07 ± 0.02	0.08 ± 0.01	0.22 ± 0.19	0.05 ± 0.03	0.10 ± 0.01	0.07 ± 0.03	0.06 ± 0.02
diethyl malate	EE25 I	0.41 ± 0.16	0.38 ± 0.19	0.44 ± 0.00	0.38 ± 0.07	0.33 ± 0.04	0.53 ± 0.07	0.38 ± 0.08
ethyl tetradecanoate	EE26	0.41 ± 0.00 a	1.10 ± 0.06 b	1.06 ± 0.08 b	0.69 ± 0.31 ab	0.88 ± 0.05 ab	0.77 ± 0.20 ab	0.73 ± 0.11 ab
ethyl 3-hydroxydodecanoate	EE27	0.13 ± 0.00	0.16 ± 0.03	0.18 ± 0.01	0.13 ± 0.04	0.18 ± 0.01	0.19 ± 0.02	0.15 ± 0.00
ethyl hexadecanoate	EE28	0.48 ± 0.01	1.13 ± 0.03	1.07 ± 0.18	0.67 ± 0.36	0.92 ± 0.13	0.80 ± 0.28	0.90 ± 0.15
ethyl 9-hexadecenoate	EE29	0.44 ± 0.08	0.56 ± 0.03	0.61 ± 0.12	0.35 ± 0.15	0.80 ± 0.16	0.83 ± 0.32	0.98 ± 0.16
ethyl hydrogen succinate	EE30 I	1.83 ± 1.57	1.10 ± 0.83	1.67 ± 0.32	1.27 ± 0.77	1.10 ± 0.70	1.40 ± 0.17	1.06 ± 0.51
Sum of ethyl esters		543.01 ± 16.98 a	1209.76 ± 40.24 d	1193.93 ± 43.66 d	883.33 ± 41.54 bc	999.31 ± 18.60 c	981.00 ± 41.46 c	761.34 ± 7.50 b
3-methyl-1-butanol propanoate	AE01	0.13 ± 0.00 a	0.13 ± 0.01 a	0.13 ± 0.02 a	0.19 ± 0.01 bc	0.16 ± 0.01 ab	0.16 ± 0.00 ab	0.21 ± 0.01 c
3-methylbutyl 2-methylpropanoate	AE02	0.06 ± 0.00	0.08 ± 0.02	0.08 ± 0.00	0.06 ± 0.01	0.07 ± 0.02	0.06 ± 0.00	0.06 ± 0.00
3-methylbutyl butanoate	AE03	0.08 ± 0.00 a	0.14 ± 0.03 ab	0.15 ± 0.03 ab	0.12 ± 0.01 a	0.26 ± 0.01 b	0.20 ± 0.07 ab	0.12 ± 0.01 a
methyl heptanoate	AE04	0.16 ± 0.00	0.17 ± 0.00	0.16 ± 0.01	0.16 ± 0.00	0.17 ± 0.00	0.17 ± 0.01	0.17 ± 0.01
propyl hexanoate	AE05	0.13 ± 0.00	0.13 ± 0.01	0.42 ± 0.34	0.14 ± 0.04	0.14 ± 0.01	0.12 ± 0.04	0.13 ± 0.00
2-methylpropyl hexanoate	AE06	0.19 ± 0.04	0.20 ± 0.00	0.20 ± 0.00	0.19 ± 0.01	0.22 ± 0.01	0.23 ± 0.03	0.22 ± 0.03
methyl octanoate	AE07	0.22 ± 0.00 a	0.31 ± 0.00 b	0.32 ± 0.03 b	0.23 ± 0.01 a	0.30 ± 0.02 b	0.33 ± 0.01 b	0.23 ± 0.01 a
3-methylbutyl hexanoate	AE08	2.22 ± 0.15	2.44 ± 0.07	2.45 ± 0.13	2.29 ± 0.07	2.58 ± 0.07	2.44 ± 0.12	2.48 ± 0.02
propyl octanoate	AE09	0.24 ± 0.01	0.29 ± 0.01	0.31 ± 0.01	0.26 ± 0.03	0.31 ± 0.01	0.30 ± 0.03	0.25 ± 0.00
2-methylpropyl octanoate	AE10	0.32 ± 0.01 a	0.49 ± 0.02 c	0.47 ± 0.04 c	0.35 ± 0.04 ab	0.48 ± 0.00 c	0.45 ± 0.04 bc	0.33 ± 0.00 a
3-methylbutyl heptanoate	AE11	0.15 ± 0.01 a	0.26 ± 0.01 ab	0.19 ± 0.01 ab	0.18 ± 0.03 a	0.38 ± 0.06 b	0.25 ± 0.11 ab	0.17 ± 0.00 a
3-methylbutyl lactate	AE12	0.40 ± 0.00	0.38 ± 0.00	0.48 ± 0.08	0.50 ± 0.06	0.58 ± 0.10	0.50 ± 0.06	0.47 ± 0.03
methyl decanoate	AE13	0.33 ± 0.24 ab	0.63 ± 0.03 b	0.67 ± 0.06 b	0.33 ± 0.01 ab	0.34 ± 0.01 ab	0.37 ± 0.02 ab	0.20 ± 0.03 a
3-methylbutyl octanoate	AE14	2.03 ± 0.14 a	3.11 ± 0.14 b	3.08 ± 0.12 b	2.18 ± 0.22 a	2.94 ± 0.06 b	2.79 ± 0.23 b	2.14 ± 0.03 a

(continue)

Annex 12. *(continue)*

2-methylpropyl decanoate	AE15	0.05 ± 0.01	0.40 ± 0.04	0.41 ± 0.02	0.20 ± 0.00	0.30 ± 0.01	0.25 ± 0.00	0.13 ± 0.00
methyl dodecanoate	AE16	0.02 ± 0.01 a	0.06 ± 0.00 c	0.06 ± 0.00 c	0.04 ± 0.00 bc	0.05 ± 0.00 bc	0.05 ± 0.00 c	0.04 ± 0.00 b
3-methylbutyl decanoate	AE17	0.25 ± 0.02 a	2.11 ± 0.01 d	1.99 ± 0.08 d	1.04 ± 0.11 bc	1.38 ± 0.08 c	1.31 ± 0.23 c	0.72 ± 0.09 b
ethyl 3-methylbutyl succinate	AE18 I	0.96 ± 0.14	1.19 ± 0.23	1.37 ± 0.04	1.20 ± 0.08	1.32 ± 0.12	1.35 ± 0.10	1.14 ± 0.08
<u>Sum of other esters</u>		7.93 ± 0.75 a	12.51 ± 0.05 d	12.95 ± 0.54 d	9.68 ± 0.70 abc	11.97 ± 0.51 cd	11.33 ± 0.88 bcd	9.18 ± 0.21 ab
1-propanol	AL01	2.54 ± 0.42	2.98 ± 0.08	2.78 ± 0.22	2.56 ± 0.35	2.65 ± 0.47	2.56 ± 0.04	2.86 ± 0.05
2-methyl-1-propanol	AL02	16.53 ± 0.72	19.59 ± 3.90	17.15 ± 0.33	16.61 ± 0.27	16.75 ± 0.89	16.33 ± 0.32	15.99 ± 0.32
3-methyl-1-butanol	AL03	205.77 ± 3.21	208.84 ± 10.04	206.98 ± 6.88	201.94 ± 6.11	207.22 ± 14.05	196.38 ± 4.91	196.72 ± 2.12
4-methyl-1-pentanol	AL04	0.07 ± 0.00	0.06 ± 0.00	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.02	0.06 ± 0.02
3-methyl-1-pentanol	AL05	0.30 ± 0.01 ab	0.30 ± 0.01 a	0.31 ± 0.02 ab	0.31 ± 0.01 ab	0.35 ± 0.02 b	0.31 ± 0.01 ab	0.31 ± 0.01 ab
1-hexanol	AL06	24.77 ± 0.24	23.65 ± 1.41	23.27 ± 2.83	23.40 ± 1.63	25.31 ± 1.99	23.53 ± 1.30	23.28 ± 0.55
trans-3-hexen-1-ol	AL07	0.25 ± 0.00	0.22 ± 0.01	0.23 ± 0.04	0.22 ± 0.01	0.24 ± 0.01	0.24 ± 0.01	0.25 ± 0.00
cis-3-hexen-1-ol	AL08	0.09 ± 0.03	0.11 ± 0.01	0.11 ± 0.00	0.08 ± 0.04	0.14 ± 0.02	0.10 ± 0.01	0.10 ± 0.02
2-butoxyethanol	AL09	1.24 ± 1.51	0.22 ± 0.18	0.52 ± 0.56	0.28 ± 0.13	0.05 ± 0.01	0.06 ± 0.03	0.06 ± 0.01
cis-2-hexen-1-ol	AL10	0.09 ± 0.00	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.07 ± 0.00
2-ethyl-1-hexanol	AL11	4.51 ± 3.67 a	13.12 ± 1.08 b	20.11 ± 1.90 c	10.48 ± 0.12 ab	12.50 ± 1.30 b	11.14 ± 0.99 ab	8.57 ± 0.11 ab
2-nonanol	AL12	0.22 ± 0.02	0.21 ± 0.02	0.22 ± 0.03	0.23 ± 0.01	0.27 ± 0.02	0.28 ± 0.01	0.25 ± 0.01
1-octanol	AL13	0.88 ± 0.02	0.78 ± 0.05	0.90 ± 0.03	0.82 ± 0.02	0.88 ± 0.10	0.79 ± 0.09	0.79 ± 0.00
1-nonanol	AL14	0.70 ± 0.04	0.64 ± 0.06	0.74 ± 0.10	0.71 ± 0.03	0.78 ± 0.09	0.75 ± 0.05	0.73 ± 0.03
1-decanol	AL15	0.27 ± 0.03 a	0.67 ± 0.05 bc	0.74 ± 0.06 c	0.48 ± 0.07 ab	0.72 ± 0.08 c	0.66 ± 0.02 bc	0.41 ± 0.02 a
phenylmethanol	AL16	0.56 ± 0.08	0.50 ± 0.10	0.61 ± 0.04	0.69 ± 0.05	0.53 ± 0.06	0.56 ± 0.00	0.61 ± 0.03
2-phenylethanol	AL17	52.61 ± 6.73	57.31 ± 11.58	67.04 ± 0.11	65.69 ± 1.39	57.90 ± 3.80	64.36 ± 3.33	59.87 ± 1.95
1-dodecanol	AL18	0.19 ± 0.10	0.25 ± 0.06	0.26 ± 0.14	0.39 ± 0.21	0.29 ± 0.04	0.26 ± 0.03	0.19 ± 0.01
<u>Sum of alcohols</u>		311.58 ± 8.03	329.52 ± 2.29	342.11 ± 8.11	325.05 ± 9.73	326.74 ± 20.36	318.43 ± 11.13	311.13 ± 0.03

(continue)

Annex 12. *(continue)*

hexanal	ALD01	0.21 ± 0.10	0.16 ± 0.01	0.19 ± 0.07	0.18 ± 0.02	0.18 ± 0.03	0.29 ± 0.14	0.26 ± 0.09
2-furfural	ALD02	0.17 ± 0.05 b	0.10 ± 0.01 ab	0.11 ± 0.03 ab	0.12 ± 0.00 ab	0.09 ± 0.02 ab	0.07 ± 0.01 a	0.07 ± 0.01 a
dodecanal	ALD03	0.62 ± 0.68	0.21 ± 0.09	0.38 ± 0.27	0.26 ± 0.08	0.14 ± 0.00	0.15 ± 0.01	0.11 ± 0.00
tetradecanal	ALD04	0.17 ± 0.19	0.06 ± 0.03	0.12 ± 0.10	0.08 ± 0.03	0.02 ± 0.01	0.02 ± 0.00	0.02 ± 0.00
<u>Sum of aldehydes</u>		1.18 ± 1.02	0.52 ± 0.14	0.79 ± 0.46	0.65 ± 0.12	0.43 ± 0.06	0.53 ± 0.15	0.47 ± 0.10
acetoin	K01	0.05 ± 0.00	0.07 ± 0.03	0.10 ± 0.03	0.14 ± 0.01	0.17 ± 0.06	0.11 ± 0.07	0.07 ± 0.00
2-methyl-4-hydroxyacetophenone	K02	0.02 ± 0.01 a	0.03 ± 0.01 ab	0.02 ± 0.01 ab	0.03 ± 0.01 ab	0.05 ± 0.00 ab	0.05 ± 0.01 b	0.05 ± 0.01 ab
<u>Sum of ketones</u>		0.07 ± 0.01 a	0.10 ± 0.03 ab	0.12 ± 0.04 ab	0.16 ± 0.02 ab	0.22 ± 0.06 b	0.17 ± 0.05 ab	0.11 ± 0.01 ab
TDN	N01	0.28 ± 0.02	0.24 ± 0.04	0.21 ± 0.01	0.17 ± 0.07	0.18 ± 0.04	0.18 ± 0.05	0.15 ± 0.03
NOR (163)	N02	0.48 ± 0.11	0.40 ± 0.02	0.47 ± 0.03	0.36 ± 0.03	0.42 ± 0.04	0.38 ± 0.05	0.35 ± 0.04
vitispirane like (192)	N03	0.87 ± 0.06 ab	1.04 ± 0.05 bc	1.10 ± 0.05 c	0.82 ± 0.01 a	0.91 ± 0.05 abc	0.86 ± 0.09 ab	0.76 ± 0.03 a
NOR (163)	N04	0.29 ± 0.07	0.23 ± 0.02	0.28 ± 0.00	0.23 ± 0.02	0.23 ± 0.00	0.21 ± 0.04	0.20 ± 0.03
NOR (138)	N05	1.30 ± 0.11	1.27 ± 0.04	1.34 ± 0.08	1.30 ± 0.06	1.49 ± 0.12	1.36 ± 0.04	1.48 ± 0.06
TDN	N06	0.30 ± 0.05	0.27 ± 0.04	0.26 ± 0.05	0.22 ± 0.05	0.21 ± 0.03	0.18 ± 0.03	0.21 ± 0.01
naphthalene	N07	0.26 ± 0.24	0.10 ± 0.02	0.16 ± 0.10	0.12 ± 0.03	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.00
β-damascenone	N08	1.21 ± 0.05 d	1.03 ± 0.08 bcd	1.07 ± 0.08 cd	0.89 ± 0.06 abc	0.77 ± 0.12 ab	0.74 ± 0.07 a	0.72 ± 0.01 a
<u>Sum of norisoprenoids</u>		4.98 ± 0.33 b	4.58 ± 0.03 ab	4.88 ± 0.04 ab	4.10 ± 0.27 ab	4.28 ± 0.39 ab	3.96 ± 0.36 ab	3.93 ± 0.07 a
β-myrcene	T01	0.15 ± 0.05	0.15 ± 0.03	0.14 ± 0.04	0.18 ± 0.00	0.18 ± 0.03	0.16 ± 0.03	0.20 ± 0.01
d-limonene	T02	1.09 ± 0.01	0.98 ± 0.08	1.02 ± 0.00	1.00 ± 0.08	1.00 ± 0.01	1.05 ± 0.03	1.08 ± 0.02
linalool like	T03	0.50 ± 0.00	0.47 ± 0.09	0.46 ± 0.03	0.32 ± 0.38	0.46 ± 0.04	0.43 ± 0.06	0.48 ± 0.02
nerol oxide	T04	0.12 ± 0.00	0.13 ± 0.01	0.13 ± 0.00	0.13 ± 0.00	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.00
geranylethyl ether	T05	0.16 ± 0.02	0.16 ± 0.03	0.16 ± 0.04	0.16 ± 0.01	0.16 ± 0.03	0.14 ± 0.02	0.14 ± 0.01

(continue)

Annex 12. *(continue)*

geranylethyl ether (isomer)	T06	0.84 ± 0.09	0.70 ± 0.08	0.65 ± 0.10	0.68 ± 0.08	0.60 ± 0.07	0.53 ± 0.07	0.67 ± 0.03
linalool	T07	3.06 ± 0.14	2.95 ± 0.15	3.13 ± 0.11	3.48 ± 0.07	3.21 ± 0.27	3.01 ± 0.05	3.49 ± 0.00
trans-γ-caryophyllene	T08	0.24 ± 0.09	0.17 ± 0.03	0.21 ± 0.02	0.23 ± 0.03	0.16 ± 0.03	0.15 ± 0.01	0.17 ± 0.02
citronellol acetate	T09	0.09 ± 0.10	0.03 ± 0.01	0.05 ± 0.00	0.04 ± 0.01	0.17 ± 0.00	0.16 ± 0.03	0.10 ± 0.00
α-terpineol	T10	0.44 ± 0.04	0.56 ± 0.03	0.61 ± 0.04	0.62 ± 0.05	0.53 ± 0.12	0.51 ± 0.03	0.54 ± 0.04
citronellol	T11	0.48 ± 0.03 a	0.57 ± 0.07 ab	0.62 ± 0.01 ab	0.64 ± 0.00 ab	0.77 ± 0.20 ab	0.84 ± 0.03 b	0.62 ± 0.04 ab
trans-nerolidol	T12	0.78 ± 0.20	0.50 ± 0.12	0.58 ± 0.04	0.47 ± 0.11	0.58 ± 0.01	0.58 ± 0.05	0.46 ± 0.02
Sum of terpenes		7.95 ± 0.24	7.37 ± 0.03	7.77 ± 0.30	7.95 ± 0.76	7.93 ± 0.81	7.70 ± 0.35	8.07 ± 0.08
phenol	PH01	0.28 ± 0.24	0.13 ± 0.04	0.19 ± 0.08	0.14 ± 0.02	0.10 ± 0.00	0.10 ± 0.01	0.09 ± 0.01
4-ethylguaiacol	PH02	1.35 ± 0.18 a	1.28 ± 0.28 a	1.58 ± 0.01 ab	2.46 ± 0.07 c	1.73 ± 0.10 ab	1.95 ± 0.12 bc	2.35 ± 0.10 c
4-ethylphenol	PH03	1.74 ± 0.43 a	1.51 ± 0.41 a	1.94 ± 0.06 a	4.30 ± 0.02 b	1.96 ± 0.01 a	2.51 ± 0.22 a	4.30 ± 0.28 b
2,4-di-tert-butylphenol	PH04	20.79 ± 2.47 b	6.15 ± 1.21 a	7.25 ± 0.16 a	7.60 ± 1.86 a	6.58 ± 0.70 a	6.31 ± 0.59 a	7.28 ± 0.29 a
Sum of phenols		24.16 ± 3.31 b	9.08 ± 1.95 a	10.96 ± 0.31 a	14.50 ± 1.92 a	10.37 ± 0.79 a	10.86 ± 0.92 a	14.03 ± 0.68 a
benzothiazole	S01	0.13 ± 0.16	0.03 ± 0.01	0.06 ± 0.06	0.04 ± 0.02	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
methionol	S02	0.59 ± 0.21	0.61 ± 0.15	0.74 ± 0.11	0.69 ± 0.02	0.62 ± 0.07	0.71 ± 0.02	0.60 ± 0.03
Sum of sulfur compounds		0.73 ± 0.37	0.63 ± 0.16	0.79 ± 0.17	0.73 ± 0.04	0.63 ± 0.07	0.72 ± 0.02	0.61 ± 0.03