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Comparative Study of the Stilbenes and Other Phenolic Compounds in Cabernet Sauvignon Wines Obtained from Two Different Vinifications: Traditional and Co-Inoculation

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Abstract: From grape cultivation to ripening and harvest timing to processing, each step of the winemaking process can be a critical point when it comes to wine quality and phenolic composition. In this study, the influence of winemaking technology on resveratrol and quercetin content, as well as other polyphenolic compounds, was investigated. Resveratrol is a non-flavonoid polyphenolic stilbene synthesized by grape skin when damaged by infectious diseases or ionizing radiation. Quercetin is a phenol found in grape skins and stems and is produced to protect grapes from UV light damage. *Trans*-resveratrol and quercetin are known to act as antioxidants, reduce the risk of atherosclerosis and type 2 diabetes, inhibit the growth of cancer cells, and prevent the release of allergic and inflammatory molecules. However, the question was whether red wine could be enriched with these phenols using a co-inoculation winemaking technology. The main new idea was to completely replace the cold maceration process with maceration with the addition of wild yeast (*Torulaspora delbrueckii*, Td). Maceration with the addition of wild yeast (Td) offers the following advantages over traditional cold maceration: (1) higher concentrations of *trans*-resveratrol (>35–40%) and quercetin (>35–40%) in the final wine, (2) the new wine has a higher potential for human health, (3) the wine has better aroma and stability due to the higher mannoprotein content, and (4) better energy efficiency in the production process. The study of stability during storage and aging also included derivatives of benzoic acid and hydroxycinnamic acid, piceid, catechin, naringenin, rutin, kaempherol, hesperetin, and anthocyanins. This study found that younger wines had higher phenolic content, while storage of the wine resulted in a decrease in total phenolic content, especially monomeric stilbenes and quercetin. This study represents a small part of the investigation of the influence of non-*Saccharomyces* yeasts on the phenolic profile of wine, which still requires extensive research with practical application. In addition, non-*Saccharomyces* yeasts such as *Kluyveromyces thermotolerans*, *Candida stellata*, and *Metschnikowia pulcherrima* could also be used in future studies.

Keywords: co-inoculation; *Torulaspora delbrueckii*; non-*Saccharomyces* yeast; phenolic compounds; *trans*-resveratrol; quercetin

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1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene) is a phenolic phytoalexin produced by grapevines in response to fungal infection [1]. It exists in *trans*- and *cis*-isomeric forms, with the former being much more abundant in wine. In red wine, resveratrol also can be found in its glycoside

form (resveratrol-3-*O*-*β*-mono-D-glucoside; piceid). *Trans*-resveratrol and piceid are the major active constituents of red wine. *Trans*-piceid is present in red wine to a greater extent than its aglycone, but hydrolysis of this glycosylated derivative may occur in the small intestine and liver, which would increase the amount of biologically active *trans*-resveratrol. The average content of piceid is ten times higher than that of resveratrol in red wine. In addition, piceid is the most abundant form of resveratrol in nature. Other glycosylated derivatives of resveratrol could possibly be present in wine, but the lack of literature data on their contribution to the total amount of resveratrol suggests their minor importance [2]. In vitro research indicates that resveratrol has chemopreventive effects against cardiovascular disease, aging, and cancer. A number of studies suggest that piceid may have similar bioactivity to resveratrol, e.g., anticarcinogenic effects, inhibition of platelet aggregation, and antioxidant activity [3,4]. For this reason, it was of interest to analyse piceid in derived wines. It acts as a pro-drug and is stable during transport from the mouth to the small intestine, where it undergoes metabolic conversion to active *trans*-resveratrol and is reabsorbed into the blood plasma. Piceid preserves resveratrol from degradation in the gastrointestinal tract [2]. In Bordeaux varieties, *trans*-resveratrol was not found in measurable amounts, but only *trans*-piceid, in amounts between 0.26 mg/L and 1.25 mg/L [5]. The influence of winemaking techniques and grape varieties on the resveratrol content, total phenolic content, and antioxidant potential of red wines was previously studied on 10 commercial Serbian red wines. It was clearly found that resveratrol content was very low (0.18–1.31 mg/L) in all studied wines [6]. It was also demonstrated that winemaking techniques influence the amounts of phenolic compounds. The highest average resveratrol and total phenolic contents were found in Merlot (4.85 mg/L; 1208 mg/L GAE) and Cabernet Sauvignon (3.78 mg/L; 1410 mg/L GAE) wines [7]. Quercetin has unique biological properties that may improve mental/physical performance and reduce the risk of infection. The first study on the pharmacokinetics of quercetin in humans showed very low oral bioavailability after a single oral dose (~2%) [8]. In red winemaking, maceration with skin and seeds during fermentation results in a higher concentration of resveratrol and quercetin in red wines than in the white wines. Resveratrol and quercetin content in wine depends on many different factors, including grape variety, harvest year, climatic conditions, UV light, winemaking technique, selected yeast strain, and aging [7,9]. The techniques of skin extraction and enzymatic hydrolysis of the glucoside forms also play an important role in the resulting resveratrol and quercetin concentrations. One way to control phenolic content in wine is through the choice of yeast strain. Not only do yeasts play a role in alcoholic fermentation, but they are also responsible for biochemical, enzymatic, and physical reactions during the process, and thus exert a significant influence on phenolic composition of the wine. Thus, resveratrol, which is present in glycoside form (piceid), can be hydrolysed by *β*-glucosidases, resulting in an increased concentration of free resveratrol [10]. Fermentation of must with yeast strains of *Saccharomyces cerevisiae* (SC) and fermentation with mixed cultures of *Saccharomyces*/non-*Saccharomyces* and lactic acid bacteria are widely used in modern winemaking [11]. On the other hand, many studies have highlighted the positive influence of non-*Saccharomyces* yeast strains on the chemical composition of wine [10,12,13]. Recent studies with non-*Saccharomyces* species described the intense effect of some strains on anthocyanin colour and subsequent stability by greatly lowering pH of wine during fermentation [14], as well as co-inoculation with lactic acid bacteria [12]. *Torulaspora delbrueckii* is probably the non-*Saccharomyces* yeast currently most commonly used for winemaking because of its good fermentation performance compared to other non-*Saccharomyces* yeasts considered for winemaking [15]. The fermentative capacity of *T. delbrueckii* allows it to be used at the beginning of the fermentation process, unlike other strictly oxidising non-*Saccharomyces* yeasts [16].

Torulaspora delbrueckii has also been reported to increase anthocyanin content during fermentation [17]. Recently, Chen et al. [18] observed an increase in total anthocyanins during sequential fermentation of *T. delbrueckii*/*S. cerevisiae* compared to fermentation with *S. cerevisiae* alone. In addition, *T. delbrueckii* has also been shown to influence the phenolic

composition of wine. In this context, Ngqumba et al. [13] observed that the influence of *T. delbrueckii* on phenolic compounds, such as flavonols and phenolic acids, of cv. Chenin blanc depends on the strain.

Regarding the influence of non-*Saccharomyces* yeasts on stilbene composition, *T. delbrueckii* obtained the best results, especially when compared to *S. cerevisiae.* Moreover, *T. delbrueckii* resulted in higher total phenolic content without increasing some negative organoleptic properties such as acetic acid content [17]. According to Minnaar et al. [19], the measured anthocyanins and flavanols in Pinotage wines produced with *T. delbrueckii* were consistently higher than those in Pinotage grape must inoculated with *S. cerevisiae*. Glycosylated anthocyanins and acetylated anthocyanins were also highest in Pinotage wines produced with *T. delbrueckii*. The main focus of this study was to find out the contribution of the co-inoculation (with mixed cultures) to increase the content of stilbenes and quercetin in wine. In addition to these compounds, which are of great benefit to human health, the enrichment of wine with other phenolics by co-inoculation with mixed cultures was also investigated. A new winemaking process could improve wine bioactivity, provide guidance to future wine producers, and identify knowledge gaps for future research.

2. Materials and Methods

2.1. Grape

The grapes used in this study came from vineyards in the village of Vinča in Topola (Republic of Serbia), which are characterised by particular microclimatic conditions and soil types (44°13′35.4″ N 20°39′11.1″ E). The Cabernet Sauvignon grape variety was harvested in the state of technological maturity. Their phytosanitary condition was 100% healthy. The sugar content of the must was 23.4 °Bx and the acidity was 6.7 g/L, expressed as tartaric acid.

2.2. Standards and Chemicals

Sigma-Aldrich (Steinheim, Germany) supplied standards of resveratrol (>99%), piceid (>99%), quercetin dihydrate (98%), rutin hydrate (95%), (+)-catechin (≥90%), kaempferol (≥97%), naringenin (95%), and hesperetin (≥95%), as well as phenolic acids: caffeic, chlorogenic (95%), *p*-coumaric, vanillic, syringic (≥95%), *trans*-cinnamic (99%), and 4-hydroxybenzoic. Alfa Aesar (Heysham Lancaster, UK) supplied gallic acid and Lach-Ner (Neratovice, Czech Republic) benzoic acid. AppliChem (cyanin-3-glucoside chloride, delphinidin-3-glucoside chloride, and malvidin-3-glucoside) and Phytolab (petunidin-3-glucoside chloride, and peonidin-3-glucoside chloride) supplied standards of anthocyanins. Promochem LGC (Wesel, Germany) supplied HPLC-grade solvents (methanol and acetonitrile), while acids were supplied by Lach-Ner (Neratovice, Czech Republic) (formic and acetic) and Roth (Karlsruhe, Germany) (hydrochloric, 35%).

2.3. Winemaking

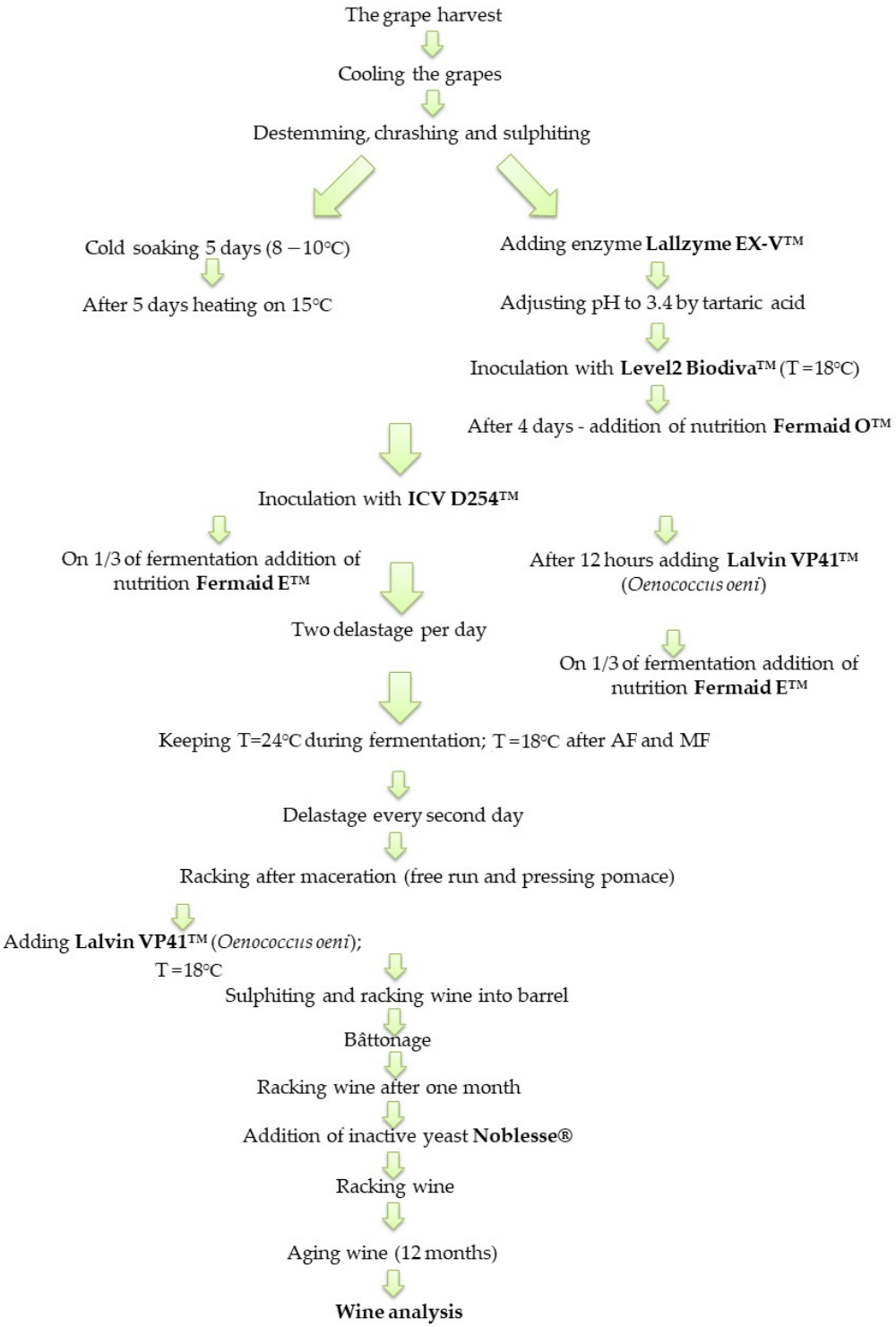
2.3.1. Traditional Processing

The first part of the current study was based on traditional winemaking technology (Scheme 1). The sample harvested in small beans (10 kg) after grape harvest was stored at 5 °C. The next step was grape destemming and gentle crashing with sulfation (50 mg/kg $K_2S_2O_5$). Cold maceration lasted 5 days at 8–10 °C with daily pumping over (1 volume of juice per day) covered with CO_2. After 5 days, the temperature was increased to 15 °C and then the sample was inoculated with 25 g/hL yeast *Saccharomyces cerevisiae* ICV D254™ (Lallemand, Cornwall, ON, Canada). After one-third of the fermentation time, the nutrient Fermaid E™ (Lallemand, Cornwall, ON, Canada) was added at a rate of 30 g/hL. During fermentation, the temperature was not allowed to exceed 24 °C. Two delastages per day were performed, one in the morning with oxygenation and the second at night (closed without spraying). After 10 days, when alcoholic fermentation was completed, the temperature was lowered to 18 °C, and delastage was performed every second day for the next 6 days (closed without spraying). The total maceration time was 21 days, and then the free run wine was separated from pomace and stored in a stainless-steel tank. This pomace

was pressed with a pneumatic press until a pressure of 0.8 bar was reached, and the liquid fraction thus obtained was mixed with the free-running wine. After 24 h, the wine was decanted from the yeast lees and the same procedure was repeated after 72 h and inoculated with the selection bacteria Lalvin VP41™ (*Oenococcus oeni*) (Lallemand, Cornwall, ON, Canada). Induced malolactic fermentation was carried out at 18 °C and stirring was done twice a week. After completion of malolactic fermentation, SO_2 was added (50 mg/L) and the wine was decanted from the lees to large barrels (3800 L). The barrels were made from Serbian–Slavonian oak (*Quercus sessiliflora*) and had been in use for 20 years. The material came from Homolje—Eastern Serbia (44°19′30″ N 21°45′28″ E, altitude 752 m). The wine matures in them for 4 years, and they are emptied and refilled every fourth year. Bâtonnage was performed twice a week. After one month (end of December 2018), the wine was racked off the lees and transferred to barrels. During the next three months, the lees were mixed (bâtonnage) twice a month. In March 2019, the wine was again racked off the lees and during the next 6 months, and Noblesse® (Lallemand, Cornwall, ON, Canada) and inactive yeast were added every second month (3 additions) (10 g/hL). During this period, yeast and Noblesse® were mixed once a month. In June 2019, the wine was racked off the lees and Noblesse® and put back into barrels for the next 12 months. The temperature and the free SO_2 content were controlled every month during the wine's storage in the barrels (temperature 12 ± 2 °C, relative humidity 75%). In addition, the analyses were performed.

2.3.2. Co-Inoculation

For the second experiment, the harvested grapes were stored at 16 °C (Scheme 1). In this case, grapes were sulfurized with 15 mg/kg $K_2S_2O_5$ during destemming and gentle crashing. The enzyme preparation Lallzyme EX-V™ (Lallemand, Cornwall, ON, Canada) was added in the amount of 3 g/100 kg. The pH was 3.58 and was adjusted from 3.58 to 3.4 by adding tartaric acid. Crushed grapes were inoculated with Level2 Biodiva™ (*Torulaspora delbrueckii*) in the amount of 25 g/hL. Temperature control (maintained at 18 °C) and two delastages per day, one in the morning and the second during the night (closed delastage without spraying), allowed a very slow fermentation. After 4 days, when the sugar content had decreased by 3 °Bx, 20 g/hL of Fermaid O™ (organic nutrition—Lallemand) was added and inoculated with the selected yeast, *Saccharomyces cerevisiae* ICV D254™, at 25 g/hL according to the manufacturer's protocol for rehydration. Twelve hours after the addition of ICV D254™, the selected bacteria, Lalvin VP41™ (*Oenococcus oeni*), was inoculated. Two delastages were performed every day, one in the morning with spraying (with oxygenation) and one at night without spraying (closed), until the end of alcoholic fermentation. At 1/3 of the fermentation time, 30 g/hL of the nutrient Fermaid E™ was added. The maximum temperature reached during alcoholic fermentation was 24 °C. After the alcoholic fermentation was completed (after 14 days), the temperature was lowered to 18 °C and the process continued until the malolactic fermentation was completed on pomace. On every second day, delastage was performed without spraying and malolactic fermentation was monitored. After 8 days, malolactic fermentation was completed (total time on pomace was 22 days). Free run wine was separated from the pomace and stored in a stainless-steel tank with sulfurization (50 mg/L). Then, the pomace was pressed on a pneumatic press until a pressure of 0.8 bar was reached, and the liquid fraction obtained was mixed with the free run wine. After 24 h, the wine was drawn off the lees and after 72 h the same procedure was applied, the SO_2 content was adjusted and the wine was stored in large barrels (3800 L). To study the influence of wine aging in bottles on polyphenolic composition of the wine, part of the wine was bottled and analysed after one year. The bottles were stored in the horizontal position and closed with a cork. Aging was carried out under cellar conditions (temperature 12 ± 2 °C, relative humidity 75%).



Scheme 1. Traditional processing and co-inoculation.

2.4. HPLC Analyses

HPLC analyses were conducted on wine samples filtered through regenerated cellulose membrane filters (0.45 µm; Sartorius, Ann Arbor, MI, USA), using an Agilent 1100 liquid chromatograph (Agilent Technologies, Santa Clara, CA, USA) configured as follows: quaternary gradient pump, autosampler (10–200 µL loop), heated column compartment equipped with a Poroshell 120 EC-C18 column (4.6 × 100 mm, 2.7 µm; Agilent, Santa Clara, CA, USA), and UV–VIS detector. For the quantification of compounds of interest, an external

calibration approach was applied, based on calibration curves prepared using mixtures of standard phenolic compounds [20]. Identification of compounds in a chromatogram of standard calibration mixture (and in wine samples) was based on comparisons of observed peak retention times with retention times recorded in chromatograms obtained for each individual compound and, in case of phenols, based on different absorption wavelengths used for detection. All samples were measured in triplicate.

2.4.1. Phenols

Phenolic compounds were analysed using the HPLC method validated by Atanacković Krstonošić et al. [21]. The wine samples (5 µL) were analysed under the following HPLC conditions: a mobile phase composed of 0.1% CH_3COOH (A) and 0.1% CH_3COOH (B) in acetonitrile run at a flow rate of 1.0 mL/min (gradient elution), a column temperature of 25 °C, and a UV-VIS detector set at the following wavelengths: 225 nm (detection of vanillic and benzoic acid), 280 nm (detection of catechin, hesperetin, naringenin, and phenolic acids: gallic, 4-hydroxybenzoic, syringic, and *trans*-cinnamic), 305 nm (detection of resveratrol, piceid, and *p*-coumaric acid,), 330 nm (detection of caffeic and chlorogenic acid), and 360 nm (detection of quercetin, rutin, and kaempferol) [20]. Figure 1 presents a HPLC chromatogram of the phenolic compounds.

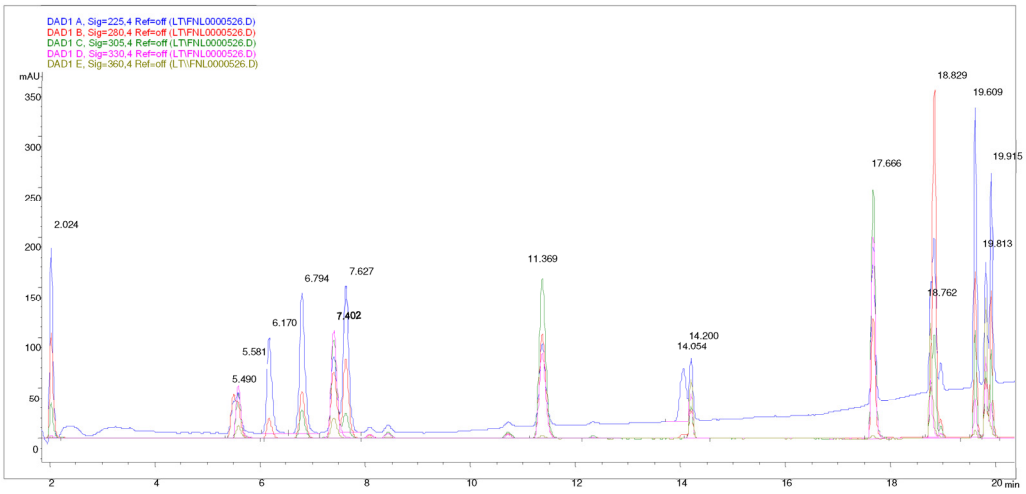


Figure 1. HPLC chromatogram of phenolic compounds (retention times (min): gallic acid 2.024, *p*-hydroxybenzoic acid 5.490, chlorogenic acid 5.581, catechin 6.170, vanillic acid 6.794, caffeic acid 7.402, syringic acid 7.627, *p*-coumaric acid 11.369, benzoic acid 14.054, rutin 14.200, resveratrol 17.666, quercetin 18.762, *trans*-cinnamic acid 18.829, naringenin 19.723, kaempferol 19.609, and hesperetin 19.915).

The stock solutions of the individual phenolics were prepared by their dissolution in dimethyl sulfoxide (DMSO) [21]. Solutions of individual standards were further mixed and diluted with 0.1% acetic acid as a way to obtain working solutions in a concentration range from 0.5 to 20/25 mg/L. The correlation coefficients of calibration curves (>0.998) confirmed excellent linearity for all phenolics. The sensitivity of the method was improved by using sample volumes increased by a factor of five compared to the standard mixture solution. Such an approach enabled a quantification limit of 0.1 mg/L and detection limit of 0.05 mg/L (based on signal-to-noise (S/N) ratios $\geq$10 and $\geq$3, respectively) and thus the determination of less-abundant phenolics in the wine samples [20].

2.4.2. Anthocyanins

HPLC conditions used for separation of anthocyanins (100 µL) were the following: the mobile phase consisted of water/formic acid/acetonitrile mixtures (A—87:10:3, B—40:10:50; flow rate was 0.8 mL/min; gradient elution), column temperature was 40 °C,

and the UV-VIS detector was set at 518 nm (according to the method proposed by the International Organization of Vine and Wine (OIV, 2013) and modified by Majkić et al. [20]). Figure 2 presents the chromatographic separation of five anthocyanins commonly present in wines.

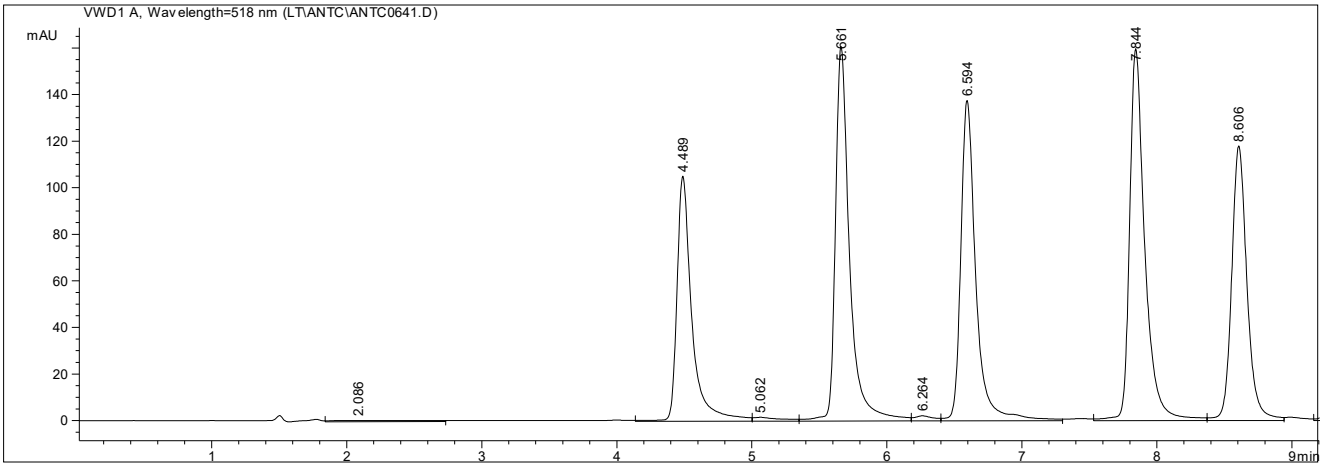


Figure 2. Chromatographic separation of anthocyanins (3-*O*-glucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin; concentration 10 mg/L).

The stock solution of each anthocyanin was prepared by its dissolution in methanol acidified with HCl to 1%. Individual standards were mixed and further diluted with the solvent mixture corresponding to the HPLC mobile phase in order to obtain calibration solutions in a concentration range of 0.1 to 100 mg/L. All five corresponding calibration curves were characterized with excellent correlation coefficients (>0.998). The lowest calibration level was set as the limit of quantification (0.1 mg/L; S/N $\geq$ 10), while the detection limit was established at 0.05 mg/L (S/N $\geq$ 3).

2.5. Determination of Titratable Acidity in Must

A volume of 25 mL was titrated with 0.25 M NaOH to determine the titratable acidity of the wine [22]. The end point of the titration (pH 7.0 $\pm$ 0.5) was indicated with a pH meter (Farnell, Denmark).

2.6. Determination of the Sugar Content in Must

The sugar content (expressed in °Bx) was measured in the grape juice using a PAL-87S refractometer (Atago, Tokyo, Japan).

2.7. Determination of Physico-Chemical Parameters in Wine

In order to determine the physico-chemical parameters of the wine, the alcohol content (%), the reducing sugar (g/L), the total extract (g/L), the extract without sugar (g/L), the total acidity as tartaric acid (g/L), the volatile acidity as acetic acid (g/L), the total and free SO_2 content, the ash (g/L), and the total phenolic content as gallic acid (g/L) were carried out in accordance with the recommendations of the OIV [23]. All analyses were carried out in triplicate.

Sensory Evaluation of Wine

The sensory evaluation was carried out by 3 trained sensory judges using the Buxbaum positive points method. This is a 20-point method that requires a detailed evaluation of each wine. In this test, the evaluator assigns scores for colour 0–2, clarity 0–2, smell 0–4, and taste 0–12 [24]. In this way, the wine can receive a maximum of 20 points.

2.8. Statistical Analysis

Results were presented as mean ± sd. Independent samples *t*-test was used to test differences between groups. All *p* values less than 0.05 were considered significant. Statistical data analysis was performed using IBM SPSS Statistics v22 (IBM Corporation, Armonk, NY, USA).

3. Results

3.1. Stilbenes

Stilbenes are mainly found in the grape skins, so yeasts with increased enzymatic activity could increase the extraction of stilbenes from the grape into the wine. By applying the co-inoculation technology, the concentration of resveratrol and piceid in Cabernet Sauvignon wine could be significantly increased (by a factor of two and four, respectively) compared to the wine produced using traditional technology (Table 1).

Table 1. Concentration of stilbenes and quercetin in Cabernet Sauvignon wines produced by traditional technology and co-inoculation.

Phenolic Compound (mg/L)	Traditional Winemaking Technology	Co-Inoculation	% Increase in Content	*p*-Value
trans-resveratrol	1.30 ± 0.09	3.10 ± 0.10	138.5	<0.001 *
Piceid	4.80 ± 0.18	9.30 ± 0.20	93.8	<0.001 *
Quercetin	0.70 ± 0.04	3.0 ± 0.08	328.6	<0.001 *

* Denotes the level of significance of 0.001.

The concentration of piceid and *trans*-resveratrol in our Cabernet Sauvignon wine was significantly higher in the wine produced via applying co-inoculation ($p < 0.001$) (9.30 ± 0.20 mg/L; 3.10 ± 0.10 mg/L) than in the wine produced with the traditional winemaking technology, which is consistent with a study by Escribano-Viana et al. [17] that showed higher levels of total stilbenes, *cis*-piceid, *trans*-piceid, and *trans*-resveratrol compared to fermentation without non-*Saccharomyces* yeasts. The same study confirmed the higher content of piceid than of free resveratrol. The higher piceid content could be attributed to incomplete enzymatic cleavage of *trans*-piceid during vinification [25]. This suggests that the *β*-glucosidase activity of yeasts could be inhibited under winemaking conditions due to the low pH, high initial glucose concentration, and low aeration at the beginning of fermentation [26]. Little is known in the literature about the effects of this specific culture mixture on stilbene content, but the study of the individual factors (yeasts, enzyme preparation, and malolactic bacteria) can be considered [11,25–27]. In contrast to traditional fermentation, in co-inoculated fermentation, malolactic bacteria were inoculated at the beginning of fermentation, resulting in a lower pH that could inhibit *β*-glucosidase activity, which could describe a higher piceid content. However, it is the bacterial enzyme activity that increases the stilbene content [28], probably in synergy with the *β*-glucosidase activity of the yeasts and the added enzymatic preparation. Obviously, the co-inoculation has increased both the free and glucosidic form of resveratrol (piceid) content, which is characterized by a higher value, probably due to the lower adsorption by yeast and yeast cells [29]. According to Sato et al. [30], the content of *trans*-piceid in Cabernet Sauvignon wine varies between 0.37–2.60 mg/L. In general, the average content of *trans*-piceid was found to be three times higher than that of *trans*-resveratrol. Kostadinović et al. [25] reported that Merlot wine produced with a 6-day maceration using French yeast contained a high concentration of *trans*-piceid (4.10 ± 0.86 mg/L), which was similar to our results obtained with traditional winemaking technology. The content of resveratrol was determined in red wines from Greece (0.550–2.534 mg/L), which was higher than in our traditionally produced wine [31], but the co-inoculation was characterised by a higher content (3.10 mg/L). Higher levels of *trans*-resveratrol were also found compared to Karaoglan wine from Turkey, whose maximum concentration was 2.68 ± 0.16 mg/L after 15 days of maceration [32]. In the

Croatian wine Crljenak kaštelanski, the resveratrol concentration ranged from 0.51 mg/L in the wine produced by the addition of Vinozym Vintage to 1.07 mg/L in the wine produced by the addition of SihazymExtro [33], which was less abundant compared to the values measured in our wine obtained by co-inoculation (Table 1). This suggests that the enzyme preparation Lallzyme EX-V™, used in our study in combination with mixed co-inoculation, contributed to a higher resveratrol content. According to Alencar et al. [34], the maximum value for *trans*-resveratrol was reached after 10 days of maceration (2.2 mg/L). In some other studies, *trans*-resveratrol concentrations of 0–2.0 mg/L were found in red wine from Queensland [35], 2.1–2.5 mg/L in Italian wines [36], and 0.56–2.86 mg/L in Italian red wines [37]. Experiments with the Tempranillo grape variety confirmed that the composition of stilbenes in wine can be modulated by the use of specific fermentation starters such as *Torulaspora delbrueckii* and other non-*Saccharomyces* strains compared to SC [17]. It is hypothesised that the enzymatic activity of yeasts improves their extraction as well as the enzymatic activities of early inoculated lactic acid bacteria. Yeasts with β-glucosidase activity not only release aroma precursors during alcoholic fermentation of grape must, but can also hydrolyse resveratrol glucosides from grapes [26]. The use of only SC and Lallzyme EX-V™ enzyme preparations on the same grape variety resulted in a lower content of *trans*-resveratrol [38]. *Torulaspora delbrueckii* produces glycerol or pyruvic acid and lower levels of acetic acid than most non-*Saccharomyces* yeasts, so it contributes to better organoleptic wine characteristics [16]. Its use as a starter for controlled slow fermentation and inoculation with SC after 4 days resulted in wines with higher stilbene content.

3.2. Quercetin

The quercetin content in our wine obtained by co-inoculation was higher than that of other Cabernet Sauvignon wines produced in Serbia reported by Radovanović et al. [39], as well as the study by Lisov et al. [38], whose results did not exceed 1.73 mg/L using different combinations of yeasts and enzymatic preparations. Similar observations were made for the Croatian autochthonous grape variety Crljenak kaštelanski obtained with different enzymes and a five-day maceration [33], but the quercetin content was lower than in our co-inoculated Cabernet Sauvignon. According to Hernández et al. [27], the amount of free flavonols in red wines could depend on the *Lactobacillus* strain carrying out malolactic fermentation. Regarding the use of mixed cultures, a recent study reported that the timing of bacterial inoculation did not result in significant changes in flavonol content [40]. The higher quercetin content in the co-inoculated wine could therefore be due to the enzymatic activity of the inoculated yeast *Torulaspora delbrueckii*. Additionally, the use of Lallzyme EX-V™ as a highly concentrated pectinase preparation with a secondary hemicellulase activity could result in a higher quercetin content [33,41]. According to Maturano et al. [42], mixed conditions also appeared to influence the expression of pectinases. The activity was significantly lower in mixed fermentations compared to pure fermentations of *T. delbrueckii* and *S. cerevisiae*, the main producers of this enzyme. The addition of Lallzyme EX-V™ pectinolytic enzyme preparation could overcome these challenges.

As reported by Artem et al. [43], the quercetin content in wines from 2016 was up to 5.94 ± 1.7 mg/L, while the wine from 2017 was not as rich in this flavonol.

3.3. Biologically Active Phenols

The concentrations of other biologically active phenols from red wine (catechin, rutin, and kaempherol, Table 2) were significantly higher in our co-inoculated wine than in the wine produced by traditional winemaking ($p < 0.05$).

Among the flavonoids, catechin was the most abundant phenolic compound, which is consistent with other reports [33,44]. The co-inoculation resulted in better extraction of catechin compared to the traditional method (Table 2), leading to higher catechin levels compared to Cabernet Sauvignon wine analysed in a previous Serbian study [39] and Monastrell wine from Spain, which ranged from 7.8 to 14.0 mg/L [41]. Prolonged contact of solids and must resulted in a higher catechin content in wine [38,43]. Thus, a 21-day

maceration promoted a high catechin content of up to 29.2 mg/L in co-inoculated wine. The combination of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* wild yeast resulted in a higher catechin content, which was consistent with the results of Ngqumba et al. [13], who experimented with the same mixture of strains for the Chenin blanc grape variety. Syrah wines produced with mixed co-inoculations of *Saccharomyces cerevisiae*/*Lactobacillus thermotolerans*/*Torulaspora delbrueckii* had lower concentrations of total flavan-3-ols compared to SC-inoculated wines [12], which is consistent with the results reported in this study for catechin.

Table 2. Concentration of other flavonoids in Cabernet Sauvignon wines produced by traditional technology and co-inoculation.

Phenolic Compound (mg/L)	Traditional Winemaking Technology	Co-Inoculation	% Increase in Content	*p*-Value
Catechin	25.3 ± 0.90	29.2 ± 1.10	15.4	0.009 **
Rutin	7.3 ± 0.20	12.2 ± 0.20	67.1	<0.001 **
Kaempherol	n.d. *	0.40 ± 0.04	100.0	

* n.d.—not detected; analysed but not detected: naringenin and hesperetin; ** indicates significance level of 0.05.

According to Maturano et al. [42], vinifications carried out with a pure or mixed culture (99% of *T. delbrueckii*) showed the highest production of all enzymes studied, with the exception of *β*-glucosidase. In mixed cultures, *S. cerevisiae* outperformed *T. delbrueckii* and was only detected up to halfway through the fermentation process. Nevertheless, its secreted enzymes could be detected throughout the fermentation process and are involved in phenol extraction, among other things.

3.4. Phenolic Acids

The concentrations of phenolic acids in Cabernet Sauvignon wines produced using traditional technology and co-inoculation are shown in Table 3.

Table 3. Concentration of phenolic acids in Cabernet Sauvignon wines produced by traditional technology and co-inoculation.

Phenolic Acids (mg/L)	Traditional Winemaking Technology	Co-Inoculation	*p*-Value
Gallic acid	58.90 ± 1.60	57.60 ± 1.20	0.323 *
Chlorogenic acid	0.80 ± 0.05	0.95 ± 0.09	0.065 *
p-hidroxybenzoic acid	0.70 ± 0.04	0.60 ± 0.02	0.018 *
Vanillic acid	10.40 ± 0.25	9.50 ± 0.40	0.030 *
Caffeic acid	3.70 ± 0.20	4.80 ± 0.25	0.004 *
Syringic acid	6.90 ± 0.30	5.40 ± 0.20	0.002 *
p-coumaric acid	3.0 ± 0.12	2.80 ± 0.15	0.146 *
Benzoic acid	2.50 ± 0.10	3.0 ± 0.10	0.004 *
t-cinnamic acid	0.10 ± 0.02	0.75 ± 0.04	<0.001 *

* Denotes the level of significance of 0.05.

The gallic acid content was in the range of its average content in red wines (41.8 ± 24.0 mg/L) [45] and higher than reported by Radovanović et al. [39] for Cabernet Sauvignon wine from Serbia (22.82 ± 0.98 mg/L). According to the literature, different winemaking techniques, such as pre-fermentative maceration, addition of enzymes, tannins, oak chips, or Ganimede autowinemaker [46], did not result in higher gallic acid content than that obtained in our wine by both traditional technology and co-inoculation. As reported by Artem et al. [43], gallic acid was the predominant phenolic acid in wines, with values ranging from 9.77 to 57.44 mg/L. According to Ngqumba et al. [13], *Torulaspora delbrueckii* strains had a positive effect on gallic acid concentration, but no significant

difference was found in our study with respect to the use of different yeast strains. The concentrations of other analysed phenolic acids detected in our study were lower compared to the results reported by Soto Vázquez et al. [46].

The content of *p*-coumaric acid in co-inoculated and traditional wine were almost identical (up to 3.0 mg/L). It was reported that the *p*-coumaric acid concentration in Syrah wine was quite low, at 0.2 mg/L [34]. However, Karaoglan wine was richer in this phenolic acid compared to our wines [32]. In contrast to our results, wines produced with a combination of *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* yeast had higher levels of *p*-coumaric acid [13].

The wines produced by co-inoculation had a higher content of caffeic acid (Table 3). This was in agreement with Ngqumba et al. [13], who found a higher level of caffeic acid in wines produced with a combination of selected and wild yeast strains.

The literature confirmed that the enzymatic preparations used had no statistically significant influence on the content of *p*-coumaric and caffeic acid [41,47].

3.5. Anthocyanins

The anthocyanin profiles of the traditional and co-inoculated wines are shown in Table 4. A statistically significant difference was found between the anthocyanin content of the co-inoculated and traditional wines ($p < 0.05$) except for malvidin 3-*O*-glucoside.

Table 4. Concentration of anthocyanins in Cabernet Sauvignon wines produced by traditional technology and co-inoculation.

Anthocyanin (mg/L)	Traditional Winemaking Technology	Co-Inoculation	% Increase in Content	*p*-Value
Delphinidin 3-*O*-glucoside	1.60 ± 0.15	2.70 ± 0.25	68.80	0.003 *
Cyaniding 3-*O*-glucoside	0.40 ± 0.04	0.80 ± 0.10	100.0	0.003 *
Petunidin 3-*O*-glucoside	2.0 ± 0.05	3.30 ± 0.15	65.0	<0.001 *
Peonidin 3-*O*-glucoside	2.0 ± 0.05	3.80 ± 0.25	90.0	<0.001 *
Malvidin 3-*O*-glucoside	24.20 ± 0.80	26.0 ± 0.95	7.40	0.066 *

* Denotes the level of significance of 0.05.

Yeasts are known to affect wine colour in several ways, including the adhesion/adsorption of pigment compounds to yeast cells. The final amount of anthocyanins in wine depends on environmental/agronomic factors and the conditions of fermentation (time and temperature), but the anthocyanin profile of different wine varieties is considered to be relatively stable [48].

As expected, malvidin-3-*O*-glucoside, the most abundant anthocyanin in grapes, was the dominant anthocyanin compound in both wines produced with traditional technology and co-inoculation (Table 4), similar to the findings of Generalić Mekinić et al. [33]. The concentrations of anthocyanins analysed in the different winemaking techniques were comparable to a study published for red wines from Spanish grape varieties [46]. According to Soto-Vázquez et al. [46], the wines produced by co-inoculation had higher anthocyanin contents than those produced with conventional vinification, pre-fermentative maceration, the addition of enzymes and tannins, oak chips, the delastage technique, and Ganimede autowinemaker. Moreover, our results were consistent with those obtained for wines produced with a combination of *Saccharomyces*, non-*Saccharomyces*, and *Oenococcus oeni* in successive inoculations compared to *Saccharomyces cerevisiae* reference wines [12]. Recently, Chen et al. [18] found an increase in total anthocyanins during the fermentation of *Torulaspora delbrueckii*/*Saccharomyces cerevisiae* in comparison with fermentation conducted

by *Saccharomyces cerevisiae* alone. Mixed fermentation cultures can change the chemical profiles of the wine. The absorption of anthocyanins could be different between species and strains [16]. In general, *Torulaspora delbrueckii* has been reported to increase anthocyanin content during alcoholic fermentation [17] and probably competes with *Saccharomyces* yeast, which adsorbs these compounds less through the cell walls. As for *O. oeni*, with a cell size several orders of magnitude smaller than that of *S. cerevisae*, its cells have a much smaller surface area [49]. In this study, malvidin-3-*O*-glucoside was the most abundant compound among the anthocyanins, possibly due to its low polarity and lower probability of being adsorbed by the yeast cells [49]. It is important to point out that added enzymatic preparation allows the complete and rapid release of anthocyanins, greater efficiency in the release of tannins and their subsequent binding with anthocyanins, and better wine stability (color and body). In recent decades, the use of enzymatic preparations has proven to be effective, so we use starter yeasts that produce extracellular hydrolytic enzymes. There is very little information on the effects of β-glucosidase activity of yeasts on grape and wine anthocyanins. Although *S. cerevisiae*, the main wine yeast, is not a good producer of β-glucosidase, other non-*Saccharomyces* wine yeasts (*Brettanomyces*, *Candida*, *Debaromyces*, *Dekkera*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Pichia*, *Rhodotorula*, *Saccharomycodes*, *Schizosaccharomyces*, *Torulaspora*, and others) are considered potential sources of this enzyme. Moreno-Arribas and Polo [50] presented that the effect of a β-glucosidase activity of *Candida molischiana* resulted in a 50% colour loss during the fermentation period.

Significantly higher levels of malvidin-3-*O*-glucoside have been reported compared to our wine [51,52]. According to Minnaar et al. [12], the content of malvidin-3-*O*-glucoside in *Saccharomyces* wines after malolactic fermentation (MLF) was significantly lower than in wines with mixed culture inoculation (SC/non-*Saccharomyces* and *O. oeni*), which is consistent with our results. According to Burns and Osborne [49], significantly higher levels of all individual anthocyanins were found in wines inoculated with *Oenococcus oeni* compared to the control (without MLF). It is known that malolactic bacteria produce enzymes such as glycosidases which act on the acylated anthocyanins and could therefore influence the anthocyanin profile of the wines [40]. When comparing the co-inoculation of yeasts and malolactic bacteria with sequential traditional MLF, a higher content of monomeric anthocyanins was found in co-inoculated wines. This suggests the involvement of bacterial enzymatic activities such as glycosidases during early inoculation [40], which is consistent with our experiment. The co-inoculation of *Saccharomyces* and non-*Saccharomyces* yeasts and the addition of malolactic bacteria during alcoholic fermentation and enzymatic preparation may contribute to a higher anthocyanin content in wine. In addition to *Saccharomyces* yeasts and enzymatic preparation, *Torulaspora* yeasts also contribute to the intensity and stability of wine colour through the production of pyruvic acid [16].

3.6. Complete Physico-Chemical Analysis of the Wine

A physico-chemical analysis was carried out which included the alcohol content, reducing sugar, total extract, extract without sugar, total acidity as tartaric acid, volatile acidity as acetic acid, total and free SO_2 content, ash content, and total phenolic content as gallic acid of wines made by traditional winemaking technology and co-inoculation. The results are shown in Table 5.

Table 5. Physico-chemical parameters of wine samples.

Parameter	Traditional Winemaking Technology	Co-Inoculation
Alcohol content (%)	13.7 ± 0.2	13.9 ± 0.1
Reducing sugar (g/L)	0.75 ± 0.2	0.62 ± 0.2
Total extract (g/L)	23.6 ± 0.2	25.2 ± 0.2
Extract without sugar (g/L)	23.6 ± 0.2	25.2 ± 0.2
Total acidity as tartaric acid (g/L)	4.69 ± 0.2	4.75 ± 0.3
Volatile acidity as acetic acid (g/L)	0.45 ± 0.3	0.62 ± 0.2
Total SO_2 (mg/L)	110.6 ± 0.2	112.4 ± 0.2
Free SO_2 (mg/L)	27.4 ± 0.2	29.3 ± 0.2
Ash (g/L)	2.8 ± 0.2	2.4 ± 0.3
Total phenolic content as gallic acid (g/L)	2.1 ± 0.1	3.9 ± 0.2

Sensory Evaluation of Wine

The wine obtained by traditional technology was characterized by a slightly more open ruby colour and a more closed aroma. The taste had a lighter structure and moderate tartness. The wine obtained by co-inoculation had a closed ruby colour and a distinct fruity smell associated with raspberry and blackberry, and the taste was fuller compared to the traditional one, with an accentuated tannic structure. The results are shown in Table 6.

Table 6. Sensory evaluation of wine.

Parameter	Traditional Winemaking Technology	Co-Inoculation
Colour	1.77 ± 0.06	2.00 ± 0.00
Clarity	2.00 ± 0.00	2.00 ± 0.00
Smell	3.33 ± 0.15	3.66 ± 0.20
Taste	10.57 ± 0.15	10.82 ± 0.15
TOTAL	17.67 ± 0.09	18.48 ± 0.08

3.7. Wine after Storage

The storage and maturation of the wine in the bottle has an influence on the polyphenol content due to the interactions between the phenolic compounds. These changes are due to combination reactions with various other compounds in the wine, as well as degradation reactions. The stability of wine pigments depends on various reactions, such as the thermal and oxidative degradation of anthocyanins, anthocyanin copigmentation reactions, and condensation reactions of anthocyanins and tannins (A-T, T-A, and condensation with an ethyl cross-bond). In addition to the oxidation and degradation reactions, polyphenols, especially tannins, are able to form stable compounds with proteins and polysaccharides during aging [53].

As shown in Figure 3a, no decrease in anthocyanins was observed during storage, which is in contrast to the reports of Ivanova et al. [54]. Statistical analysis also showed a significant increase in certain anthocyanins (delphinidin-3-*O*-glucoside and peonidin-3-*O*-glucoside). Aging had no significant effect on the other anthocyanins tested. A smaller decrease in anthocyanin content was observed in wines which were macerated longer [54], as they are stabilized by participation in complexes or polymers with flavan-3-ols. In contrast to other studies [55,56], our wines did not undergo degradation of the monomeric anthocyanins. During wine storage, there is a chemical equilibrium between monomeric and copigmented anthocyanins. A decrease in the concentration of monomeric anthocyanins due to polymerization reactions alters this chemical balance. Another possibility would be the dissociation of copigmented anthocyanins, which increases the concentration of monomeric anthocyanins [57], which can be related to our results. Similarly, in a study by Carew et al. [58], no significant decrease in average anthocyanin content was found for any yeast treatment between bottling and 6 months of bottle age. According to

Castillo-Sánchez et al. [55], the anthocyanin contents in young red wines depend strongly on the type of vinification, but these differences are significantly smaller in aged red wines. During storage, the anthocyanins are polymerized with other compounds and cannot be determined by HPLC. The differences between the anthocyanin contents in aged red wines determined by HPLC and spectrophotometric methods were greater than the differences observed between red wines at the time of bottling and aged wines [55].

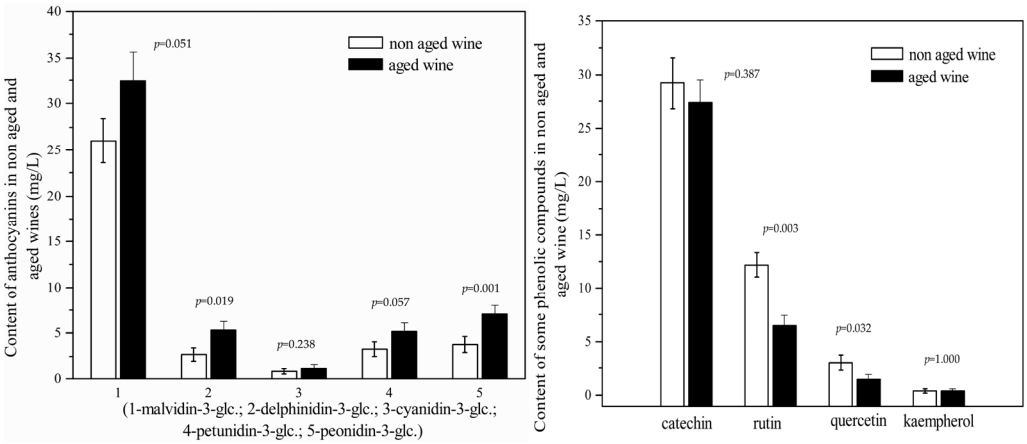


Figure 3. (**a**) Content of anthocyanins in wine after storage in the bottle; (**b**) content of catechin, rutin, quercetin, and kaempherol in wine after storage in the bottle.

A decrease in the content of catechin, rutin, quercetin and kaempherol (Figure 3b) was observed. Among these phenols, the changes caused by wine aging were significant only for quercetin and rutin ($p < 0.05$). According to other studies, a decrease in flavan-3-ols was observed, partly due to polymerization with anthocyanins and partly due to the formation of procyanidins [56,59]. The decrease in flavonols is related to the fact that they can be oxidized by coupled reactions or act as co-pigments with anthocyanins in co-pigmentation processes. A decrease in flavonol glycosides and an increase in the corresponding aglycones was observed, which is attributed to enzymatic hydrolysis [60]. Quercetin was under the influence of oxygenation, indicating its high reactivity with oxygen [61], which could be a reason for the degradation in our wine during the two-year maturation in the barrel before storage in the bottle.

Our results also showed a decrease in the content of phenolic acids (Figure 4a) during wine storage, which is in agreement with Suprun et al. [62]. As for the phenolic acids analysed, only benzoic acid, chlorogenic acid, and *p*-hidroxybenzoic acid change significantly ($p < 0.05$) during aging. Bautista-Ortín et al. [41] reported that the content of *p*-coumaric acid increased by one third after 8 months of bottle storage. In our study, the increase in *p*-coumaric acid could be due to the enzymatic hydrolysis of *trans*-coumaric acid and especially *trans*-caftaric acid in favour of caffeic acid and coumaric acid, which is in agreement with García-Falcón et al. [63]. The increased coumaric acid content can also be attributed to the degradation of cumarylanthocyanins during storage of the wines [63].

As shown in Figure 4b, aged wine contained a lower concentration of piceid (a bound form of resveratrol), which is due to its hydrolysis. This is also confirmed by the statistical data processing. As expected [64], the concentration of free *trans*-resveratrol in our wine increased with storage time, which is due to the natural acid hydrolysis of sugar–resveratrol complexes at storage temperature, but this increase was not significant ($p > 0.05$). Six months of storage resulted in a 1.2-fold decrease in total stilbene content [62]. In contrast to our results, Suprun et al. [62] reported that after storage, the content of *trans*-resveratrol decreased from 14.1% to 12.4% of the total stilbenes detected.

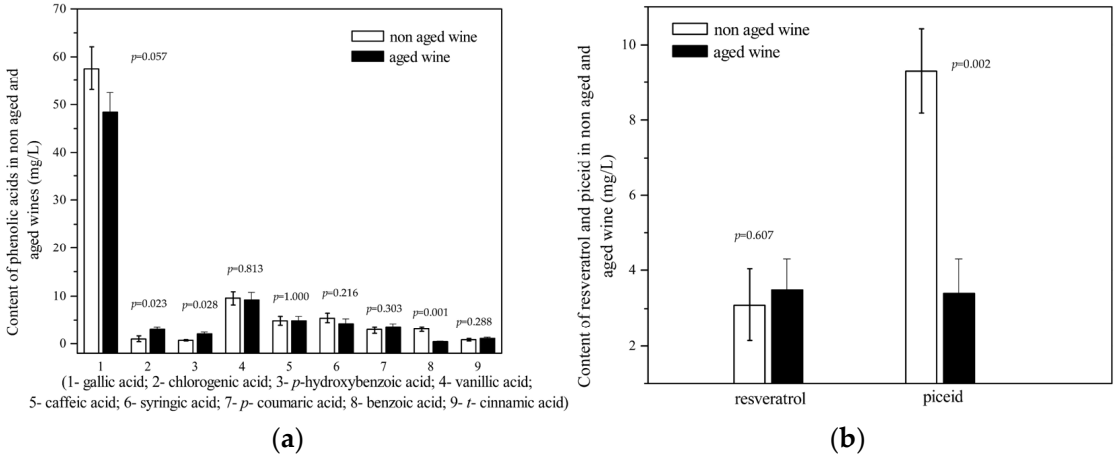


Figure 4. (**a**) Content of phenolic acids in wine after storage in the bottle; (**b**) content of resveratrol and piceid in wine after storage in the bottle.

4. Conclusions

The proposed co-inoculation protocol is a good choice for the vinification of Cabernet Sauvignon wines. Interestingly, the concentrations of resveratrol, piceid, and quercetin were higher in these wines made by co-inoculation than in the wines made with the traditional technology—cold maceration. It is therefore clear that our modified red wine production technology has achieved its objective: it has produced a wine rich in biologically active phenols and with numerous health benefits. Mixed culture co-inoculations with *Saccharomyces cerevisiae* cultures together with non-*Saccharomyces* and *O. oeni* represent a practical way to improve the phenolic composition of wine. Overall, *Torulaspora delbueckii* appears to be a good non-*Saccharomyces* yeast strain to be used in pre-fermentative maceration with Lallzyme EX-V™ enzyme and is able to increase the content of anthocyanins and stilbenes, as well as other bioactive compounds. The use of non-*Saccharomyces* yeast strains in co-inoculation with malolactic bacteria and the use of pectolytic enzyme preparations could replace cold maceration and thus improve the quality and health properties of the wine because of the extraction of the mentioned compounds (mainly anthocyanins and skin phenols).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12051020/s1, Figure S1. Structures of analyzed phenolic compounds (a–p); Figure S2. Structures of analyzed anthocyanins (a–e).

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