Impacts of added oenological tannins on red wine quality to counteract Botrytis infection in Merlot grapes

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ABSTRACT
The contamination of grape berries by Botrytis cinerea can drastically damage wine quality, in particular causing colour degradation. In musts obtained from botrytised grapes, SO₂ addition is the main means of avoiding oxidation damage due to laccases excreted by the pathogen. However, consumers are becoming increasingly reluctant to accept SO₂ addition to wine. Oenological tannins are used for fining wines because of their colloidal properties, and for wine colour stabilisation due to their ability to condense with anthocyanins. They are also known for their antioxidant and antioxidasic properties. They were thus investigated in the present study for their potential as an alternative to SO₂ against laccase oxidation.

The impact of various types of oenological tannins on musts and wines was studied once added to musts obtained from the Merlot cultivar, comprising 20 % and 50 % botrytised grapes. Laccase activity, antioxidant capacity, composition of phenolic compounds, spectrophotometric and CIELAB colour parameters were assessed in the musts and wines. Sensory analyses were also performed on 3-month-old wines to evaluate the visual, olfactory and gustative consequences of tannin addition. At a 50 % botrytisation rate, the addition of any type of oenological tannins (at a concentration of 100 g/hL) had no effect on laccase activity and did not protect phenolic compounds. However, at the same concentration and at a 20 % botrytisation rate, proanthocyanidin tannins from grape skin were found to be the most promising tannins with simultaneous protective effects, such as an inhibitory effect on laccase enzymes, protection of colour from complete degradation and preservation of some procyanidin compounds.

Oenological tannins are promising candidates for protecting wines from Botrytis damage and they induce a differential effect according to their origin and structure. They could be used to reduce the amount of SO₂ that is added during vatting. More research is needed to confirm and better understand the mode of action of various tannins at levels lower than the 20 % botrytised rate tested in the present study.

KEYWORDS
Red wines, oenological tannins, laccase activity, phenolic compounds, colour, sensory analysis.
INTRODUCTION

Grey mold resulting from the pathogenic fungus Boletus cinerea is a major grapevine disease, which is particularly prevalent in areas with temperate oceanic climates (Dubos, 1999; Elmer and Michailides, 2004; Martinez et al., 2005). The pathogen causes major economic losses; not only does it significantly affect grapevine yield, but it induces biological and chemical changes within the maturing grape berries that have a negative impact on the organoleptic qualities of the wine (Ky et al., 2012). Boletus cinerea is a necrotrophic pathogen belonging to the Sclerotiniaceae family (Ascomycota). It infects various hosts (up to 1400 plant species), possibly owing to an adaptation to those hosts, since the genetic structure of the population show a clear differentiation depending on the host plant of origin (Elad et al., 2016; Walker et al., 2015). In the vineyard, the fungus preferentially develops on berries, although it may also infect leaves and inflorescences, with significant variations in severity of infection depending on both the ontogenic resistance of the host tissue, particularly the grape berry, and the transposon subpopulation of the pathogen (Elmer and Michailides, 2004; Martinez et al., 2005; Martinez et al., 2008; Deytieux-Belleau et al., 2009).

The infection of grape berries by B. cinerea is accompanied by the excretion of metabolites (glycerol, gluconic acid and polysaccharides) and enzymes in the host cells, such as pectinases, proteases, tyrosinases and laccases. Laccases are multi-copper glycoproteins that catalyse the oxidation of mono- and/or di-phenolic substrates in the presence of oxygen. They are stable at the wine pH (Claus et al., 2014). These o-diphenol and p-diphenol dioxygen oxido-reductases can oxidize a wide range of polyphenols (Du Toit et al., 2006; Dubernet et al., 1977). Anthocyanins and tannins, which are naturally present in grape berries, are used as substrates by laccases (Oliveira et al., 2011). These phenolic compounds play a major role in the organoleptic quality of wine since they contribute to colour stabilisation and mouthfeel balance in terms of both astringency and bitterness (Noble, 2002; Santos-Buelga and Freitas, 2009). In the presence of oxygen, laccases oxidise these substrates leading to the formation of quinones, which can then polymerise and form brown compounds (Li et al., 2008; Pourcel et al., 2007; Salguès et al., 1986). This phenomenon, called “oxidasic haze”, is characterised by a degradation and/or instability of wine colour (Dubernet et al., 1975; Cheynier et al., 1995).

When this haze occurs, the pink/purple colour of red grape musts develops brick hues and white grape musts turn from yellow to brown. Following grape berry infection by B. cinerea, colour degradation due to the oxidation of polyphenols begins in the maturing berries and continues in the must, leading to the deterioration of organolectic quality. The olfactory and taste quality of the wines obtained from these botrytised musts is also affected (Ky et al., 2012). Red wines contaminated at 5 % by B. cinerea will already suffer irreversible consequences on their colour and organolecipe quality. Moreover, grey mould can affect their olfactory quality, creating aromatic defects, such as mushroom and/or earthy notes, which mask floral and fruity notes (La Guerche, 2004).

In the vineyard, synthetic fungicides are most frequently used to control the disease. Alternative control methods have been developed, particularly the use of biocontrol agents, such as antagonist microorganisms, including yeasts and bacteria strains (Calvo-Garrido et al., 2019; Pertot et al., 2017). Essential oils, mineral oils, plant hormones and plant extracts have also been tested as preventatives (Jacometti et al., 2010). In the cellar, oxidasic haze is usually prevented by sorting healthy grapes from contaminated ones and by adding SO2 (doses up to 50 mg/L), which has antioxidasic and antioxidant properties (Du Toit et al., 2006; Ribèreau-Gayon et al., 2006). Thermovinification can also be applied, allowing laccase enzymes to be denatured and inactivated by heat (Ribèreau-Gayon et al., 2006; Steel et al., 2013). However, this process is energy-expensive and may affect certain thermolabile compounds (i.e., aroma compounds), thus reducing the final quality of the wine. There are thus currently few alternatives to fungicides and to SO2 addition to treat musts obtained from mature grapes infected by the pathogen.

Oenological tannins are divided into two classes: hydrolysable tannins and condensed tannins (Versari et al., 2013). The latter, also called proanthocyanidins, mostly include procyanidins, prodelphinidins and profisetinidins (derivatives of fisetinidol present in quebracho and mimosa). Hydrolysable tannins include gallotannins (polymers of D-glucose and gallic acid) and ellagitannins (polymers of glucose and ellagic acid and/or hexahydroxydiphenic acid); for example, gallotannins are present in nutgall and tara, and ellagitannins are present in oak, chestnut and myrobalan. All oenological tannins exhibit colloidal properties used for the clarification of
musts and/or wines, but the properties of each group of tannins also differ depending on their chemical structure and composition. They may be used i) to prevent protein haze (white wines) by precipitating excess proteins (Organisation Internationale de la Vigne et du Vin, 2021), ii) to chelate metals (iron and copper), and/or iii) to stabilize the colour of wines (Canuti et al., 2012). These added tannins combine with anthocyanins from wine to form new, more stable pigments. From an organoleptic point of view, they contribute to the equilibrium of astringency and bitterness in wines. Furthermore, the use of oenological tannins in the pre-fermentation treatment of thermo-vinified Syrah musts improves their aromatic complexity and sensory properties (Chen et al., 2016). Tannins are also known for their antioxidant properties and can trap free radicals (anti-radical activity), particularly in grape seeds (Xia et al., 2010). Finally, oenological tannins can consume oxygen, with ellagitannins being the fastest consumers (Pascual et al., 2017), and they are also involved in anti-oxidase activity (Obradovic et al., 2005).

Very few studies have investigated the antioxidasic properties of tannins in general. A preliminary study on Merlot and Cabernet-Sauvignon red musts and wines showed an efficient and stable laccase inhibition owing to gallotannins and a mixture of proanthocyanidins with ellagitannins (Dumeau et al., 2004); laccase activity was inversely proportional to the tannin dose. More recently, Vignault et al. (2019) confirmed that supplementation with oenological tannins mitigated the damage caused by the presence of laccase in white wines. This study thus aims to better characterise and compare the antioxidasic effectiveness of various oenological tannins used as alternatives to SO₂ addition during vatting in botrytised musts. Their ability to reduce and/or inhibit laccase damage was quantified in red musts and wines, and the chemical and sensory impacts on the wines produced were evaluated.

MATERIALS AND METHODS

1. Experimental materials

1.1. Chemicals

Deionised water was purified with a Milli-Q® water system (Millipore, Bedford, MA, USA). Acetonitrile and methanol were of high-performance liquid chromatography (HPLC) grade and purchased from Sigma Aldrich (Saint Louis, USA), as were the following: (+)-catechin, malvidine-3-O-glucoside, gallic acid, Folin–Ciocalteu’s phenol (2N), sodium metabisulphite, sodium carbonate, phloroglucinol, L-ascorbic acid, hydrochloric acid (37 %), formic acid (98 %), 2,2’azobis(2-methylpropionamidine) dihydrochloride (AAPH, 97 %), disodium phosphate, sodium dihydrophosphate, fluorescein, trolox, polyvinylpolypyrrolidone (PVPP, 110 µm), sodium acetate, syringaldazine, 2,2-diphenyl-1-picrylhydrazyl (DPPH), sodium hydroxide and acetic acid.

Healthy grape bunches (approximately 66 kg) were randomly selected and then inoculated as described hereafter. The inoculum originated from a 7-day-old culture in Petri dishes of the B. cinerea strain 213. This single-spore isolate was selected from a collection belonging to UMR SAVE, Bordeaux, since it is a highly virulent strain belonging to the \textit{transposa} type (Martinez et al., 2003; Martinez et al., 2005; Martinez et al., 2008). Fruits were processed in 2-kg batches of grape berries. They were inoculated until the complete fruit surface was covered by spraying a mycelio-spore suspension at the rate of 1.4 x 10⁵ propagules/mL, with fungal propagules comprising a third of conidia and two thirds of mycelium fragments about 60-200 µm in length. To promote grey mould development, inoculated grape bunches were incubated in the dark at 13 °C for 16 days in a climatic phytotronic CONVIRON chamber (at 95-100 % relative humidity). Visual sorting of berries was then performed to remove undesirable rotten berries following other fungal development (e.g., \textit{Penicillium spp.}, identifiable via a blue-green colour). Botrytised grapes were used for micro-winemaking.

2. Micro-winemaking

Micro-winemakings (7 kg destemmed grapes per tank) were conducted in duplicate for each treatment and control.
2.1. Vatting

Typical infected and symptomatic berries (rotten berries often totally covered by *Botrytis* mycelium and/or sporulation) were added to healthy berries with a proportion of either 20 or 50 % (ratios were based on number of infected berries rather than on their weight), thus ending up with 7-kg batches of grapes. A control with 100 % healthy grapes was also prepared. Every batch was mechanically crushed and destemmed, then collected in a 10-L aluminium tank.

A 3 g/hL dose of SO$_2$ was added to the control tanks only (botrytisation rate 0 %) using a Bisulfite solution at 183 ± 3 g/L of SO$_2$ (Laffort, Bordeaux, France). Commercial tannins from Laffort and IOC were chosen for their various botanical origins and structures: grape seed (Gse), grape skin (Gsk), quebracho (Q), oak (O) and nutgall (N).

In the tanks with botrytised grapes, tannins were added at 100 g/hL, or not at all to obtain controls (20 % and 50 % *Botrytis* controls). The various treatments are presented in Figure 1. There were 13 tanks (1 x 2 with a botrytisation rate of 0 %, 6 x 2 with a botrytisation rate of 20 % and 6 x 2 with a botrytisation rate of 50 %) in duplicate with about 5 L of musts in each.

2.2. Alcoholic fermentation

*Saccharomyces cerevisiae* yeasts (Zymaflore$^\text{®}$FX10, Laffort) were prepared by rehydration in warm water (37 °C) for 15 minutes. Rehydrated yeasts were applied to each fermentation tank in order to reach concentrations of 20 g/hL. An alcoholic fermentation activator (Thiazote$^\text{®}$, Laffort) was added along with the leaven when the nitrogen concentration was lower than 140 mg/L and

![FIGURE 1. Experimental process of micro-winemaking from 20 % and 50 % botrytised grapes, with or without tannin addition.](image-url)

(Ø = no addition of tannins; Gse = grape seed tannins; Gsk = grape skin tannins; O = oak tannins; Q = quebracho tannins; N = nutgall tannins; AF = alcoholic fermentation; MLF = malolactic fermentation; VAT = vatting; AF1, AF2, AF3 = 1, 2, 3 days after beginning of alcoholic fermentation respectively; MAC = after post-alcoholic fermentation maceration; WINE = 3 months after bottling).
during fermentation (when a density of 1.06 kg/L had been reached) to complete nutriments at 200 mg/L. Fermentations were conducted at a constant temperature of 25 °C and temperature and density were monitored daily in each tank. The cap was punched down daily and O₂ (2 to 4 mg/L) was added when the density had reached 1.02. When the density was stable (0.993-0.997) in each batch and concentrations of reducing sugars were lower than 2 g/L, a 2-day post-fermentation maceration was conducted at 30 °C under CO₂.

2.3. Malolactic fermentation

Wines were separated from the pomaces by moderate manual pressing and poured into 5-L glass bottles. Malolactic fermentation was conducted by inoculating a commercial lactic acid bacteria, *Oenococcus oeni* (Lactoenos® SB3 Direct, Laffort), at 1 g/L. The decrease in malic acid concentrations was monitored with an enzymatic kit (R-Biopharm, Saint Didier au Mont d’Or, France). When they were lower than 0.2 g/L, finished wines were monitored with an enzymatic kit (R-Biopharm, Nieuwegein, Netherlands). C*ab* and h*ab* were calculated as follows: \[ C_{*ab} = \sqrt{(a^* + b^*)^2} \] and \[ h_{ab} = \arctan (b^* / a^*) \]. Delta E (ΔE) was also calculated to determine the colour difference between two wines: \[ \Delta E = \sqrt{(\Delta L_{*ab}^2 + \Delta a_{*ab}^2 + \Delta b_{*ab}^2)} \]. Values of ΔE higher than 3 are detectable with the naked eye (García-Marino et al., 2010).

5. Colour analyses

5.1. CIELAB parameters

Lightness (L*), position in a red-green axis (a*) and position in a yellow-blue axis (b*) (CIE, 1986) were measured with a Konica Minolta CM-5 apparatus. Chromatic parameters (Glories, 1984) were calculated as follows:

\[ C' = 10 * (A_{420nm} + A_{520nm} + A_{620nm}); \]

\[ \text{Hue} = A_{420nm}/A_{520nm}; \]

\[ \text{yellow} = (10 * 100 * A_{420nm})/C' \]

\[ \text{red} = (10 * 100 * A_{520nm})/C' \]

\[ \text{purple} = (10 * 100 * A_{620nm})/C' \]

6. Global phenolic compound analyses

6.1. Total polyphenol index (TPI)

Samples were previously diluted with a dilution factor (DF) of 50 (VAT-samples) or 100. The absorbance at 280 nm was measured in a 1-mm path length cuvette by a UV-Vis Helios Alpha™ spectrophotometer (Thermo Fisher Scientific Inc., Waltman, MA, USA). Chromatic parameters (Glories, 1984) were calculated as follows:

\[ \Delta E = \sqrt{(\Delta L_{*ab}^2 + \Delta a_{*ab}^2 + \Delta b_{*ab}^2)} \].

6.2. Folin-Ciocalteu index (FCI)

FCI was determined according to Singleton and Rossi (1965) with the following solutions placed in 50-mL volumetric flasks: 2.5 mL of Folin–Ciocalteu solution, 0.5 mL of sample or standard solution, 10 mL of Na₂CO₃ solution and water up to the 50-mL mark. After 30 min, absorbance was measured at 760 nm on a UV-vis spectrophotometer. Gallic acid was used as standard at concentrations ranging from 100 to 800 mg/L in order to express FCI in mg of gallic acid equivalents per litre of must or wine.

6.3. Total anthocyanins (TA)

Total anthocyanins were determined according to Ribèreau-Gayon and Stonestreet (1965). Solution A containing 250 µL of sample,
250 μL of ethanol acidified with 0.1 % HCl and 5 mL of 2 % HCl (v/v) was prepared beforehand. In two tubes, 1 mL of solution A was added to 400 μL of distilled water in tube 1 and 400 μL of 15 % potassium bisulfite in tube 2. After 20 min, the absorbance at 520 nm from both tubes was measured in a 1-cm optical path cuvette. Malvidin-3-O-glucoside was used as standard and TA was expressed in mg/L malvidin-3-O-glucoside equivalent.

7. Antioxidant capacities and laccase activity

ORAC and DPPH analyses were performed using a BMG Labtech FLUOstar Omega plate reader (Champigny s / Marne, France).

7.1. ORAC

All solutions were prepared in 7.4 pH phosphate buffer at 37 °C. In a 96-well black plate, 30 μL of diluted musts or wines (1/2000) or trolox (2.5 to 40 μM), 180 μL of 117 nM fluorescein and 90 μL of 40 mM AAPH solution were added to each well. The fluorescence (excitation = 485 nm and emission = 520 nm) was measured every minute for 90 min at 37 °C. For each kinetic, the area under the curve (AUC) was calculated and then from the standard curve \( \text{AUC} = f (\text{trolox concentration}) \), the antioxidant capacity of each sample was determined as being the concentration mM trolox equivalent (González-Centeno et al., 2012).

7.2. DPPH

According to Milat et al. (2019), 190 μL of 60 μM DPPH in methanol and 10 μL of the diluted sample (1/25 for the wines and 1/10 for the musts) or solutions of trolox (0.1-1 mM) were deposited in a 96-well transparent plate. The absorbance at 515 nm of each well was measured at 25 °C after 30 min. The result was expressed in mM trolox equivalent.

8. Laccase activity

Laccase activity was determined according to Grassin and Dubourdieu (1986). Five mL of sample were deposited in a syringe containing 0.8 g of PVPP to remove the phenolic compounds. In a 1-cm optical path cuvette, sample (500 μL) passed through PVPP was added, as well as 700 μL of 0.1M acetate buffer with a pH of 5.5 and 300 μL of syringaldazine 0.006 % (w/v) in ethanol. The absorbance at 530 nm was measured every minute for 5 min. We determined slope “a” (ΔA_{530nm}min^{-1}) in the linear part of the curve. Then laccase activity was calculated as follows: \( A_{\text{laccase}} = a \times 46.15 \text{ nmol/(min.mL)} \) (= laccase units, LU).

The relative laccase activity for each sampling time was also calculated:

Relative \( A_{\text{laccase}}(\%) = (\text{Sample Activity} / \text{Control Activity}) \times 100 \), the control laccase activity being that of the corresponding untreated botrytised must. Values under 100 % showed that the addition of tannins had had an inhibitory effect.

The measurements were made in duplicate.

9. HPLC analyses

9.1. Anthocyanin analyses

Musts and wines were filtered (0.45 μm) and directly injected for HPLC analyses. HPLC-UV analyses were performed by means of a Thermo Scientific Accela (Thermo Fisher Scientific, Waltham, MA, USA) with an Accela 600 pump module and a UV-Visible diode array detector and Xcalibur Software according to González-Centeno et al. (2017). Separation was performed on a reverse-phase C18 Nucleosil column (250 x 4.6 mm, 5 μm). The injected volume was 20 μL. The mobile phases were water/formic acid (95:5, v/v) (solvent A) and acetonitrile/formic acid (95:5, v/v) (solvent B), at a flow rate of 1 mL/min. Initial solvent B was set at 10 %. The mobile phase gradient was as follows: 35 % B at 25 min, 100 % B at 35 min, 100 % B from 35 to 40 min, 10 % B at 41 min, and then 10 % B for 4 min before the next injection. Eluting peaks were monitored at 520 nm. The mean peaks were identified by comparison with injected external standards and previous results (Chira, 2009). Concentrations were expressed as milligrams of malvidin 3-O-glucoside equivalents per litre.

9.2. Proanthocyanidin monomer and oligomer analyses

Musts and wines were filtered (0.45 μm) and directly injected for HPLC analyses. The equipment used for HPLC analysis consisted of a Thermo-Finnigan UV-Visible detector (UV-vis 200), a Thermo-Finnigan autosampler and a Thermo-Finnigan (San Jose, CA, USA) ternary pump coupled to a Xcalibur data treatment system. Separation was performed on a reverse-phase Lichrosphere 100-RP18 (250 mm x 2 mm, 5 μm; Merck, France) column according to González-Centeno et al. (2012). The mobile phases were water-acidified with formic
acid 0.5 % (solvent A) and acetonitrile-acidified with formic acid 0.5 % (solvent B). The gradient at a flow rate of 1 mL/min was 3 % B for 3 min, 3 to 5 % B for 11 min, 5 to 10 % B for 8 min, 10 to 14 % B for 4 min, 14 to 25 % B for 14 min, 25 to 100 % B for 1 min, 100 % B for 7 min, 100 to 3 % B for 2 min, and 3 % B for 5 min. Eluting peaks were monitored by a UV-detector at 280 nm and a fluo-detector ($\lambda_{\text{excitation}} = 280$ nm, $\lambda_{\text{emission}} = 320$ nm). Identification of mean peaks was performed by comparison with injected external standards and previous results (Chira, 2009). Calibration curves were established using the catechin as external standard. The results were expressed as milligrams of catechin equivalents per litre.

9.3. Determination of mean degree of polymerisation (mDP)

Proanthocyanidin mDP was determined for wines by phloroglucinolysis (Drinkine et al., 2007). The oligomeric and polymeric products were depolymerised in the presence of a nucleophilic agent (phloroglucinol) in an acid medium. Briefly, wine (5 mL) was evaporated to dryness using a rotary evaporator and then resuspended in 10 mL of milliQ water. The 10 mL sample (or 5 mL of musts) was passed through a pre-activated C18-SPE column (60 mL) and then washed with 60 mL of milliQ water. The samples were then eluted with 60 mL of methanol and the eluate was dried using a rotary evaporator and resuspended in 2 mL of methanol (extract 1). In an Eppendorf tube, 100 μL of extract 1 and 100 μL of methanol solution containing phloroglucinol at 50 g/L, ascorbic acid at 10 g/L and 0.1 N HCl were added. The tube was placed for 20 min in an oven at 50 °C, after which 1 mL of a 40-mM sodium acetate solution was added. The new extract obtained was filtered onto a 0.45-μm pore diameter syringe filter before injection. Reaction products were analysed by HPLC-MS on an Accela series (Thermo-Scientific, Illkirch-Graffenstaden, France), equipped with a pump module and a UV detector. Separation was performed on a reverse-phase Waters XTerra®RP C18 (Waters, Milford, MA, USA) (100 mm x 4.6 mm, 3.5 μm) column at room temperature. A binary gradient system was employed using 1 % (v/v) aqueous acetic acid (solvent A) and MeOH (solvent B) at a flow rate of 1 mL/min. The elution conditions were as follows: 5 % B for 25 min; 5-20 % B for 20 min, 20-32 % B for 15 min, and 32-100 % B for 2 min. The column was then washed with 100 % B for 5 min and re-equilibrated with 5 % B for 5 min before the next injection. The mDP, % of prodelphinidin (% P) and % of galloylation (% G) were estimated using the response factors of proanthocyanidin cleavage products at 280 nm and calculated as described by Chira et al. (2012). The mDP was obtained by dividing the sum of all subunits (flavan-3-ol monomers and phloroglucinol adducts, expressed in moles) by the sum of all flavan-3-ol monomers (expressed in moles).

10. Sensory analyses (wines)

Sensory analyses were carried out three months after the wines had been bottled. Replicate bottles of each treatment were mixed once they had been tasted by an expert to ensure the replicates were similar and did not have any major defects. The wines were evaluated by 18 to 22 trained judges from the Oenology Department of the University of Bordeaux. They were all selected on the basis of interest and experience in red wine sensory analysis. All analyses were performed at 20 °C with controlled hygrometry (ISO 8589:2007) in a specific room with individual tasting booths. Depending on the type of test, wine (20 mL) was tasted either in clear glasses to evaluate colour, or in black glasses to evaluate olfactory and taste components (NF V 09 110). Samples were presented randomly, numbered with 3-digit codes and balanced (triangular tests) or randomised (ranking tests and profiles).

10.1. Triangle tests

Several triangular tests (ISO4120: 2007) were carried out to determine significant differences between

i) the main control wine [0 % Botrytis, Ø tannins] and the other control wines [20 % Botrytis and 50 %, Ø tannins], and

ii) between control wines [20 % and 50 % Botrytis, Ø tannins] and the corresponding wines supplemented with tannins.

During the sessions, the judges were asked to observe, smell and/or taste the samples in the defined order and indicate one seemed different, even if they were not sure of their answer. The panel was also asked to freely note one or more descriptor(s) in order to differentiate the samples. The descriptors were retained only in the event of a correct answer from the judge.

10.2. Ranking tests
Ranking tests (ISO8587: 2007) were conducted in two sessions of six wines each (for each botrytisation rate). The panel of tasters classified the six tested wines according to the "overall quality" of the product (visual, aromatic and taste qualities), knowing that they were young wines (three-months old) made from Merlot. Ratings were assigned from 1 (the most qualitative) to 6 (the least qualitative). Results were processed using a Friedman test on the sum of the ranks assigned by the judges. The smallest significant difference was then calculated and compared to the absolute value of the difference in the sum of the ranks (for two wines), to determine which products were significantly different from each other.

11. Statistical analyses

For all duplicated tanks and all sampling times, classical oenological analyses and laccase activity were assessed in duplicate and chemical analyses in triplicate. Statistical analyses were performed on the mean of these replicates using the RStudio software (Version 1.1.442 - © 2009-2018 RStudio, Inc.). Error bars on the histograms corresponded to the standard deviation.

To reveal possible effects of "botrytisation level", "tannins" or "sampling time" main factors, one-way or two-way ANOVA analyses were performed with α = 0.05. Normality (Shapiro-Wilks test) was verified for most variables with α = 0.05. An ANOVA was also performed when the Shapiro-Wilks test gave a $p$-value $> 0.001$. Post-hoc tests (Tukey) were applied when significant differences were revealed.

Principal component analysis (PCA) was also performed using the R-Commander and FactoMineR packages with the following variables: i) oenological parameters (reducing sugars, pH, titratable acidity, volatile acidity, malic, tartaric and gluconic acids), ii) total anthocyanins (TA) and polyphenol contents (TPI and FCI), iii) colour (CI, hue, % purple, L*, a*, b*, C*ab, h*ab), iv) antioxidant capacities (ORAC and DPPH), and v) laccase activity. The correlated variables with $R^2 > 0.9$ (density, TAV, % yellow, % red, lactic acid) were eliminated before PCA. The "botrytisation rate" was used as the illustrative variable represented on figures by different colours (black for 0 %, red for 20 % and green for 50 %).

RESULTS AND DISCUSSION

1. Characterisation of musts and three-month wines after bottling

1.1. Musts

The wines were obtained from healthy grapes (0 % *Botrytis*) and from a mix of healthy grapes and totally infected grapes to reach a final 20 % or 50 % botrytisation rate. Table 1 presents the oenological and chemical parameters of the control (0 %), 20 % and 50 % botrytised musts and their corresponding 3-month-old wines.

The classic oenological parameters (pH, density, concentrations of reducing sugars and total sugars) varied slightly according to the level of botrytisation of the musts, although significant differences were found. Volatile acidity content increased by 17 % with a botrytisation level (trend) of 0 to 20 %, and significantly by 36 % with a 20 % to 50 % botrytisation level. Beside the possible production of acetic acid by *Botrytis*, the highest volatile acidity at higher botrytisation levels can also be explained by the action of acetic bacteria, most likely present in damaged grapes (Ribéreau-Gayon *et al.*, 2012).

The greatest differences were observed for phenolic compounds. The total phenolic compounds in botrytised musts decreased significantly to levels approximately two-fold less significant (TPI) or even three-fold less significant (FCI) than in the healthy musts. Anthocyanins were also drastically degraded in both 20 % and 50 % botrytised musts. Their concentration decreased significantly from 108.2 (control must) to less than 10 mg/L malvidin-3-O-glucoside eq. in the botrytised musts (anthocyanins identified and quantified by HPLC in botrytised musts in trace amounts). These results may be due to the presence of laccase with corresponding activities of 25.8 LU and 42.9 LU in 20 % and 50 % botrytised musts respectively (Table 1). Hence, there was sufficient laccase activity in the 20 % botrytised must to degrade all the anthocyanin content in the healthy musts. The major anthocyanin compound, malvidin-3-O-glucoside, present in the healthy must at about 35 mg/L, was thus nearly absent from both botrytised musts. These results are in agreement with those obtained by Ky *et al.* (2012). Moreover, the results in Table 1 show that the molecular tannins were significantly affected by enzymatic oxidation. Like the anthocyanins, the tannins had probably been oxidised since they are also substrates of laccases (Oliveira *et al.*, 2011).
Furthermore, since the chromatic characteristics and antioxidant capacities of botrytised musts were also markedly affected, the consequences of the degradation of phenolic compounds by laccase were again directly observable (Table 1); for example, the spectrophotometric “hue” parameter ($A_{420\text{ nm}} / A_{520\text{ nm}}$) was approximately twice as high in botrytised musts as in healthy musts, indicating a major oxidative degradation of botrytised musts.

The presence of laccase produced by $B.\ cinerea$ thus had a considerable negative impact on the phenolic compounds of the musts as soon as they were in the vat, the degradation having begun in the grape berries (Ky et al., 2012). The oxidation of polyphenols by laccase therefore directly affected the visual characteristics and antioxidant properties of the musts, which is expected to impact the final quality of the wines.

1.2. General description of 3-month-old wines

In contrast to the wines from healthy Merlot grapes, the botrytised control wines obtained from the botrytised musts were damaged, their colour and phenolic compounds having been drastically affected (Table 1). The botrytised wines were less intense (CI); they had a stronger yellow hue (increasing % yellow and decreasing % red) and a brickish colour. These characteristics increased with botrytisation rate. Tannin and anthocyanin contents decreased with botrytisation level.

All wine variables (oenological, chromatic, spectrophotometric, fluorometric and chromatographic parameters of the phenolic compounds and laccase activity) are represented in a PCA plot (Figure 2), with three main axes accounting for over 83 % of the overall variability.

**FIGURE 2.** Plot of principal component analysis obtained from healthy or botrytised (20 % and 50 %) 3-month-old wines, with or without added tannins.

A) plot of axes 1 and 2, B) plot of axes 1 and 3. Gse = grape seed tannins, Gsk = grape skin tannins, Q = quebracho tannins, O = oak tannins, N = nutgall tannins. Groups corresponding to the level of botrytisation are highlighted in green (50 %), red (20 %) and black (0 %).
**TABLE 1.** Physicochemical characterisation of control (0 %), 20 % and 50 % botrytised musts and 3-month-old wines.

| % Botrytis | 0 % | 20 % | 50 % | 0 % | 20 % | 50 % |
|------------|-----|------|------|-----|------|------|
| **Oenological parameters** | | | | | | |
| pH | 4.8 a* ± 0.0 | 4.8 a ± 0.0 | 4.7 b ± 0.0 | 3.7 ± 0.0 | 3.8 ± 0.0 | 3.8 ± 0.0 |
| Titratable acidity (g/L H₂SO₄ eq) | 3.4 ± 0.0 | 3.7 ± 0.2 | 3.7 ± 0.3 | 3.5 ± 0.1 | 3.2 ± 0.1 | 3.6 ± 0.2 |
| Density (g/dm³) | 1105 a ± 0 | 1104 b ± 0 | 1106 c ± 0 | 991 a ± 0 | 993 ab ± 0 | 995 b ± 1 |
| Alcohol (% v/v) | - | - | - | 12.8 a ± 0.1 | 12.2 b ± 0.0 | 12.3 b ± 0.0 |
| Volatile acidity (g/L H₂SO₄ eq.) | 0.45 a ± 0.00 | 0.53 a ± 0.00 | 0.72 b ± 0.04 | 0.34 a ± 0.01 | 0.52 b ± 0.02 | 0.90 c ± 0.20 |
| **Sugars** | | | | | | |
| Reducing sugars (g/L) | 167.4 a ± 0.0 | 162.8 b ± 0.4 | 164.6 ab ± 1.3 | 1.6 ± 0.1 | 1.4 ± 0.1 | 1.6 ± 0.3 |
| Total sugars (g/L) | 194.4 a ± 0.0 | 192.2 b ± 0.1 | 197.8 c ± 0.0 | 6.2 a ± 0.3 | 7.7 ab ± 0.2 | 10.2 b ± 1.1 |
| **Organic acids** | | | | | | |
| Tartaric acid (g/L) | 3.8 a ± 0.0 | 3.4 a ± 0.0 | 2.6 b ± 0.2 | 2.0 ± 0.1 | 2.2 ± 0.0 | 2.4 ± 0.3 |
| Malic acid (g/L) | 2.0 a ± 0.0 | 2.8 b ± 0.1 | 2.6 b ± 0.1 | 0.4 ± 0.0 | 0.2 ± 0.0 | 0.3 ± 0.0 |
| Lactic acid (g/L) | - | - | - | 1.9 a ± 0.0 | 1.7 b ± 0.0 | 1.5 c ± 0.0 |
| **Phenolic compounds** | | | | | | |
| TPI | 12.4 a ± 0.7 | 5.4 b ± 0.3 | 7.6 b ± 0.7 | 45.8 a ± 1.5 | 43.9 a ± 0.6 | 37.4 b ± 1.7 |
| FCI (mg/L gallic acid eq.) | 436 a ± 1 | 137 b ± 10 | 186 b ± 25 | 1932 a ± 62 | 1692 b ± 45 | 1424 c ± 59 |
| TA (mg/L malvidin-3-O-glucoside eq.) | 155.1 a ± 0.6 | 0.2 b ± 0.2 | 4.9 c ± 0.2 | 547.4 a ± 1.1 | 347.6 b ± 36.4 | 124.3 c ± 16.6 |
| Molecular anthocyanins (mg/L malvidin-3-O-glucoside eq.) | | | | | | |
| delphinidin-3-O-glucoside | 8.5 ± 0.4 | < 2 | < 2 | 29.5 a ± 0.9 | 14.1 b ± 2.5 | 5.3 c ± 0.1 |
| cyanidin-3-O-glucoside | 10.4 ± 0.3 | < 2 | < 2 | 8.6 a ± 0.6 | 7.1 a ± 0.2 | 4.7 b ± 0.0 |
| petunidin-3-O-glucoside | 7.8 ± 0.2 | < 2 | < 2 | 31.5 a ± 0.0 | 17.8 b ± 2.5 | 7.3 c ± 0.6 |
| peonidin-3-O-glucoside | 23.2 ± 0.6 | < 2 | < 2 | 30.9 a ± 1.2 | 21.9 b ± 0.1 | 10.5 c ± 0.4 |
| malvidin-3-O-glucoside | 34.9 ± 0.6 | < 2 | < 2 | 128.6 a ± 3.3 | 85.3 b ± 8.4 | 31.6 c ± 5.0 |
| peonidin-3-O-(6-O-acetyl)-glucoside | 5.5 ± 0.0 | - | - | 12.9 a ± 0.5 | 8.1 b ± 0.7 | 4.8 c ± 0.2 |
| malvidin-3-O-(6-O-acetyl)-glucoside | 10.7 ± 0.5 | - | - | 40.0 a ± 0.3 | 21.7 b ± 3.8 | 8.1 c ± 0.6 |
| peonidin-3-O-(6-O-p-coumaroyl)-glucoside | 3.0 ± 0.1 | - | - | 8.0 a ± 0.3 | 6.0 b ± 0.3 | 4.2 c ± 0.1 |
| malvidin-3-O-(6-O-p-coumaroyl)-glucoside | 4.2 ± 0.3 | - | - | 16.7 a ± 0.1 | 10.7 b ± 1.6 | 5.2 c ± 0.3 |
| **Total anthocyanins identified** | 108.2 a ± 1.4 | < 10.0 b ± 0.0 | < 10.0 b ± 0.0 | 306.6 a ± 1.9 | 192.6 b ± 19.9 | 81.5 c ± 7.3 |
**Molecular tannins (mg/L catechin eq.)**

|     | B1 (dimer) | B3 (dimer) | catechin | B4 (dimer) | B2 (dimer) | epicatechin | T (trimer) | Total tannins identified |
|-----|------------|------------|----------|------------|------------|-------------|------------|--------------------------|
|     | 0.01 ± 0.0 | 4.6 ± 0.4  | 10.0 ± 0.7 | 0.7 ± 0.1  | 1.4 ± 0.1  | 4.9 ± 0.1   | 17.7 ± 0.7 | 39.4 ± 1.8               |
|     | -          | -          | < 2       | -          | -          | -           | 3.8 b ± 0.0 | 5.9 a ± 0          |
|     | -          | -          | < 2       | -          | -          | -           | 3.7 b ± 0.1 | 6.9 ab ± 0      |
|     | 0.9 ± 0.1  | 17.0 ± 6.5 | 68.4 a ± 1.9 | 5.7 a ± 1.1 | 10.2 ± 1.2 | 54.0 a ± 0.8 | 13.3 ± 0.1 | 3.2 ab ± 0.1      |
| %  G | -          | 5.8 ± 1.6  | 42.6 b ± 4.6 | 0.8 b ± 0.5 | 3.6 b ± 1.0 | 19.2 b ± 8.8 | 6.9 ab ± 0.8 | 3.5 ± 0.1       |
| %  P | -          | 0.6 ± 0.5  | 5.1 c ± 2.9 | 0.3 b ± 0.0 | 0.8 b ± 0.5 | 1.7 b ± 1.3 | 4.0 b ± 2.8 | 2.8 b ± 0.2      |
| %  C | -          | -          | -         | -          | -          | -           | -          | 25.1 ± 3.1       |

**Antioxidant capacity**

|     | B1 (dimer) | B3 (dimer) | catechin | B4 (dimer) | B2 (dimer) | epicatechin | T (trimer) | Total tannins identified |
|-----|------------|------------|----------|------------|------------|-------------|------------|--------------------------|
|     | -          | -          | -        | -          | -          | -           | -          | -                        |
| %  G | -          | -          | -        | -          | -          | -           | -          | -                        |
| %  P | -          | -          | -        | -          | -          | -           | -          | -                        |
| %  C | -          | -          | -        | -          | -          | -           | -          | -                        |

**Spectrophotometric parameters of colour**

|     | CI | Hue | % yellow | % red | % purple | L* | a* | b* | Ca.b | ha.b |
|-----|----|-----|----------|-------|----------|----|----|----|------|------|
|     | 0.7 ± 0.3 | 1.1 a ± 0.2 | 47.5 a ± 3.7 | 44.8 a ± 3.3 | 7.6 ± 0.5 | 79.0 ± 5.7 | 18.8 ± 9.4 | 15.2 a ± 0.4 | 24.6 ± 7.0 | 41.0 a ± 15.1 |
|     | 0.7 ± 0.0 | 2.6 b ± 0.0 | 65.6 b ± 0.0 | 25.7 b ± 0.3 | 8.7 ± 0.3 | 86.9 ± 0.6 | 3.1 ± 0.5 | 24.7 b ± 1.2 | 24.9 b ± 1.2 | 82.9 b ± 0.7 |
|     | 0.8 ± 0.1 | 2.6 b ± 0.0 | 65.6 b ± 0.2 | 25.4 b ± 0.7 | 9.0 ± 0.0 | 83.2 ± 1.4 | 6.5 ± 1.7 | 29.2 ± 0.4 | 29.9 c ± 0.7 | 77.5 ab ± 3.0 |
|     | 9.3 a ± 0.2 | 0.6 a ± 00 | 32.1 a ± 0.1 | 56.7 a ± 0.0 | 11.1 a ± 0.1 | 14.6 a ± 0.1 | 46.2 a ± 0.0 | 24.6 a ± 0.1 | 52.3 a ± 0.0 | 28.0 a ± 0.1 |
|     | 8.0 b ± 0.0 | 0.8 a ± 01 | 38.0 b ± 2.0 | 50.5 b ± 2.1 | 11.5 a ± 0.1 | 15.3 a ± 0.3 | 45.1 a ± 0.4 | 25.2 a ± 0.0 | 51.6 b ± 0.3 | 29.2 a ± 0.2 |
|     | 5.5 c ± 0.0 | 1.2 b ± 00 | 48.8 c ± 0.7 | 41.0 c ± 0.6 | 10.2 b ± 0.1 | 27.0 b ± 0.0 | 50.9 b ± 0.3 | 39.3 b ± 0.8 | 64.3 c ± 0.2 | 37.7 b ± 0.7 |

**CIELAB parameters**

|     | L* | a* | b* | Ca.b | ha.b |
|-----|----|----|----|------|------|
|     | 79.0 ± 5.7 | 18.8 ± 9.4 | 15.2 ± 0.4 | 24.6 ± 7.0 | 41.0 ± 15.1 |
|     | 86.9 ± 0.6 | 3.1 ± 0.5 | 24.7 ± 1.2 | 24.9 b ± 1.2 | 82.9 b ± 0.7 |
|     | 83.2 ± 1.4 | 6.5 ± 1.7 | 29.2 c ± 0.4 | 29.9 c ± 0.7 | 77.5 ab ± 3.0 |
|     | 14.6 a ± 0.1 | 46.2 a ± 0.0 | 24.6 a ± 0.1 | 52.3 a ± 0.0 | 28.0 a ± 0.1 |
|     | 15.3 a ± 0.3 | 45.1 a ± 0.4 | 25.2 a ± 0.0 | 51.6 b ± 0.3 | 29.2 a ± 0.2 |
|     | 27.0 b ± 0.0 | 50.9 b ± 0.3 | 39.3 b ± 0.8 | 64.3 c ± 0.2 | 37.7 b ± 0.7 |

**Laccase activity**

|     | Laccase activity nmol/(min*mL) |
|-----|--------------------------------|
|     | -                              |
|     | 25.8 a ± 0.4                   |
|     | 42.9 b ± 2.0                   |
|     | 0.5 ± 0.1                      |
|     | 6.9 b ± 1.0                    |

* Different letters show significant differences between different botrytisation levels for the same variable (p-values < 0.05); a lack of letter indicates no significant difference. TPI = total polyphenol index, FCI = Folin-Ciocalteu index, TA = total anthocyanins, mDP = mean degree of polymerisation, % G = % of galloylation, % P = % of prodelphinidins, % C = % of procyanidins, CI = colour intensity.
In the PCA plot, the first PCA axis classifies the wines according to their botrytisation rate very clearly (increasing from right to left). However, two wines were differentiated owing to positive axis-2 coordinates; i.e., the wines at 20 % and 50 % botrytisation levels with added nutgall tannins (N). These wines had higher TPI, ORAC and DPPH values than the other wines. The plot of 1 and 3 axes shows differences between wines supplemented with the various tannins, especially within the 20 % botrytisation group. Wines with Gse and Gsk tannins were the furthest from the control wines without added tannins, suggesting that their addition had a positive impact on the final phenolic content (Figure 2B). Wines obtained from 50 % botrytised grapes were more grouped, showing lower variability. As a preliminary result, the added oenological tannins at 100 g/hL may have had less effect on the studied variables in the 50 % botrytised wines than in the 20 % botrytised wines.

2. Impact of tannin addition on the laccase activity of botrytised wines

Laccase activity was measured during the various steps of the winemaking process (i.e., VAT, AF1, AF2, AF3, and MAC) and in 3-month-old wines for each botrytised treatment.

2.1. Laccase activity during micro-winemaking

In musts (VAT) and wines at the end of alcoholic maceration (MAC), laccase activity was lower with the 20 % botrytised treatment than with the 50 % one (Figure 3A). According to both MAC and VAT data (two-way ANOVA with "Botrytis rate" and "Tannin" as main factors), botrytisation level had a highly significant effect ($p$-value = $5\times10^{-7}$) on laccase activity. Without any added tannin, laccase activity in 50 % botrytised musts ($\approx43$ laccase units) was 1.65-fold higher than in 20 % botrytised musts ($\approx26$ laccase units) (Figure 3A). With the addition of tannins, a decrease in laccase activity was also observed in the wines at the end of post-fermentation maceration compared to the musts.

In the 20 % botrytised musts and wines (Figure 3A), the two-way ANOVA with "Tannin" and "Time" (VAT, MAC) as main factors showed significant effects of both "Time" ($p$-value=$5.6\times10^{-7}$) and "Tannin"($p$-value=$0.015$), and of their interaction ($p$-value = $0.031$).
FIGURE 3. Laccase activity in botrytised (20 % and 50 %) musts and wines, either with or without added tannins.

Gse = grape seed tannins, Gsk = grape skin tannins, Q = quebracho tannins, O = oak tannins, N = nutgall tannins.

A - Laccase activity in musts (VAT) and post-alcoholic fermentation maceration wines (MAC) in laccase units. For each botrytisation level, the various letters show significant differences between samples for the two-way ANOVA with “Tannin” and “Time” (VAT, MAC) as main factors.

B - Relative laccase activity (%) in musts (VAT) and wines (AF1, AF2, AF3, and MAC) compared to controls (wines without tannins) at each step of alcoholic fermentation. At the 20 % botrytisation level, the various letters show significant differences between tannins for the two-way ANOVA, with “Tannin” and “Time” (VAT, J1, J2, J3 and MAC) as main factors considering all “Time” data, since the “Time” effect was not significant.

C - Laccase activity in 3-month-old wines after bottling in laccase units.
Besides precipitation, they can also inhibit laccase activity without protein precipitation (Vignault et al., 2020), making the active site of the enzyme inaccessible due to tannin-protein interactions.

2.2. Laccase activity in 3-month-old wines

Laccase activity was observed in all wines obtained from 50 % botrytised grapes (Figure 3C). Only the "Botrytis" effect was significant ($p$-value = 7.93*10^{-7}). Free SO$_2$ added before bottling (20 mg/L) can usually inhibit and completely destroy laccase after a few days, as long as there is free SO$_2$ remaining. Nevertheless, if SO$_2$ combines before the complete destruction of the enzyme, laccase activity can persist, since the combined form of SO$_2$ does not show any antioxidasic properties (Ribéreau-Gayon et al., 2012). Laccase activity in 50 % botrytised wines was between 5 and 11 laccase units (no significant differences between wines: $p$-value = 0.22), which is enough for a wine to be at significant risk of oxidasic haze. Indeed, the colour of red wines with a residual activity greater than 3 laccase units per millilitre is unstable and evolves into haze (Grassin and Dubourdieu, 1989). Wines obtained from 20 % botrytised grapes had little or no laccase activity, with no significant differences between tannins. However, the trend confirms that Gsk, Gse and N tannins might lead to lower laccase activity in wines compared to the control wine. These results confirmed those observed during winemaking.

**FIGURE 4.** Total anthocyanins and epicatechin concentration in botrytised (20 % and 50 %) 3-month-old wines after bottling, either with or without added tannins. Gse = grape seed tannins, Gsk = grape skin tannins, Q = quebracho tannins, O = oak tannins, N = nutgall tannins. Different letters show significant differences: A and B for the "Botrytis" effect (Figures A), and a, b, c, d for the "interaction Botrytis*Tannin" effect (Figure B).
**TABLE 2.** *P*-values from two-way ANOVA of colour and phenolic compounds in 3-month-old wines only when results were significant with $\alpha = 0.05$ (*P*-values < 0.05).

| Variables                          | $P$-values (when < 0.05) |
|------------------------------------|--------------------------|
|                                    | Botrytis effect | Tannin effect | Interaction effect |
| **Total phenolic compounds**       |              |              |                   |
| TPI                                | $6.9 \times 10^{-9}$  | $1.2 \times 10^{-9}$ | -                  |
| FCI                                | $1.7 \times 10^{-6}$  | $1.7 \times 10^{-4}$ | -                  |
| TA                                 | $5.4 \times 10^{-12}$ | -              | -                  |
| **Anthocyanin**                    |              |              |                   |
| Malvidin-3-$O$-glucoside           | $1.3 \times 10^{-10}$ | -            | -                  |
| **Flavan-3-ol mono- and oligomers**|              |              |                   |
| B3 (dimer)                         | $6.9 \times 10^{-8}$  | $1.4 \times 10^{-3}$ | $8.6 \times 10^{-3}$ |
| Catechin                           | $6.6 \times 10^{-9}$  | $1.6 \times 10^{-3}$ | -                  |
| B4 (dimer)                         | $4.3 \times 10^{-6}$  | $1.6 \times 10^{-2}$ | $4.9 \times 10^{-2}$ |
| B2 (dimer)                         | $3.0 \times 10^{-6}$  | $1.0 \times 10^{-2}$ | -                  |
| Epicatechin                         | $1.5 \times 10^{-6}$  | $7.8 \times 10^{-3}$ | $4.5 \times 10^{-2}$ |
| T (trimer)                         | $2.0 \times 10^{-6}$  | $3.8 \times 10^{-2}$ | -                  |
| mDP                                | $1.6 \times 10^{-5}$  | $1.2 \times 10^{-5}$ | -                  |
| % G                                | $6.0 \times 10^{-4}$  | -            | -                  |
| % P                                | -                  | $7.7 \times 10^{-8}$ | -                  |
| **Antioxidant capacities**         |              |              |                   |
| ORAC                               | $3.2 \times 10^{-5}$  | $1.1 \times 10^{-6}$ | -                  |
| DPPH                               | $7.5 \times 10^{-7}$  | $4.7 \times 10^{-6}$ | -                  |
| **Spectrophotometric chromatic parameters** |              |              |                   |
| CI                                 | $3.4 \times 10^{-11}$ | -            | -                  |
| % yellow                           | $6.3 \times 10^{-10}$ | -            | -                  |
| % red                              | $1.3 \times 10^{-9}$  | -            | -                  |
| % purple                           | $1.7 \times 10^{-8}$  | $4.6 \times 10^{-2}$ | -                  |
| Hue                                | $9.8 \times 10^{-9}$  | -            | -                  |
| L*                                 | $1.8 \times 10^{-9}$  | -            | -                  |
| a*                                 | $2.4 \times 10^{-5}$  | -            | -                  |
| b*                                 | $1.4 \times 10^{-9}$  | -            | -                  |
| C<sub>ab</sub>                     | $4.6 \times 10^{-8}$  | -            | -                  |
| h<sub>ab</sub>                     | $1.0 \times 10^{-9}$  | -            | -                  |

"-" = no significant effect. TPI = total polyphenol index, FCI = Folin-Ciocalteu index, TA = total anthocyanins, mDP = mean degree of polymerisation, % G = % of galloylation, % P = % of prodelphinidins, CI = colour intensity.
3. Impact of tannin addition on phenolic compounds, colour and antioxidant capacities of botrytised wines

3.1. Impact on phenolic compounds and antioxidant capacities

The ANOVA results of the phenolic compounds and antioxidant capacities (Table 2) showed a significant "Botrytis" effect, except for the variable "% P". Total anthocyanins, malvidin-3-O-glucoside, and % G were significantly influenced by the botrytisation rate only and not by tannin addition. Figure 4A shows that total anthocyanin (TA) concentrations decreased by approximately 35 and 70 % on average with 20 and 50 % botrytisation respectively.

A significant degradation of anthocyanins in wines induced by botrytisation ranging from 5 to 20 % has been demonstrated by Ky et al. (2012).

Significant "Botrytis" and "Tannin" main effects were found for other variables (Table 2): TPI, FCI, catechin content, T, mDP, % purple, and ORAC and DPPH antioxidant capacities. These variables were thus not only affected by the botrytisation rate, but also by the addition of tannins, without having a protective effect on these variables.

Finally, several variables (i.e., epicatechin, B4 and B3 procyanidin dimers) were significantly affected by the botrytisation rate, the addition of tannins and the interaction of "Botrytis" and "Tannin" factors (Table 2). For example, Figure 4B shows that the level of epicatechin, one of the major

![Graph showing CIELAB parameters (L*, a*, b*, delta E) of 3-month-old wines after bottling, with or without added tannins.](image)

**FIGURE 5.** CIELAB parameters (L*, a*, b*, delta E) of 3-month-old wines after bottling, with or without added tannins.

Gse = grape seed tannins, Gsk = grape skin tannins, Q = quebracho tannins, O = oak tannins, N = nutgall tannins.
procyanidin monomers, was the lowest in the 50 % botrytised wines. The addition of tannins did not prevent the damage of Botrytis laccase when the grapes were contaminated at 50 %. However, despite oak and quebracho tannins not preventing epicatechin degradation, at 20 % Botrytis contamination tannins from grape seed, grape skin and even nutgall had a protective effect on laccase for this compound (Figure 4B) and for B4 and B3 procyanidin dimers (data not shown).

3.2. Impact on colour

Regarding the spectrophotometric and CIELAB chromatic parameters, the ANOVAs showed that botrytisation level had a significant effect on all "colour" variables (Table 2). No "Tannin" effect or interaction effect was observed (except a "Tannin" effect for % purple), showing that 20 % and 50 % botrytised wines were nearly similar in colour respectively. However, the "Tannin" effect for % purple corresponded to the fact that, at the 20 % botrytisation rate, the Gsk wine and N wine were significantly different from each other at $\alpha = 0.1$ for this variable. In Figure 5, CIELAB parameters showed that $L^*$ and $b^*$ values were higher in 50 % than in 20 % botrytised wines; the wines therefore became lighter and yellower as botrytisation rate increased. Moreover, the colour differences ($\Delta E$) between 20 % and 50 % botrytised wines were also calculated from parameters $L^*$, $a^*$ and $b^*$ (Figure 5). In general, values greater than or equal to 3 indicate that the differences between two wines are visually perceptible (García-Marino et al., 2010). 20 % and 50 % botrytised wines were thus visually different, with a $\Delta E$ ranging between 14 and 26. Interestingly, $\Delta E$ differed among the 50 % botrytised wines: the wine with added oak tannins was significantly different from all the others, with more oxidized wine colour characteristics (slightly higher $L^*$ and $h_{ab}$). At 20 % botrytisation, only the wine with grape skin tannins ($\Delta E = 4.4$) was noticeably different from the control. The 20 % botrytised wines with quebracho and oak tannins were similar, although both were different from the three other wines with grape skin, grape seed and nutgall tannins (close colour with $\Delta E < 2$). The added tannins thus produced differential effects: Gsk tannin (and even Gse and N tannins) improved the colour of the 20 % botrytised wine, whereas oak tannin addition caused further colour degradation in the 50 % botrytised wine, which had already been degraded by the pathogen.

4. Impact of tannin addition on the sensory quality of botrytised wines

4.1. Wines without added tannins

The visual and smell/taste of the control wines obtained from 20 % and 50 % botrytised grapes were compared with the control wine made from healthy grapes (0 % Botrytis) without the addition of any tannin (Table 3). Significant differences in the visual features were observed: botrytised wines were more orange in colour, and were even brick/brown and less purplish at 50 % botrytisation. From an olfactory/taste point of view, differences were also significant (4 out of 18 judges did not perceive the differences between the 0 % and the 20 %, as opposed to 1 out of 18 between the 0 % and the 50 %). Botrytised wines were described as being oxidized and jammy at 20 % botrytisation, while adjectives such as acescent, dusty, old and oxidized were used at 50 % botrytisation.

4.2. Effect of added tannins on visual quality of 20 % and 50 % botrytised wines

The 50 % botrytised wines with added tannins were compared visually to the 50 % botrytised control with no added tannins. The triangular tests (Table 3) showed that the wines supplemented with grape skin (Gsk 50 %) and oak (O 50 %) tannins were significantly different ($\alpha = 0.001$) from the control. The Gsk-supplemented 50 % wine was described as being less orange in colour and more intense than the control. The O-supplemented 50 % wine appeared to be lighter and more orange. Ranking tests (Table 4) revealed that the addition of nutgall tannins to 50 % botrytised musts tended to intensify the colour slightly. However, the addition of grape seed, quebracho and oak tannins caused a significant opposite effect. These results with oak tannins were in agreement with the descriptors used by judges in the triangular tests.

Triangular tests also showed significant differences with the addition of grape skin ($\alpha = 0.05$) and oak tannins ($\alpha = 0.001$) in all wines made from 20 % botrytised grapes. The addition of grape skin tannins resulted in a darker wine than that of the control. This result confirmed those obtained for the 50 % botrytised wines and the $\Delta E$ results (Figure 5). Regarding overall visual quality (Table 4), the judges ranked the 20 % Botrytis wine with added grape skin tannins first. The 20 % Botrytis control wine was ranked last. Furthermore, the judges suggested that the addition of oak tannins may have a positive impact on colour in 20 % botrytised wines (triangular tests).
TABLE 3. Triangle tests from healthy 20 % and 50 % botrytised 3-month-old wines, with or without added tannins.

| Triangle test | Number of panellists | Number of correct answer | Comments from correct answers: B compared to A |
|---------------|---------------------|--------------------------|---------------------------------------------|
|               | A vs B              | Visual test | Olfactory/ Taste test | Visual aspect | Olfactory/Taste description |
| 0 % vs 20 %   | 18                  | 17***        | 14***                      | + orange, + evolved, - purple oxidized, jammy fruits, ethyl acetate, old, undergrowth, dust, oxidized |
| 0 % vs 50 %   | 18                  | 18***        | 17***                      | orange, brown + ample |
| 20 % vs 20 % Gse | 18        | 6            | 7                          | + purple sour |
| 20 % vs 20 % Gsk | 18        | 10*          | 13***                      | + dark |
| 20 % vs 20 % Q | 18                  | 6            | 6                          | + vegetable |
| 20 % vs 20 % O | 18                  | 14***        | 8                          | + intense, - orange, + purple |
| 20 % vs 20 % N | 18                  | 4            | 9                          | mushroom, earthy |
| 50 % vs 50 % Gse | 18       | 9            | 12**                       | - intense, - dark - acidic, + astringent, + fruity, pepper |
| 50 % vs 50 % Gsk | 22        | 18***        | 12*                       | - orange, + red, + intense, + limpid dried prune, + acidic, + astringent, - bitter, - mushroom |
| 50 % vs 50 % Q | 22                  | 5            | 12*                       | - intense, - red isoamyl acetate, candy, nougat, rose |
| 50 % vs 50 % O | 18                  | 17***        | 14***                     | - intense, - red, + evolved, + orange, - limpid |
| 50 % vs 50 % N | 22                  | 11           | 9                          | + intense, + dark, -yellow + vegetal, + astringent, dried prune |

*α=0.05; **α=0.01; ***α=0.001
Gse = grape seed tannins, Gsk = grape skin tannins, Q = quebracho tannins, O = oak tannins, N = nutgall tannins.

TABLE 4. Ranking of 20 % and 50 % botrytised 3-month-old wines, with or without added tannins regarding visual, aromatic and gustative quality.

| Ranking test | Number of judges | Control | Gse | Gsk | Q | O | N | F1 | Significance |
|--------------|------------------|---------|-----|-----|---|---|---|----|--------------|
|               |                  |         |     |     |   |   |   |    | Botrytisation rate 50 % |
| Visual quality | 20               | 492     | 93  | 58  | 66 | 113| 38| 51.2| 0.1%3 |
| Differences between wines | a, b | c | a, b | b | c | a | 5% |
| Aromatic quality | 20               | 72      | 59  | 56  | 84 | 50 | 71 | -5.2 | Not significant |
| Differences between wines | a | a | a | a | a | a | 5% |
| Gustative quality | 20               | 77      | 55  | 52  | 72 | 70 | 88 | 1.2 | Not significant |
| Differences between wines | a | a | a | a | a | a | 5% |

|               |                  |         |     |     |   |   |   |    | Botrytisation rate 20 % |
| Visual quality | 18               | 93      | 58  | 40  | 50 | 77 | 60 | 29.0| 0.1%3 |
| Differences between wines | c | a, b | a | a | b, c | a, b | 5% |
| Aromatic quality | 18               | 69      | 63  | 56  | 73 | 50 | 67 | 5.9 | Not significant |
| Differences between wines | a | a | a | a | a | a | 5% |
| Gustative quality | 18               | 54      | 56  | 50  | 66 | 49 | 82 | 13.3| 5%3 |
| Differences between wines | a | a | a | a | b | b | 5% |

Gse = grape seed tannins, Gsk = grape skin tannins, Q = quebracho tannins, O = oak tannins, N = nutgall tannins.

1F calculated as described in ISO 8587:2007 for Friedman test. F = (12 × ∑ pi = 1 Ri2)/(n×p×(p+1))−3×n×(p+1) with n representing number of tasters, p representing number of modalities and Ri representing sum of ranks for modality i. 2Sum of ranks. 3α=0.01 when F > 20.52 and at 5% when F > 11.07.
This result was not confirmed by the ranking tests (Table 4).

4.3. Impact of added tannins on the aromatic and taste quality of 20 % and 50 % botrytised wines

In terms of smell/taste, the 50 % botrytised wine supplemented with oak tannins was perceived as being significantly (α = 0.001) different from the control; i.e., it was described more positively than the control by the judges (Table 3). Although significant, there were fewer differences between the control and the wines with i) added grape seed tannins (α = 0.01) - described as being fruitier, ii) added grape skin tannins (α = 0.05) - described as expressing more prune and fewer mushroom flavours, and iii) added quebracho tannins (α = 0.05) - perceived as being more oxidized. Regarding the overall aromatic and taste qualities, the ranking tests showed no significant differences (Table 4). For 20 % botrytised wines, the triangular tests showed that only the 20 % botrytised Gsk-supplemented wine was perceived as being significantly different from the corresponding control (Table 3). Although the differences between these two wines were not significant in terms of overall flavour and taste quality (Table 4), the Gsk-supplemented 20 % botrytised wine was ranked better than the control, as was the O-supplemented 20 % botrytised wine. Finally, the N-supplemented 20 % and 50 % botrytised wines were ranked last for gustative quality.

CONCLUSIONS

Oenological tannins are authorised by the OIV (International Organization for Vine and Wine) for stabilising wines, fining and avoiding protein haze. They may also be used for their antioxidant and anti-oxidase properties that make them exploitable for new applications. The present study demonstrates for the first time the antioxidasic effects of oenological tannin supplementation during the vatting of grapes (Merlot) contaminated by Botrytis cinerea. It determined their impact on the preservation of the quality of red wines. High botrytisation rates (20 % and 50 %) were used to carry out micro-winemaking in order to determine the effects of the addition of these tannins. The results revealed that at 50 % of botrytisation, laccase activity was so great that only the botrytisation effect was measurable, making it difficult to demonstrate a tannin effect on the oxidative action of laccases. At this level of botrytisation, all the wines exhibited a residual laccase activity greater than 5 laccase units per millilitre, despite the addition of sulphur dioxide at 20 mg/L before bottling. In these wines, high residual laccase activity can lead to oxidasic haze in the presence of oxygen. However, tannin addition in 20 % botrytised musts had beneficial effects, providing significant potential for protection from laccase oxidation. Proanthocyanidic tannins (grape seed and grape skin) and gallotannins (nutgall) significantly lowered laccase activity in the tannin-supplemented wines compared to the control wine. Added grape tannins helped to maintain concentrations of monomeric epicatechin equivalent to those in 0 % botrytised wines, whereas they were strongly affected in the 20 % botrytised control wine.

Regarding organoleptic properties, the wines obtained from botrytised grapes were visually different from the healthy control wine, with more orange hues and a less intense colour due to laccase activity. The tested tannins produced differential effects in the wines, in particular reduced colour degradation following the addition of grape skin tannins. In the olfactory/taste ranking tests, the wines were not perceived as being significantly different, except the 20 % botrytised wine with nutgall tannins, which was ranked last; i.e., with the lowest quality.

Condensed tannins from grape berries are therefore promising for their inhibitory action on laccase activity, and for their organoleptic effect on wines made from botrytised grapes. Future research should investigate the effect of combining different tannins to improve their action. Another perspective would be to test the effect of tannins on botrytised musts at more realistic rates (e.g., 5, 10 and/or 15 %), and to test their effect on B. cinerea strains with different levels of aggressiveness, which belong to different subpopulations usually found in vineyards.

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