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This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1826908 since 2023-02-22T16:31:53Z

Published version:

DOI:10.1016/j.foodres.2021.110874

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23 April 2024

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PII: S0963-9969(21)00774-2
DOI: https://doi.org/10.1016/j.foodres.2021.110874
Reference: FRIN 110874

To appear in: *Food Research International*

Received Date: 29 August 2021
Revised Date: 2 December 2021
Accepted Date: 4 December 2021

Please cite this article as: Gambino, G., Ferrero, L., Scalzini, G., De Paolis, C., Alessandra Paissoni, M., Río Segade, S., Giacosa, S., Boccacci, P., Rolle, L., Impact of oenological processing aids and additives on the genetic traceability of 'Nebbiolo' wine produced with withered grapes, *Food Research International* (2021), doi: https://doi.org/10.1016/j.foodres.2021.110874

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Impact of oenological processing aids and additives on the genetic traceability of 'Nebbiolo' wine produced with withered grapes

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Abstract

'Nebbiolo' is a well-known grapevine variety used to produce prestigious monovarietal Italian red wines. Genetic traceability is an important tool used to protect the authenticity of high-quality wines. SNP-based assays are an effective method to reach this aim in wines,

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but several issues have been reported for the authentication of commercial wines. In this study, the impact of the most common commercial additives and processing aids used in winemaking was analysed in 'Nebbiolo' wine using SNP-based traceability. Gelatine and bentonite had the strongest impact on the turbidity, colour and phenolic composition of wines and on residual grapevine DNA. The DNA reduction associated with the use of bentonite and gelatine (> 99% compared to the untreated control) caused issues in the SNP-based assay, especially when the DNA concentration was below 0.5 pg/mL of wine. This study contributed to explaining the causes of the reduced varietal identification efficiency in commercial wines.

Keywords: Grapevine; wines; oenological additives, Sfursat; genetic traceability; SNPs

1. INTRODUCTION

'Nebbiolo' (*Vitis vinifera* L.) is an important Italian winegrape variety used to produce high-quality wines. It is diffused in north-western Italy, where it is used to produce well-known worldwide DOCG (*Denominazione di Origine Controllata e Garantita*) wines, such as Barolo, Barbaresco, Roero, Gattinara, Ghemme, and Sfursat (Raimondi et al., 2020). 'Nebbiolo' wines play an important role in the Italian wine market due to their high economic value (Miglietta & Morrone, 2018). The wine market is often plagued by fraud, which can occur in many forms, and adulteration is defined as the fraudulent alteration of wine composition. However, other types of fraud have increasingly spread in recent years. Among them, the misrepresentation on the label regarding the origin and variety of wine is very common (Holmberg, 2010). Thus, there is the need to protect 'Nebbiolo' wines from fraud that could damage the image and market of these premium wines. To protect the

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authenticity and verify the truthfulness of what is claimed on the label, models that allow the recognition of wines are needed.

During the past two decades, several authors have studied the authenticity of wines, to identify traceability methods to associate the chemical composition of wine with its varietal, geographic, and productive origin(Versari et al., 2014; Villano et al., 2017; Solovyev et al., 2021). Nevertheless, chemometric approaches are often expensive in terms of time and resources, and they present some inaccuracies caused by the great influence of viticultural and winemaking methods on the qualitative and quantitative composition of wine (Versari et al., 2014). Therefore, the results cannot be considered reliable if the models are applied to commercial wines (Zhang et al., 2010). Biological traceability techniques based on a genetic approach appear very interesting. By extracting DNA from wine and using variety-specific molecular markers, it is possible to discriminate musts and wines (Siret et al., 2000; Pereira et al., 2012). However, the results can be very different depending on the wines, the DNA extraction technique, the type of marker, and the amplification technique used. Single sequence repeats (SSRs) represent the most common markers used in grapevine for fingerprinting (This et al., 2004). Several authors used SSRs as markers for genetic traceability and varietal recognition starting from residual DNA in musts and wines (Boccacci et al., 2012; Pereira et al., 2012; Recupero et al., 2013; Siret et al., 2000; Zambianchi et al., 2021). However, due to DNA degradation in the winemaking process, the results of amplification are often not reliable (Catalano et al., 2016). Indeed, several studies have reported issues with using these methods for assessing the traceability of commercial wines (Agrimonti & Marmiroli, 2018; Recupero et al., 2013).

After the first sequencing approach (Jaillon et al., 2007), several projects involving the sequencing or re-sequencing of grapevine cultivars, including 'Nebbiolo', have been

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performed (Gambino et al., 2017). The comparison between different available genomes allowed the identification of several mutations and polymorphisms between different genotypes, such as single nucleotide polymorphisms (SNPs). SNPs are particularly interesting because they are spread throught the grapevine genome and have the potential to become a valid alternative to SSRs for cultivar identification (Cabezas et al., 2011). Therefore, SNPs have also been used for genetic traceability of varieties in wine (Barrias et al., 2019; Pereira et al., 2017; Fanelli e al., 2021) since they can be detected in low-quality fragmented DNA (Catalano et al., 2016). However, while SSRs are the optimal markers for fingerprinting in grapevine and a limited number of markers is sufficient for varietal identification, many SNP markers are required, which can be analysed by sequencing or hybridisation techniques that are not applicable in wine (Cabezas et al., 2011; Myles et al., 2011). In wine, the most effective approach is the analysis of a limited number of SNPs using qPCR, which allow the identification of a specific cultivar within a group of genotypes (Catalano et al., 2016; Pereira et al., 2017). For example, SNPs for the authentication of 'Nebbiolo' were identified, and a way for assessing the molecular traceability of this cultivar in experimental wines, based on the SNP TaqMan® assay was developed (Boccacci et al., 2020). Two markers, SNP_15082 and SNP_14783, were sufficient to distinguish 'Nebbiolo' from a group of more than 1,100 genotypes. Nevertheless, the efficiency of the assay decreased at the end of malolactic fermentation and in commercial wines due to the reduction of the amplification efficiency and to the increased presence of PCR inhibitors.

After malolactic fermentation, wine can undergo several winemaking practices before bottling, which may modify its composition. In winemaking, the use of additives and oenological adjuvants or processing aids to enhance wine stability is well diffused, and several oenological products have been allowed for this purpose (OIV, 2016a). To produce

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high-quality wines, stabilisation and clarification are essential. Different products can be employed as fining agents; among them, the most frequently used are bentonite, chitosan, vegetable proteins, animal proteins, and polyvinylpolypyrrolidone (Castro Marin et al., 2020; Ficagna et al., 2020; Río Segade et al., 2020). The main products used as stabilisers are potassium polyaspartate, yeast mannoproteins, and Arabic gum (Bosso et al., 2020; Rinaldi et al., 2019).

These problems for the amplification efficiency of DNA in commercial wines (Boccacci et al., 2012; Boccacci et al., 2020; Recupero et al., 2013; Zambianchi et al., 2021) are probably associated with aging, clarification, fining agents, and/or DNAse yeast activity (Catalano et al., 2016). However, to date, no work has analysed in detail these procedures and agents, which may potentially drastically reduce the quality and quantity of DNA in the wine after malolactic fermentation (Faria et al., 2008; Siret et al., 2000; Siret et al., 2002). The effect of the most common additives on wine's chemical composition has been widely studied, but to the best of our knowledge, there are no studies about their effect on wine DNA traceability. Therefore, this study aimed to determine the impact of the most common commercial additives and processing aids on the SNP-based traceability of 'Nebbiolo' wine.

2. MATERIALS AND METHODS

2.1 Plant Material

Young leaves of 'Nebbiolo', 'Barbera', and 'Freisa' were collected, and DNA was extracted using a Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Thorold, Canada) following the manufacturer's instructions. Accessions were genotyped with six SSR markers (This et al., 2004) to confirm their cultivar identity, together with ampelographic observations.

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2.2 Experimental vinification

Partially dehydrated 'Nebbiolo' grapes from the Valtellina wine region (Sondrio, Italy) were crushed in a TEMA de-stemmer–crusher (Enoveneta, Piazzola Sul Brenta, Italy) in December 2019, and 10 mg/L SO_2 was added to the grape must. After 24 h, the must was inoculated with *Saccharomyces cerevisiae* active dry yeast (ACTIFLORE® BO213, Laffort, Bordeaux, France) at the dose suggested by the producer (30 g/hL). Maceration lasted for 14 days; the cap was punched down once the first day, and two punches down were carried out daily until the 6th day. During the second week of fermentation, two pumpings per day were performed in the first two days, while only one per day was carried out in the following days. At the end of maceration, free-run wine was obtained, and then the pomace cap was gently pressed using a PMA 4 pneumatic press (Velo SpA, Altivole, Italy). Malolactic fermentation was induced by the inoculation of *Oenococcus oeni* (MalotabsTM, Lallemand Inc., Montreal, Canada). After malolactic fermentation, 50 mg/L SO_2 were added, and the wine was subsequently racked to remove the lees. The first control wine (CONTR20) was sampled in 0.5 L bottles and frozen for two weeks at –20°C before DNA extraction, as described below in Section 2.5. Every 6 months the wine was racked and of 10 mg/L SO_2 was added, and in March 2021, the wine was used for the oenological treatments.

2.3 Wine treatment with oenological additives and processing aids

In March 2021, 10 winery treatments were tested (Table 1), with three replicates each, on the same 'Nebbiolo' wine. The most common additives and processing aids used in winemaking were selected for this experiment. For each treatment, the preparation was carried out according to the instructions reported on the product's technical sheet (Table 1). The dose used was calculated as 85% of the maximum dose suggested by the producer. In

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each sample, a small quantity of water was added to reach the same final volume of the treatment that required more water in the preparation phase (bentonite). The treatment time was kept constant at 7 days for all treatments according to previous experience, and to information available in the literature (Table 1). At the end of the treatment, each trial was racked with a small laboratory peristaltic pump, avoiding the collection of lees deposited on the bottom of the container. The clear wine was collected for chemical analysis, and a 0.5 L bottle was frozen for two weeks to enhance nucleic acid precipitation.

2.4 Chemical-physical analysis of 'Nebbiolo' wines

After treatment, 250 mL of wine was collected to carry out the chemical-physical analysis. Total acidity was determined by titrimetry according to the OIV-MA-AS313-01 method, while pH was evaluated by potentiometry using an InoLab 730 calibrated pHmeter (WTW, Weilheim, Germany) following the OIV-MA-AS313-15 method (OIV, 2016b). Ethanol, glycerol, and organic acids (malic, lactic, tartaric, citric and acetic acid) were determined by HPLC (Agilent Technologies, Santa Clara, USA) with a diode array detector set to 210 nm, following the method proposed by Schneider et al., (1987). Turbidity was analszed using a turbidimeter (Model TB1, Velp Scientifica, Usmate, Italy) and expressed in nephelometric turbidity units (NTU) in accordance with the OIV-MA-AS2-08 method (OIV, 2016b). Total and free-SO_2 were quantified by titration after the extraction using a Solfotech apparatus (Exacta + Optech Labcenter Spa, San Prospero, Italy) according to the OIV-MA-AS323-04A method (OIV, 2016b).

Wine phenolic composition and colour parameters were evaluated following the methods reported by Petrozziello et al. (2018) using a UV-1800 spectrophotometer (Shimazdu Corporation, Kyoto, Japan). Total anthocyanins (TA) and total flavonoids (TF) were

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quantified by diluting the sample with ethanol:water: 37% hydrochloric acid (70:30:1, v/v) and subsequently measuring absorbance at 536–540 nm and 280 nm, respectively. TA were quantified as mg/L of malvidin-3-O-glucoside chloride, and TF were expressed as mg/L of (+)-catechin. The total polyphenol index (TPI) was evaluated by measuring absorbance at 280 nm in a sample diluted in water, and it was expressed in mg/L of (−)-epicatechin, as reported by Scalzini et al. (2020). Wine colour parameters were evaluated through the acquisition of the visible spectra (380–780 nm) of the undiluted samples using 2-mm optical path cuvettes. Subsequently, colour intensity (CI) ($A_{420}+A_{520}+A_{620}$) and hue (A_{420}/A_{520}) were calculated on an optical path of 10-mm, following the OIV-MA-AS2-07B method (OIV, 2016b). Wine colour was also evaluated by CIELab parameters, according to the OIV-MA-AS2-11 method (OIV, 2016b). L* represents lightness, whereas a* and b* are red/green and yellow/blue colour coordinates, respectively. The total colour difference (ΔE*) between the control and treated samples was calculated as follows: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. Then, the CIELab coordinates were converted to RGB values.

2.5 Grapevine DNA extraction from wines

The total DNA from wine was extracted with two different methods: i) Plant/Fungi DNA Isolation Kit (Norgen Biotek Corp., Thorold, Canada) (Norgen protocol) and ii) the cetyltrimethylammonium bromide (CTAB)-based method by Siret et al. (2002) with several modifications (SirM protocol).

The wine aliquots for DNA extraction were collected from wine conserved at –20°C and homogenised by inverting the bottle several times. Each replicate was extracted from 50 mL (Norgen) and 100 mL (SirM) wine pellets obtained after centrifugation at 4,000 *g* at 4°C for 1 h. In the Norgen protocol, before the extraction, the pellet was frozen in liquid nitrogen

and ground using a TissueLyser II (Qiagen, Hilden, Germany). All DNA extractions were performed by following the manufacturer's instructions, excluding the RNase step, and the final elution occurred in 45 µL of elution buffer. In the SirM protocol, DNA was extracted according to a modified CTAB-based method by Siret et al. (2002) and following some modifications proposed by Agrimonti and Marmiroli (2018). The pellet obtained after centrifugation, as reported above, was dissolved in 5 mL TEX buffer (20 mM EDTA, pH 8.0; 1.4 M NaCl; 1M Tris–HCl, pH 8.0; 3% CTAB: and 1% β-mercaptoethanol) and incubated at 65°C for 1 h, with mixing every 10–15 min. Then, 1 volume of chloroform:isoamyl alcohol (24:1) was added and homogenised. After centrifugation at 8,000 *g* for 10 min at 4°C, the supernatant was mixed with 0.1 volume of 10% CTAB and extracted again with 1 volume of chloroform:isoamyl alcohol. The DNA-containing upper phase was precipitated overnight at –25 °C with 2 volumes of ethanol. Then, DNA was collected by centrifugation at 10,000 *g* for 30 min at 4 °C, resuspended in 250 µL TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0), and treated with 20 µL Proteinase K (20 mg/mL) at 48°C for 30 min. Then, 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added and samples were centrifuged at 11,000 *g* for 15 min at 4 °C. DNA was precipitated with 2 volumes of ethanol and 2.5 M ammonium acetate (7.5 M) at –25°C for at least 2 h. After centrifugation at 22,000 *g* for 30 min at 4°C, the pellets were washed twice with 500 µL 70% ethanol and resuspended in 45 µL TE buffer. Final purification was performed with the NucleoSpin® Plant Kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. DNA quantity and quality were estimated by determining the spectrophotometric absorbance of the samples at 230, 260, and 280 nm and the ratios of A_{260}/A_{280} and A_{260}/A_{230}. A NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) was used. DNA was stored at −20 °C.

2.6 Grapevine DNA quantification by qPCR and determination of PCR inhibitors

To quantify grapevine DNA, all DNA samples were analysed by qPCR amplification of the 9-cis-epoxycarotenoiddioxygenase gene (*VvNCED2*, VIT_10s0003g03750), using the primers and TaqMan® FAM-labelled probe reported by Savazzini and Martinelli (2006). The presence of PCR inhibitors in the extracted DNA was evaluated according to Boccacci et al. (2020), by adding TaqMan® Exogenous Internal Positive Control (EIPC) reagents (Thermo Fisher Scientific) into the qPCR mixture. The qPCR reaction was performed in a final volume of 10 µL, consisting of 2.5 µL DNA, 5 µL TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.3 µM of each primer and 0.2 µM of FAM probe, 0.2 µL EIPC DNA, 1 µL EIPC mix (containing premixed forward, reverse primers, and VIC probe specific for EIPC), and 0.1 µL sterile water. Amplification cycles were characterised by an initial denaturation step at 95°C for 10 min, followed by 55 cycles of 95 °C for 15 s, and 60 °C for 1 min. A calibration curve of the *VvNCED2* TaqMan® assay was constructed with samples of 'Nebbiolo' DNA extracted from leaves and, obtained by serial dilution. Grapevine DNA quantification took place by plotting the Ct values obtained from the DNA extracted from wines together with the standard curve using the CFX96 Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The percentage of PCR inhibition was calculated from a calibration curve with serial dilution of EIPC, according to Boccacci et al. (2020). All samples were analysed in duplicate.

2.7 SNP genotyping protocol and data analysis

DNA extracted from 'Nebbiolo' wines was analysed by SNP_15082 and SNP_14783. As reported by Boccacci et al. (2020), 'Nebbiolo' alleles and non-'Nebbiolo' alleles were

marked with different dyes (FAM and VIC) (Table S1). ‘Barbera’ and ‘Freisa’ were selected as examples of homozygous and heterozygous non-‘Nebbiolo’ cultivars (Boccacci et al., 2020), respectively, which were necessary to product allelic discrimation plots. The qPCR reaction for TaqMan® SNP assays was performed in a final volume of 10 µL, consisting of 2.5 µL DNA, 5 µL TaqMan® Environmental Master Mix 2.0 (Thermo Fisher Scientific), 0.25 µL 40X TaqMan® SNP Genotyping Assay mix (containing pre-mixed forward and reverse primers, VIC probe, and FAM probe), and 2.25 µL sterile water. The amplification profile was the same as that reported in Section 2.6. Allelic discrimination plots were constructed using the CFX96 Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). All samples were analysed in duplicate.

2.8 Statistical analyses

Statistical analyses were performed using R statistic software (R Foundation for Statistical Computing, Vienna, Austria). For each variable, one-way analysis of variance (ANOVA) with the Tukey HSD post-hoc test was used to evaluate significant differences among treatments. The normality and homoscedasticity ANOVA assumptions were tested using Shapiro–Wilk’s and Levene’s tests, respectively. When the ANOVA assumptions were not met, the Kruskal–Wallis non-parametric test with Conover's All-Pairs Rank Comparison Test was performed. Differences were considered statistically significant at p-value < 0.05.

3. RESULTS AND DISCUSSION

3.1 Impact of treatments on chemical-physical parameters of ‘Nebbiolo’ wines

The chemical composition and colour characteristics of the ‘Nebbiolo’ wine used in this experiment are reported in Table S2. The impact of additives and processing aids on

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‘Nebbiolo’ wine turbidity, phenolic composition, and colour parameters is reported in Table 2. Bentonite (BEN), gelatine (GEL), polyvinylpolypyrrolidone (PVPP), and yeast hulls (YST) strongly decreased wine turbidity, while mannoprotein (MAN), chitosan (CHT), and Arabic gum (ARG) slightly increased the NTU level compared to the untreated control (CONTR). BEN, a commercial product mainly composed of a natural clay known as montmorillonite, is widely used as a fining agent in wine due to its ability to adsorb and precipitate proteins. In our study, BEN had the greatest impact on wine turbidity (–80%). These results agree with those of Ficagna et al. (2020), in which ‘Merlot’ wine clarification with BEN showed the most intense reduction in turbidity, while PVPP and vegetable proteins (VEG) treatments led to a minor reduction in the NTU level. GEL also had a great impact in terms of turbidity reduction (–43%), reported by González-Neves et al., (2014).

The wine phenolic composition changed after treatment with different processing aids and additives. ‘Nebbiolo’ wines treated with grape skin tannin (TAN) showed a higher TPI and TF content compared to CONTR, whereas GEL, PVPP, CHT and BEN treated wines showed significantly lower TPI values. The TA content was not significantly affected by the treatments with TAN, ARG, MAN, and CHT, whereas GEL, PVPP, VEG, potassium polyaspartate (POL), YST and BEN caused a decrease in anthocyanin content. ‘Nebbiolo’ wines treated with GEL showed the lowest TA values, which were about 15% lower compared to CONTR. GEL and PVPP treatments significantly affected the TF content with respect to CONTR, leading to a reduction of 12.3% and 4.3%, respectively, while TAN increased this parameter by 4.4%. Instead, other treatments did not significantly change the TF content. Among the treatments tested in our study, GEL had the strongest impact on wine phenolic composition, showing the lowest TPI, TF, and TA values (Table 2). Our results agree with the literature; BEN and GEL strongly affected the phenolic composition of wine

through a reduction of anthocyanins and tannins in ‘Tannat’ red wine (González-Neves et al., 2014). In ‘Nebbiolo’ wine, a significant reduction of about 9% was observed in TPI values after GEL treatment due to the removal of oligomeric and polymeric flavanols (Río Segade et al., 2020). PVPP treatment also caused a strong decrease (> 55%) in flavanol content (Ficagna et al., 2020). In addition to protein removal, BEN can bind other positively charged molecules, like anthocyanins, leading to a loss of colour, while VEG has a minor impact on the anthocyanin content (Ficagna et al., 2020). Other additives, such as CHT, can marginally decrease phenolic composition as a side effect (Castro Marin & Chinnici, 2020). Wine colour can decrease as a secondary effect of treatment with fining agents (Río Segade et al., 2020). In our experiment, only TAN treatment significantly ($p < 0.05$) increased the colour intensity (CI), while the lowest values were shown in wines treated with GEL, followed by BEN and PVPP. Regarding hue values, VEG-, YST-, and CHT-treated wines did not show any significant differences compared to CONTR. BEN and TAN treatments showed higher hue values, while GEL and PVPP showed the lowest values. However, the changes in hue induced by the treatments were minimal, resulting in values in the range 0.74–0.76. The reduction of wine CI and the increase of hue values are likely due to the lower TA values reported after the treatment with fining agents, which led to a reduction of the red colour component (A_{520}). In contrast, the decrease observed in hue values after GEL and PVPP treatments could be due to their higher effectiveness in removing the flavanic component, as mentioned above.

To further explore the colour of treated wines, CIELab characterisation of ‘Nebbiolo’ wines was performed. L* (lightness), a*, and b* (red/green and yellow/blue colours, respectively) values are reported in Table 2, while their colour outcome (after conversion in 24-bit RGB values for publication purposes) is available in Figure 1. A slight increase in L*, a*, and b*

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values was shown after treatment with CHT, YST, PVPP, and VEG, while a more prominent increase in these parameters interested BEN and GEL treatments, which was visually confirmed by representation in Figure 1; only TAN showed a significant ($p < 0.05$) decrease in L* values with respect to CONTR. From the obtained CIELab data, the ΔE* parameter was calculated for all treatments compared to CONTR (Figure 1). GEL gave the highest ΔE* value (8.01), followed by BEN (2.98). Wines treated with BEN and GEL reached a visual perceived colour reduction, whereas all other treatments did not approach a value of three ΔE* units, which is the threshold estimated to allow a visual recognition of wine colour difference by the human eye (Pérez-Magariño & González-Sanjosé, 2003). Therefore, BEN and GEL had the strongest impact on the turbidity, colour, and phenolic composition of 'Nebbiolo' wines. These results confirmed that the ΔE* were higher than 5, which were obtained when 'Nebbiolo' wines were treated with a GEL fining agent (Río Segade et al., 2020).

3.2 DNA extraction from wine after application of oenological additives

The DNA yield and quality ratio of DNA extracts were initially estimated through a spectrophotometric analysis using NanoDrop. The spectrophotometric quantification of wine extracted using the Norgen protocol is reported in Table S3, while the quantification results of wines extracted with the SirM method are reported in Table 3. In general, low-quality DNA was found in all wine samples using both extraction methods. Concerning the Norgen protocol, no significant differences in DNA yield were discovered between CONTR and the treatments; only CONTR20 ('Nebbiolo' wine sampled in 2020, one year before application of oenological additives) showed a high DNA yield (Table S3). In addition, significant differences were found in wine samples extracted with the SirM method;

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CONTR20 showed the highest DNA yield, while CONTR had the lowest concentration. In contrast with the results obtained after the Norgen protocol, all wines treated with different additives and processing aids showed a significantly higher DNA yield compared to CONTR (Table 3). The quality of the extracted DNA was estimated from traditional absorbance ratios (A_{260}/A_{280} and A_{260}/A_{230}). 'Nebbiolo' wines extracted using both protocols did not show any significant differences in terms of A_{260}/A_{280} and A_{260}/A_{230} ratios. However, DNA extracted with the SirM protocol showed higher quality than DNA extracted with the Norgen protocol, likely due to the application of a more intense DNA cleaning operation using phenol and chloroform (Table 3, Table S3). The presence of polysaccharides and phenolic substances (including tannins), which are extremely common in grapes, negatively affects the quality of DNA extracted from wine.

Several previous works (Savazzini & Martinelli, 2006; Vignani et al., 2019) reported the presence of yeast DNA and phenolic substances in the DNA extracted from the wine, which can decrease the precision of the measurement; thus, spectrophotometric quantification is often not reliable for the quantification of grapevine DNA in wine. Consequently, we adopted a more specific quantification of grapevine DNA based on *VvNCED2* amplification using TaqMan® probes, as previously suggested (Boccacci et al., 2020; Savazzini & Martinelli, 2006; Vignani et al., 2019). No amplification of *VvNCED2* was observed in DNA samples collected in 2021 and extracted with the Norgen protocol, only in CONTR20, collected in 2020 after malolactic fermentation, *VvNCED2* was amplified in qPCR. The grapevine DNA present in the samples was probably too limited and/or too impure to allow amplification during qPCR of *VvNCED2*, while after malolactic fermentation the protocol was more efficient, as reported previously (Boccacci et al., 2020). Instead, the DNA extracted with the SirM protocol was successfully amplified using *VvNCED2* TaqMan®

probes (Figure 2C). Values of grapevine DNA and its percentage ratio, with respect to the total DNA yield measured by NanoDrop, are reported in Table 3. In general, the data obtained with Nanodrop quantification were overestimated. CONTR had the highest concentration of grapevine DNA with 6.73 ± 1.13 ng/mL of wine; this amount corresponds only to the 0.29% of the DNA yield quantified by NanoDrop. In the other treatments, the percentage of grapevine DNA was lower. Most of the DNA yield quantified by Nanodrop is likely not from grapevine. Spectrophotometric quantification is a non-reliable method to quantify DNA extracts from wine, regardless of the extraction method. The use of dehydrated grapes did not influence the extraction of DNA from wine; moreover, these data confirmed the overestimation previously reported in 'Nebbiolo' wines produced with fresh grapes (Boccacci et al., 2020). The results of previous work showed that grapevine DNA can be up to 25 times less than the DNA estimated with a spectrophotometer in the musts, and 20,000 times less in the wine after 1 year.

Most of the treatments showed a lower grapevine DNA concentration with respect to CONTR. All the clarification treatments played a role in removing DNA from the wine, but the intensity of the reduction differed depending on the treatment. Interestingly, the products with the highest chemical-physical impact (Table 2) caused the highest loss of DNA compared to CONTR (Table 3). Samples treated with BEN or GEL had the strongest reduction, with a DNA loss of 99.56% and 99.67%, respectively. Whereas, POL, ARG, PVPP, and YST did not have an impact on grapevine DNA extraction with respect to CONTR. Moreover, CONTR20 had 41.92% more grapevine DNA compared to CONTR. Nevertheless, the reduction that occurred during one year of storage in stainless steel casks with three rackings after malolactic fermentation was not significant. Interestingly, the loss of DNA caused by aging was lower compared to the loss due to treatment with fining agents

(i.e. BEN and GEL). Thus, fining operations play the most important role in decreasing residual DNA in 'Nebbiolo' wine. Moreover, the literature reports greater efficiency in reducing the turbidity of wine after the use of mixes of different fining agents (Ficagna et al., 2020). Therefore, it can be expected that the combined effect of these treatments may strongly reduce DNA quality and quantity in the wine, explaining why several authors did not successfully find traces of DNA in commercial wines (Boccacci et al., 2012; Boccacci et al., 2020; Catalano et al., 2016).

3.3 SNP genotyping in 'Nebbiolo' wines

The combination of allelic calls of two specific 'Nebbiolo' SNPs (SNP_15082 and SNP_14783) is enough to distinguish 'Nebbiolo' from more than 1,100 genotypes. In a precedent study, the TaqMan® assay based on these two SNPs allowed the recognition of 'Nebbiolo' musts and wines with high sensitivity (Boccacci et al., 2020). Nevertheless, as reported by several studies (Baleiras-Couto & Eiras-Dias, 2006; Siret et al., 2002), due to the lack of quality and DNA integrity, commercial wines and aged wine also showed a reduced identification efficiency for 'Nebbiolo'.

TaqMan® assays for the detection of SNP_15082 and SNP_14783 were applied to the DNA extracted with the Norgen protocol, only CONTR20 correctly amplified both alleles, while all other samples collected in 2021 lacked amplification or had incorrect calls using the genotyping assays (Table S3). These results confirm that the problems observed with the amplification of *VvNCED2* are likely due to the extremely low quality of DNA extracted from wine. Commercial kits, which are extensively used in the extraction of plant material, are not reliable tools for DNA extraction from aged wine. The use of commercial kits is fully effective only with musts and young wines (Boccacci et al., 2020); nevertheless, in aged

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wine and clarified samples, the quality of DNA is too low and can cause incorrect amplification during the TaqMan® assay. The genotyping of DNA extracted with the SirM protocol was more successful (Table 3, Figure 2A, B, D). CONTR and CONTR20 correctly amplified both alleles. Several authors (Boccacci et al., 2020; Catalano et al., 2016) reported aging time as one of the causes of the reduction of identification efficiency in commercial wines, as was also confirmed in this work using an ineffective extraction method (Table S3). However, according to our results using SirM protocol, the reduction in the quantity of DNA that occurs over time is not alone responsible for the incorrect amplification of commercial wines. Despite the aging of the wine and the low quality of the DNA, this TaqMan® assay was confirmed to be very robust and effective in identifying 'Nebbiolo' wines in experimental conditions.

Interestingly, there is a clear correlation between the treatments and the success of genotyping; ARG, TAN, CHT, YST, MAN, VEG, POL, and PVPP treatments did not have any effect on the assay. All repetitions had correct amplification and allelic discrimination was always possible with a precision of 100%. Nevertheless, BEN and GEL resulted in incorrect or absent SNP amplification (Table 3, Figure 2A, B, D). This is probably due to the low quantity of residual DNA in the wine. Indeed, according to the *VvNCED2* quantification results, the TaqMan® assay used in our study loses efficacy if the samples have less than 0.5 pg of DNA per mL of wine. This threshold can be deduced from the CHT treatment, with 0.56 ± 0.32 pg of DNA per mL of wine being the treatment with the least amount of DNA in which the genotyping assays worked correctly (Table 3). Serial dilutions of DNA extracted from CONTR, ARG, TAN, CHT, YST, MAN, VEG, POL, and PVPP confirmed that under 0.5 pg of DNA per mL of wine, both TaqMan® assays lost their effectiveness in all treatments showing incorrect or absent of SNP amplification. In addition

to the low DNA concentration, the presence of PCR inhibitors in the extracted DNA can influence the PCR efficiency and the results of the TaqMan® assay. The amplification efficiency, verified by adding an EIPC in all DNA extracts, was 100% in all samples extracted with SirM protocol without significant differences. This result suggests that the amplification issues in BEN and GEL treatments were uniquely caused by the low quantity of DNA in wine after treatment with fining agents and not by the presence of PCR inhibitors in the extracts.

The use of BEN and GEL represent very common practices widely used in the production of most commercial red wines, including 'Nebbiolo' wines. Moreover, it is not unusual to use these products together. Considering our results, one of these fining agents alone can reduce the grapevine DNA by 99%; therefore, their effect, alone or combined, on the residual DNA can explain why the TaqMan® assay and other molecular assays do not properly work on aged commercial wines (Baleiras-Couto & Eiras-Dias, 2006; Boccacci et al., 2012; Boccacci et al., 2020; Catalano et al., 2016; Recupero et al., 2013). To the best of our knowledge, this is the first time an experiment has investigated the causes of the reduced efficiency of genetic traceability in wine.

4. CONCLUSIONS

In this study, we investigated the impact of the most common additives and processing aids used in winemaking on the efficiency of the TaqMan® assay for the varietal authentication of 'Nebbiolo' wines. As already reported by Boccacci et al. (2020), using two SNP markers (SNP_14783 and SNP_15082) it is possible to identify 'Nebbiolo' from a group of 1157 non- 'Nebbiolo' genotypes. Nevertheless, the winemaking process can affect the precision of varietal identification. All oenological operations at reaching the clarity and stability of

the wine after malolactic fermentation can reduce the quality and the amount of DNA in the wine. In the present study, the results showed an impact of fining agents on turbidity and phenolic composition in line with other studies reported previously. BEN and GEL had the strongest impact on turbidity, phenolic composition, and colour parameters. The efficiency of the TaqMan® assay for varietal identification was also confirmed in aged wines; indeed, under experimental conditions, recognition was possible in 2-years-old wine with 100% precision. Identification was also possible for most of the wines treated with additives or processing aids. Nevertheless, recognition failed in wines treated with BEN and GEL. 'Nebbiolo' wines that have undergone these treatments showed the lowest concentration of grapevine DNA. Therefore, there is a clear correlation between the efficiency of the assay and the quantity of DNA in the wine. These results allowed us to identify a threshold DNA concentration (0.5 pg/mL of wine) below which the TaqMan® assay loses efficiency. Moreover, one year of aging in stainless steel did not significantly affect either the DNA quantity or the identification efficiency. This study contributed to explaining the reasons for the decreased identification efficiency in commercial wines and confirmed the need for future improvements of DNA extraction techniques from wine. Furthermore, these data suggest how an integrated molecular approach using different markers (SSRs and SNPs) with different characteristics could probably be useful for wine authentication by overcoming the limitation of a single class of molecular markers (Vignani et al., 2019; Fanelli et al., 2021).

Declaration of Competing Interest

The authors declare that there is no any conflict of interest in this work.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Author contributions

Giorgio Gambino: Conceptualization, Supervision, Funding acquisition, Methodology, Visualization, Writing - review & editing. **Lorenzo Ferrero**: Investigation, Formal analysis, Data curation, Validation, Visualization, Writing - original draft. **Giulia Scalzini**: Investigation, Methodology, Formal analysis, Validation. **Camilla De Paolis**: Investigation, Formal analysis, Data curation, Validation. **Maria Alessandra Paissoni**: Investigation, Formal analysis, Data curation, Validation. **Susana Río Segade**: Investigation, Methodology, Validation, Writing - review & editing. **Simone Giacosa**: Investigation, Methodology, Validation, Writing - review & editing. **Paolo Boccacci**: Investigation, Methodology, Validation, Writing - review & editing. **Luca Rolle**: Conceptualization, Supervision, Funding acquisition, Writing - review & editing

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Figure legends

Figure 1. 'Nebbiolo' wine colour detected after the treatment with different additives and processing aids. BEN: bentonite; GEL: gelatine; VEG: vegetable protein; PVPP: polyvinylpolypyrrolidone; YST: yeast hulls; CHT: chitosan; MAN: mannoprotein; ARG: Arabic gum; POL: potassium polyaspartate; TAN: grape skin tannin. Each colour was acquired by spectrophotometry, expressed in CIELab coordinates, and then converted to RGB values. The untreated control (CONTR) sample was extended on the top side of the bar to facilitate comparisons with treated wines.

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Figure 2. SNP genotyping in 'Nebbiolo' wines extracted with SirM method and previously treated with different oenological additives and processing aids. (A) Scatterplot of TaqMan® SNP_14783 genotyping assay with ‘Nebbiolo’ wines. (B) Scatterplot of TaqMan® SNP_15082 genotyping assay with ‘Nebbiolo’ wines. (C) Standard curve of *VvNCED2* TaqMan® probe used to quantify grapevine DNA in 'Nebbiolo' wines. DNA from ‘Nebbiolo’ leaves was used as a calibrator for the standard curve. (D) Relative fluorescence unit (RFU) of the TaqMan® SNP_14783 probe tagged with VIC dye (allele G ’Nebbiolo’). The yellow line in the amplification plot indicates the RFU level of ‘Barbera’ (non-‘Nebbiolo’ control), above which, it was possible to detect ‘Nebbiolo’ wines. The control DNA from ‘Nebbiolo’, ‘Barbera’ and ‘Freisa’ were extracted from leaves. CONTR: untreated control; BEN: bentonite; GEL: gelatine.

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Table 1. 'Nebbiolo' wine samples and treatments used for the study. CONTR20: untreated wine sampled in 2020, one year before application of treatments; CONTR: untreated wine sampled in 2021 at the time of application of treatments.

Sample	Treatment	Product	Used Dose	Reference
CONTR20	-	-	-	-
CONTR	-	-	-	-
BEN	Bentonite	Gelbentonite Dal Cin, Concorezzo, Italy	25.5 g/hL	Ficagna et al., 2020
GEL	Gelatine of animal origin	Premium Gel Grado 1 Vason, S. Pietro in Cariano, Italy	25.5 g/hL	Cosme et al., 2007
VEG	Vegetable protein	Vegecoll Laffort, Bordeaux, France	4 g/hL	Río Segade et al., 2020 Ficagna et al., 2020
PVPP	Polyvinylpolypyrrolidone	PVPP Alea Evolution, Molinella, Italy	25.5 g/hL	Cosme et al., 2012 Ficagna et al., 2020
YST	Yeast hulls	Aleavit Help Alea Evolution	32 g/hL	Costa et al., 2019
CHT	Chitosan	Chitogel AEB, Brescia, Italy	25.5 g/hL	Castro Marin & Chinnici, 2020
MAN	Yeast mannoprotein	Oenoless MP Laffort	25.5 g/hL	-
ARG	Arabic gum	Arabique L30 Alea Evolution	85 mL/hL	-
POL	Potassium polyaspartate	Zenith Uno Enartis, Trecate, Italy	85 mL/hL	-
TAN	Grape skin tannin	Protan Raisin AEB	25 g/hL	-

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Table 2. Turbidity, phenolic composition and colour parameters of 'Nebbiolo' wines treated with different additives and processing aids. CONTR: untreated control; BEN: bentonite; GEL: gelatine; VEG: vegetable protein; PVPP: polyvinylpolypyrrolidone; YST: yeast hulls; CHT: chitosan; MAN: mannoprotein; ARG: arabic gum; POL: potassium polyaspartate; TAN: grape skin tannin. CONTR20: untreated wine sampled in 2020, data not available. Data are mean values ± SDs of three replicates. Values followed by different letters within a column are significantly different ($p < 0.05$, one-way ANOVA with Tukey HSD post-hoc test). L*: lightness; a*: red/green colour coordinate; b*: yellow/blue color coordinate.

	Turbidity	**TPI – Total phenolic index**	**TA - Total anthocyanins**	**TF -Total flavonoids**					
Sample	**NTU**	**mg (-)-epicatechin/L**	**mg malvidin-3-glucoside chloride/L**	**mg (+)-catechin/L**	**Colour intensity (AU)**	**Hue**	**L***	**a***	**b***
CONTR 20	-	-	-	-	-	-	-	-	-
CONTR	11.59±0.04 cd	3070±17 bcd	146±1 a	1051±8 b	8.25±0.01 bc	0.75±0.00 c	16.6±0.1 ef	47.49±0.09 ef	2781±0.10 ef
BEN	2.30±0.61 g	2974±22 efg	140±2 bcd	1042±8 bc	7.82±0.01 f	0.76±0.05 a	18.1±0.1 b	49.01±0.08 b	29.93±0.09 b
GEL	6.58±1.31 e	2739±37 h	124±0 e	922±6 e	6.97±0.03 g	0.74±0.01 d	20.9±0.1 a	51.43±0.07 a	33.31±0.12 a
VEG	9.93±1.16 d	3010±42 cdef	139±3 d	1019±17 bcd	8.08±0.01 d	0.75±0.00 c	17.2±0.1 d	48.05±0.09 d	28.61±0.08 d
PVPP	4.15±0.27 f	2917±16 g	137±2 d	1005±11 d	7.89±0.02 e	0.74±0.01 e	17.6±0.1 c	48.39±0.07 c	29.16±0.08 c
YST	7.21±0.49 e	2996±9 def	139±2 bcd	1029±9 bcd	8.06±0.00 d	0.75±0.00 c	17.2±0.0 d	48.09±0.02 d	28.69±0.03 d
CHT	14.55±0.24 a	2963±25 fg	145±4 ab	1035±23 bcd	8.06±0.00 d	0.75±0.01 c	17.2±0.0 d	48.04±0.04 d	28.62±0.05 d
MAN	14.56±0.61 a	3081±33 bc	143±1 abcd	1018±4 bcd	8.30±0.03 ab	0.75±0.02 b	16.5±0.1 fg	47.27±0.14 f	27.57±0.17 f
ARG	13.61±0.37 ab	3097±22 ab	144±2 abc	1014±19 bcd	8.24±0.01 c	0.75±0.03 b	16.6±0.0 ef	47.47±0.03 ef	27.82±0.03 ef
POL	12.58±0.04 bc	3048±22 bcde	139±1 cd	1023±9 bcd	8.23±0.03 c	0.75±0.04 b	16.7±0.1 e	47.50±0.10 e	27.86±0.11 e
TAN	12.35±0.01 bc	3163±25 a	142±0 abcd	1097±6 a	8.35±0.00 a	0.76±0.05 a	16.4±0.0 g	47.28±0.06 ef	27.56±0.06 f

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Table 3. DNA purity and yield measured by NanoDrop; yield evaluated by a standard curve with FAM-labelled endogenous gene *VvNCED2*. Percentage ratio between DNA quantification by *VvNCED2* and the yield measured by NanoDrop. Loss of DNA after treatment expressed as percentage ratio between the DNA (quantified by *VvNCED2*) of the control and the treated wine. The ratio was calculated as follows: (DNA of the control - DNA of' wine after treatment)/ DNA of the control. Allelic profile of genotyping assay SNP_14783, SNP_15082. For each treatment replicate, one sample was extracted (R1, R2, and R3). For each sample, genotyping was performed twice (1st and 2nd repetition). '-' in the allelic profile denotes an incorrect allelic call; '+' indicates samples that correctly amplified, and 'nd' stands for 'not detected'. Data are means of 3 replicates ± standard deviation. Values followed by different letters within a column are significantly different ($p < 0.05$, Kruskal-Wallis test with Conover's Comparison test). CONTR20: untreated control sampled one year before the application of additives; CONTR: untreated control; BEN: bentonite; GEL: gelatine; VEG: vegetable protein; PVPP: polyvinylpolypyrrolidone; YST: yeast hulls; CHT: chitosan; MAN: mannoprotein; ARG: arabic gum; POL: potassium polyaspartate; TAN: grape skin tannin.

Sample	**NanoDrop Quantification**			***VvNCED2* quantification DNA yield [pg/mL of wine]**	**% Grapevine DNA**	**DNA treatment / DNA CONTR (%)**	**SNP_14783**			**SNP_15082**			**SNP_14783**			**SNP_15082**		
	DNA yield [ng/mL of wine]	**A_{260}/A_{280}**	**A_{260}/A_{230}**				**1st repetition**						**2nd repetition**					
							R1	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**	**R1**	**R2**	**R3**
CONTR20	55.23±31.13 a	2.05±0.04 a	2.29±0.09 a	9.55±0.97 a	0.01±0.00 ef	+41.92±12.58 a	+	+	+	+	+	+	+	+	+	+	+	+
CONTR	2.42±0.50 e	1.40±0.17 a	0.72±0.02 a	6.73±1.13 ab	0.29±0.07 a	-	+	+	+	+	+	+	+	+	+	+	+	+
BEN	4.27±0.11 abcd	1.44±0.03 a	0.66±0.03 ab	0.03±0.04 g	0.00±0.00 f	-99.56±0.35 fg	-	-	-	-	+	-	nd	-	-	nd	nd	-
GEL	4.97±0.55 ab	1.52±0.03 a	0.65±0.03 ab	0.02±0.04 g	0.00±0.00 f	-99.67±0.36 h	nd	+	-	nd	-	nd	-	nd	+	+	nd	-
VEG	4.69±0.55 ab	1.48±0.03 a	0.67±0.02 ab	1.30±0.78 efg	0.03±0.02 cdef	-80.65±12.24 defg	+	+	+	+	+	+	+	+	+	+	+	+
PVPP	4.41±0.45 abc	1.43±0.06 a	0.61±0.09 ab	1.90±0.47 abcd	0.04±0.01 bcde	-71.67±6.67 cde	+	+	+	+	+	+	+	+	+	+	+	+
YST	3.80±0.52 bcde	1.45±0.09 a	0.65±0.02 ab	4.13±0.68 abc	0.11±0.02 ab	-38.57±8.27 abc	+	+	+	+	+	+	+	+	+	+	+	+
CHT	3.02±0.12 cde	1.88±0.26 a	043±0.08 b	0.56±0.32 fg	0.02±0.01 def	-91.65±5.20 efg	+	+	+	+	+	+	+	+	+	+	+	+
MAN	4.57±0.85 ab	1.52±0.06 a	0.50±0.26 ab	1.47±0.26 cdef	0.03±0.01 bcde	-78.09±3.77 cdef	+	+	+	+	+	+	+	+	+	+	+	+
ARG	3.50±0.05 bcde	1.58±0.13 a	0.69±0.03 ab	2.51±1.49 abcd	0.07±0.04 abcd	-62.76±21.13 bcd	+	+	+	+	+	+	+	+	+	+	+	+
POL	4.76±1.19 ab	1.60±0.08 a	0.66±0.07 ab	3.97±1.92 abcd	0.10±0.07 abc	-41.00±29.33 abc	+	+	+	+	+	+	+	+	+	+	+	+
TAN	2.67±0.52 de	1.99±0.44 a	0.60±0.11 ab	1.77±0.39 cdef	0.07±0.01 abc	-73.69±6.17 cde	+	+	+	+	+	+	+	+	+	+	+	+

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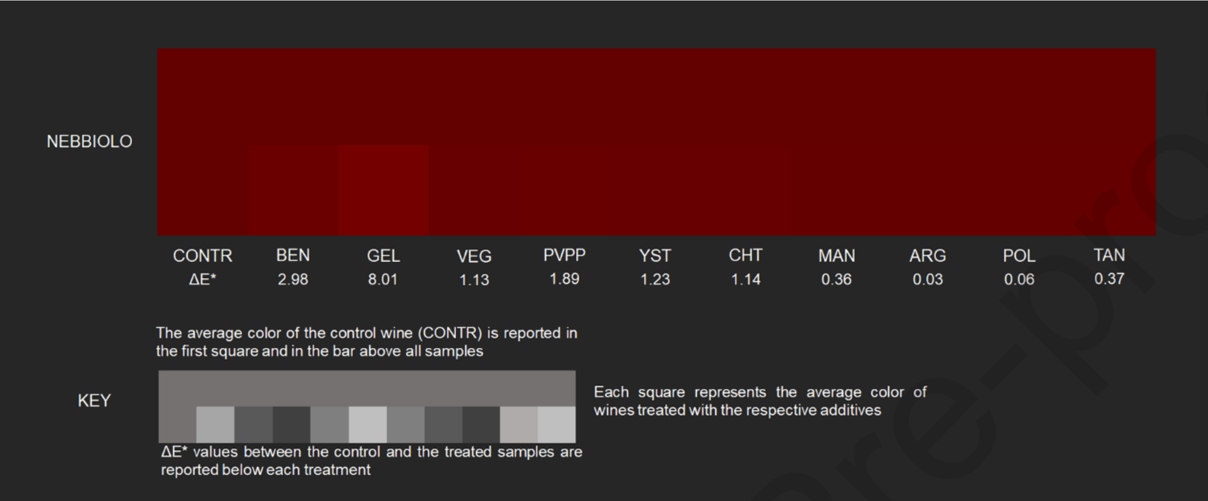

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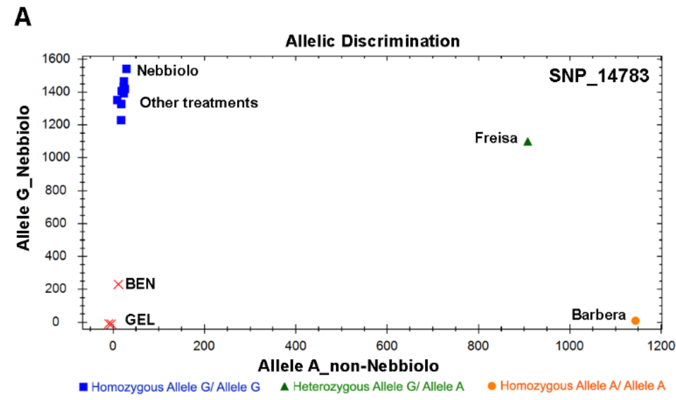

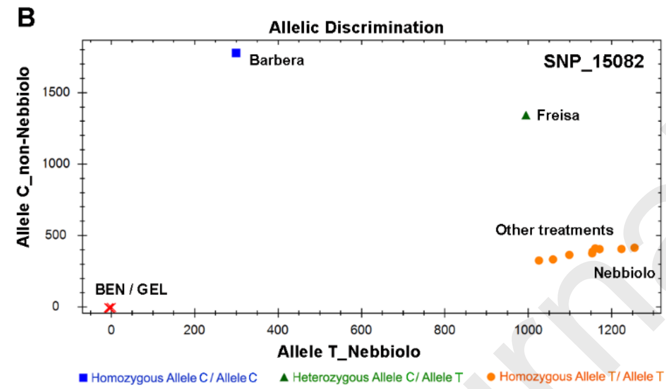

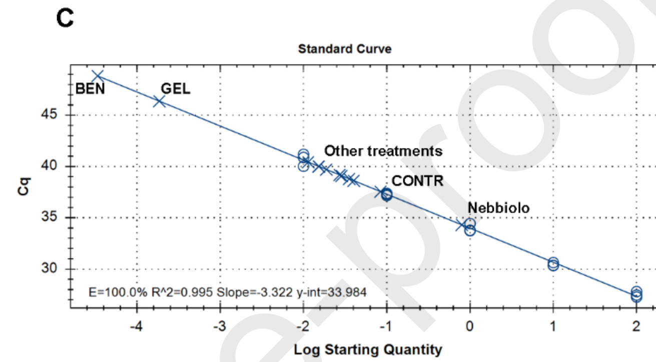

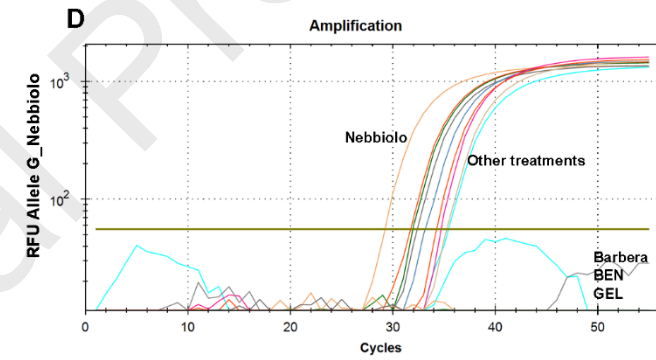

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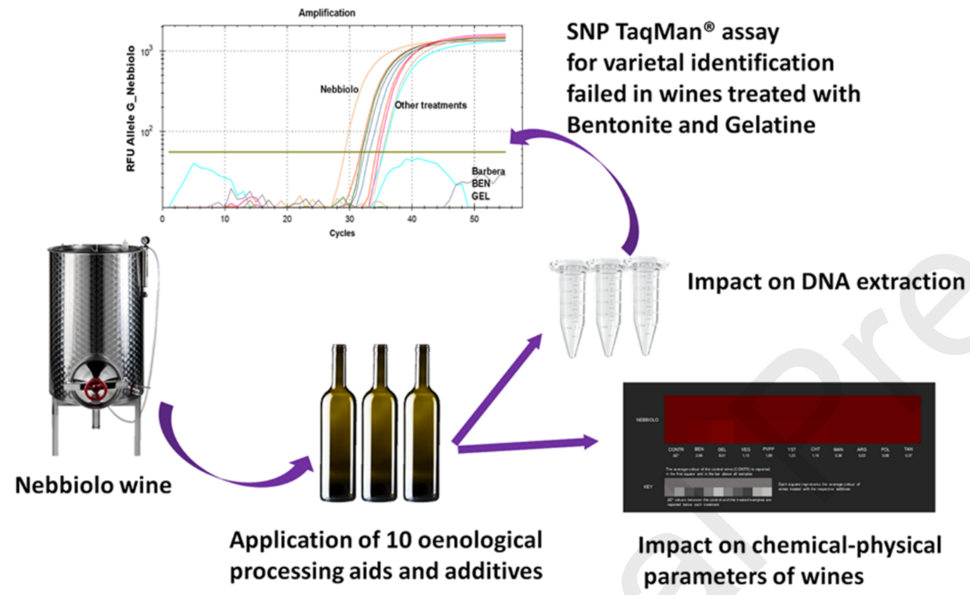
Graphical abstract



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Impact of oenological processing aids and additives on the genetic traceability of 'Nebbiolo' wine produced with withered grapes

Giorgio GAMBINO, Lorenzo FERRERO, Giulia SCALZINI, Camilla DE PAOLIS, Maria Alessandra PAISSONI, Susana RÍO SEGADE, Simone GIACOSA, Paolo BOCCACCI, Luca ROLLE

Highlights

- Oenological processing aids and additives played a role in removing DNA from wine
- Treatments with the highest oenological impact caused the highest loss of DNA
- Loss of DNA caused by aging is lower compared to the loss linked to fining agents
- SNP-based assay failed in ‘Nebbiolo’ wines treated with bentonite and gelatine

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Credit Author Statement

Giorgio Gambino: Conceptualization, Supervision, Funding acquisition, Methodology, Visualization, Writing - review & editing. **Lorenzo Ferrero**: Investigation, Formal analysis, Data curation, Validation, Visualization, Writing - original draft. **Giulia Scalzini**: Investigation, Methodology, Formal analysis, Validation. **Camilla De Paolis**: Investigation, Formal analysis, Data curation, Validation. **Maria Alessandra Paissoni**: Investigation, Formal analysis, Data curation, Validation. **Susana Río Segade**: Investigation, Methodology, Validation, Writing - review & editing. **Simone Giacosa**: Investigation, Methodology, Validation, Writing - review & editing. **Paolo Boccacci**: Investigation, Methodology, Validation, Writing - review & editing. **Luca Rolle**: Conceptualization, Supervision, Funding acquisition, Writing - review & editing

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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