UNIVERSITÀ CATTOLICA DEL SACRO CUORE
Sede di Piacenza

Dottorato di ricerca per il Sistema Agro-alimentare

Ciclo XXXV

S.S.D. AGR07-AGR16

Applications of biomolecular methods along the PDO wines chain production

Coordinatore:
Ch.mo Prof. Paolo Ajmone Marsan

Tesi di Dottorato di:
Sara Zambianchi
N. Matricola: 4915101

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A chi mi ha insegnato
che c'è più audacia nel sapersi rialzare
rispetto al solo librare.
Perché nella vita bisogna stringere i denti,
avere fisso l'obiettivo e non mollare mai!
A Voi, Mamma e Papà!

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Introduction

In today's social environment, wine sector, which boasts great economic value, is focused on consumer demands for quality, reliability, and safety. Authentication of quality is essential to confirm the correctness of wine production and to combat fraudulent practices: some products have been recognized as being of great value by obtaining Protected Designation of Origin (PDO), or Protected Geographical Indication (PGI), guaranteeing a higher income for the producer, high added value for the company, and a high-quality product for the consumer (Recupero et al., 2013).

Thus, in this context, authenticity has become a major issue as mislabelling of wines represents trade fraud. In the sphere of *"Made in Italy"*, certainly wine represents one of the most complex and appreciated alcoholic beverages, its quality is highly dependent on several factors and among them the grape variety is of primary importance.

The latter factor turns out to be strongly linked to the production culture of so-called "Old World" countries, where the oenological tradition is lost in the mists of time and of which Italy and France are the greatest ambassadors (Bottero and Dalmasso, 2011). According to our oenological history, the quality of wine is closely linked to the name of the cultivar used to produce it, so it is important that wineries follow the Production Regulations related to their geographical area, that regulate the grape varieties used in their wines whether they are monovarietal or derived from the union of several cultivars but in the correct percentages. Sometimes, the irregular addition of wines derived from other grape varieties is used to enhance the sensory characteristics of the final product and to decrease production costs (Galimberti et al., 2013).

In light of these findings, technical approaches and legislative guidelines for traceability of production have been developed to ensure the origin of the product and to identify those fraudulent practices aimed at deceiving the final consumer and tainting the identity of the product or even the entire designation. (EC Regulation No. 178/2002) (Faria et al., 2000; Woolfe and Primrose, 2004; Baleiras-Couto and Eiras-Dias, 2006).

Thanks to wine traceability whomever can verify the origin and composition of each batch of wine within the production chain, starting from grapes in the vineyard and

arriving at the packaged bottle through all the oenological steps considered of interest by the consumer (Catalano et al., 2016).

Wine authenticity has been widely studied because is a product that can be easily adulterated due to its chemical composition and its availability throughout the world (Faria et al., 2000; Boccacci et al., 2020).

The field of research in this area is constantly rejuvenating continuing technological and scientific developments in molecular genetics and genomics have provided a major boost to nucleic acid-based diagnostics. Methods based on the analysis of metabolites such as volatile compounds, amino acids and proteins, polyphenols, anthocyanins, even minerals, and recently, stable isotope analysis, have been developed to authenticate the geographic origin of wines (Agrimonti and Marmiroli, 2018; Catalano et al., 2016; Recupero et al., 2013; Boccacci et al., 2012; Pereira et al., 2012).

Some of these approaches have also been applied for varietal identification of wines; however, the metabolic composition of grapes and wines depends on environmental conditions and cultural practices, which, on the other hand, do not influence genotype. Therefore, varietal control is more accurate and efficient when DNA-based methodologies are used (Maul et al., 2021).

The information required for genetic analysis often goes beyond species characterization, having to refer very frequently to specific varieties or populations in cultivation. For many plant species and plant-derived products, the market price and the quality attributed by the consumer, depend to a large extent on the variety grown, so in this context, identification of vine variety using techniques based on the extraction of DNA present in samples is an established practice (Recupero et al., 2013; Boccacci et al., 2012).

Among the various analytical techniques, those that rely on the use of DNA are widely accepted and validated. These techniques analyse DNA directly recovered from the food matrix by available genetic markers such as microsatellites (SSRs). SSRs have proven to be the best for grapevine DNA typing because of their high degree of polymorphism, species specificity, reproducibility and simple data interpretation (Barrias et al., 2019; Pereira et al., 2012).

Due to the extensive use of this technology, large international databases of SSR profiles used as references for grapevine varietal identification are now available

(VIVC - Vitis International Variety Catalogue, VitisDB -Italian Vitis Database, etc.) (Maul et al., 2021).

Therefore, the development of methods based on genetic analysis would make it possible to trace a specific wine at all levels and stages of the winemaking process.

In this regard, in the literature, for what concerns must at various stages of fermentation and final wine, it is reported that high-quality DNA is usually recoverable from the initial musts and in high quantity (Savazzini and Martinelli, 2006).

These two parameters, however, decrease rapidly, because of nucleic acid degradation, during fermentation process, to the point that, as early as mid-fermentation, obvious signs of the presence of DNA are usually not detectable (only very faint signals can usually be seen). The progressive degradation of nucleic acids can cause problems in terms of traceability. Major problems are usually highlighted in the more advanced stages and in wine at the end of the fermentation process (Boccacci et al., 2020).

These problems are most amplified when operating in real-world situations, which allow for the assessment of totally different circumstances, difficulties and situations than those instituted in a research laboratory. Quality of the sample being analysed, in a real-world case, depend on a myriad of factors such as the storage of the goods before arrival at the plant, sampling on large volumes, the possibility of maintaining accurate traceability on large masses, the adoption of oenological practices that cannot be adopted in microvinification cases, and much more (Zambianchi et al., 2021).

Another important moment, not yet investigated in the literature, concerns the storage time in the case of pre-bottling stage and packaged wine.

Determining how long wine traceability is possible after the winemaking process can be of extreme interest to control-bodies in order to prevent or detect possible fraud. That traceability is possible up to the end of winemaking is well known, while less certainty has been obtained for later stages during the storage period, before and after bottling, when many authors reported a high degree of difficulty or impossibility in successfully analysing wine DNA (Garciaa-Beneytez et al., 2002; Siret et al., 2002; Catalano et al., 2016).

In a broader overview, safeguarding the quality of a product is not just about verifying what is stated on the label, or whether the percentages of the grapes involved participate in the quantities specified in the specifications but, rather, it means evaluating all those characteristics that make a finished bottle palatable and sought after to the consumer's palate (Pafundo et al., 2010; Galimberti et al., 2013).

Therefore, it is of great interest and topicality to be able to assess how the *"microbial terroir,"* i.e. the set of microbial populations that colonize the vine, can affect the quality of the wine in the finished bottle since several studies have shown that wine characteristics are correlated with the regional compositions of the microbial community (Bubeck et al., 2020).

The issue of sustainability and respect for different terroirs have now become pillars of modern agriculture, which seeks to be ever more at the forefront of environmental friendliness and a trump card in achieving products that are identifiable with the terroir of production.

Scientific research, in these areas, has made great strides in providing powerful tools for a more conscious use of the means normally employed in terms of phytosanitary defence, simpler soil tillage, or vegetation management, to arrive at a profound revision of the most common oenological practices so as to emphasize the quality of the product (De Filippis et al., 2019; Bubeck et al., 2020).

This attention derives from the knowledge that a wine of excellent value can only come from grapes of the highest quality grown in constantly monitored vineyards to enhance every peculiarity, in which quality is the must, and proceeding along the supply chain, in wineries in which logic is followed to enhance the product, up to that point, obtained.

Knowing the mechanisms of action of the microorganisms involved and understanding how they can positively or negatively influence the quality of the finished product would be a great help in getting to the bottle with the best result (Lopez-Rituerto et al., 2012; Rocchetti et al., 2018).

In light of what has been said so far, the present doctoral project has attempted to respond to the issues, needs and concerns regarding the entire wine chain production. In particular, it consisted of two main, closely integrated research strands concerning:

1) the application of DNA analysis for the traceability of quality wine production (recognition of varieties used for wine production). To consolidate the traceability work, special attention was paid to the delicate phase of wine storage in order to assess how long it was possible to trace the productions.

2) metagenomic analysis of the microbiome and microbiota of grapes, musts (at various stages of processing) and wines, produced under different cultural regimes, in a niche production area such as the Buttafuoco area of Oltrepò Pavese.

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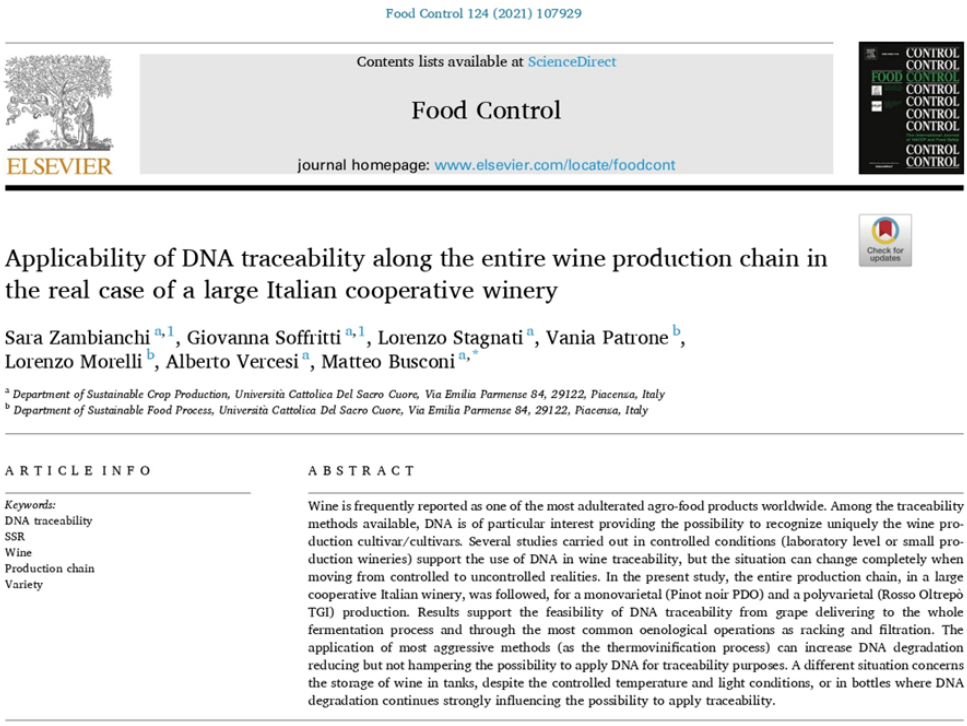
Chapter 1

Published article 1: Applicability of DNA traceability along the entire wine production chain in the real case of a large Italian cooperative winery



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Applicability of DNA traceability along the entire wine production chain in the real case of a large Italian cooperative winery

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ABSTRACT

Wine is frequently reported as one of the most adulterated agro-food products worldwide. Among the traceability methods available, DNA is of particular interest providing the possibility to recognize uniquely the wine production cultivar/cultivars. Several studies carried out in controlled conditions (laboratory level or small production wineries) support the use of DNA in wine traceability, but the situation can change completely when moving from controlled to uncontrolled realities. In the present study, the entire production chain, in a large cooperative Italian winery, was followed, for a monovarietal (Pinot noir PDO) and a polyvarietal (Rosso Oltrepò TGI) production. Results support the feasibility of DNA traceability from grape delivering to the whole fermentation process and through the most common oenological operations as racking and filtration. The application of most aggressive methods (as the thermovinification process) can increase DNA degradation reducing but not hampering the possibility to apply DNA for traceability purposes. A different situation concerns the storage of wine in tanks, despite the controlled temperature and light conditions, or in bottles where DNA degradation continues strongly influencing the possibility to apply traceability.

Abstract

Wine is frequently reported as one of the most adulterated agro-food products worldwide. Among the traceability methods available, DNA is of particular interest providing the possibility to recognise uniquely the wine production cultivar/cultivars. Several studies carried out in controlled conditions (laboratory level or small production wineries) support the use of DNA in wine traceability, but the situation can change completely when moving from controlled to uncontrolled realities. In the present study, the entire production chain, in a large cooperative Italian winery, was followed, for a monovarietal (Pinot noir PDO) and a polyvarietal (Rosso Oltrepò PGI)

production. Results support the feasibility of DNA traceability from grape delivering to the whole fermentation process and through the most common oenological operations as racking and filtration. The application of most aggressive methods (such as the thermovinification process) can increase DNA degradation reducing, but not hampering the possibility to apply DNA for traceability purposes. A different situation concerns the storage of wine in tanks, despite the controlled temperature and light conditions, or in bottles, where DNA degradation continues strongly influencing the possibility to apply traceability.

Highlights

1. First application of DNA traceability in a real large scale cooperative winery
2. DNA traceability is easily doable in uncontrolled conditions until musts
3. Standard oenological processes do not interfere with traceability
4. Thermovinification reduces but does not hamper traceability
5. Storage in tanks reduce the possibility to apply DNA traceability

Keywords: DNA traceability, SSR, wine, production chain, variety

1. Introduction

Vitis vinifera subsp. *vinifera* L. (or *Sativa*) is an ancient crop that is used to produce a wide range of products such as table fruit, wine and spirits, juice and raisins. An enormous bio-diversity of varieties can be used in the production of wine, even if a small number of them is of commercial importance (Işĉi et al., 2009), further, depending on the production regions, only specific cultivars can be planted in vineyards and used to produce wines.

In the European Union, the wine quality categories are separated into PDO (Protected Designation of Origin) and PGI (Protected Geographical Indication). Although cultivar information in wine labelling is not mandatory according to European law, it has become an important aspect of the consumer product perceived value, in a market characterized by fierce competition (Recupero et al., 2013). Labelling is more rigorous in those areas where viticulture has an old production tradition such as Italy, France,

and other European countries with respect to other areas such as America, Australia, New Zealand and South Africa (Catalano et al., 2016). In this context, the possibility to certify wine production from grapes to bottled wine, along the whole production chain, from the harvest and through the subsequent processing and oenological operations, would be a value added for the final consumer.

In recent years, food safety, food quality, and food traceability have played a great role in the agro-food sector due to information and the media. Cases of food adulteration have a big impact on consumption and consumer confidence. For that reason, governments have different national guidelines for the production and preservation of food and the European Food Safety Authority at the European level. The need of reliable food traceability systems has been tackled by scientific research hence producing different analytical approaches to the problem to satisfy this need (Bottero and Dalmasso, 2011).

Food authenticity validation relies mostly on the analysis of secondary metabolites, proteins, and, lastly on DNA sequences. In the agro-food sector, DNA analysis can be extremely useful because the information required for genetic characterization analysis, often, goes beyond species identification, frequently referring to particular varieties. For many plant species and many products of plant origin, such as wine, the market price and the quality attributed by the consumer largely depend on the cultivated variety (Galimberti et al., 2013). Being the DNA unique across different individuals and not influenced by the environment and cultural practices, the attribution of species and, in particular, variety is more accurate when DNA-based methodologies are used with respect to other types of analysis (Catalano et al., 2016). Due to recent advances in molecular biology, DNA markers have become the most effective instrument in the analysis of the DNA of plant cultivars and are also used to track the raw materials in food industry processes (Stagnati et al., 2020; Soffritti et al., 2016; Scarano et al., 2011; Kumar et al., 2009).

Among the different kinds of molecular markers available, SSR (Simple Sequence Repeats) and, recently, SNPs (Single Nucleotide Polymorphism) are considered as the marker of choice for food traceability (Scarano and Rao, 2014). Concerning microsatellites, scientific community, with the partnership of Organization for Vine and Wine (OIV), has developed a set of 9 SSR molecular markers for variety identification. These markers, VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62 and VrZAG79, have been used and approved in the

frame of the European projects Genres081 and GrapeGen06, in particular: the use of these markers as a standard set was recommended by the participants of the European project Genres081 (This et al., 2004). Presently they are considered as the minimum marker set for cultivar identification. The genetic profiles obtained using this set of markers, for 5428 cultivars are available on the Vitis International Variety Catalogue VIVC website (Maul et al., 2021).

The possibility of tracing wine productions by analysing the DNA of the production cultivars directly from wines or musts, at different times of the supply chain, is a hot topic that has been addressed in many studies over nearly twenty years, from early reports such as (Faria et al., 2000) up to the most recent ones such as (Gambino et al., 2022; Boccacci et al., 2020).

Grapevine DNA present in grape juice, and subsequently in wine, originates mainly from berries; these fruits are also rich in polysaccharides, tannins, and polyphenols (Cabanis et al., 1999). During the fermentation process, grape juice undergoes extensive transformations and biochemical modifications while, after fermentation, wine is further processed with many treatments (such as fining and filtration). All these modifications, processes, and treatments affect the DNA content of wine but, even if not very concentrated and highly degraded, it can be isolated and used to identify the variety, or varieties, used for wine production (Savazzini and Martinelli, 2006).

Several studies have been carried out by using molecular markers to analyse DNA extracted from must or wine for traceability purposes.

The majority of studies were based on microsatellites, both of nuclear and chloroplast origin (Agrimonti and Marmiroli, 2018; Catalano et al., 2016; Recupero et al., 2013; Boccacci et al., 2012; Pereira et al., 2012; Corrado et al., 2011; Caramante et al., 2010; Baleiras-Couto and Eiras-Dias, 2006; Garcia-Beneytez et al., 2002; Siret et al., 2002) and, recently, on SNPs (Boccacci et al., 2020; Catalano et al., 2016).

Concerning the starting material, on which to perform DNA extraction, many studies used samples of musts and wines obtained by micro vinification (Barrias et al., 2019; Pereira et al., 2012; Baleiras-Couto and Eiras-Dias, 2006; Siret et al., 2002), eliminating, in this way, various oenological practices commonly adopted in wineries (repeated racking, filtration, clarification, heat treatment, etc.) that may affect quantity and quality of DNA present in samples.

Few studies were based on samples obtained by local producers representing small or medium-sized production realities (Boccacci et al., 2020; Catalano et al., 2016; Di

Rienzo et al., 2017; Recupero et al., 2013; Boccacci et al., 2012), while the majority has been based on monovarietal musts or wines, prepared through micro-vinification, or on artificial mixtures arranged at laboratory scale by mixing known amounts of different varieties (Boccacci et al., 2020; Siret et al., 2000; Bigliazzi et al., 2012). With few exceptions (Boccacci et al., 2020; Catalano et al., 2016), the sampling steps during the production process have been few and are usually limited to the fermentation phase. From these studies, it is possible to obtain that: i) the DNA extracted from musts and, especially from wines, is highly degraded and the quality is very low (ratios A_{260}/A_{280} and A_{260}/A_{230} are usually very low); ii) the genetic profile of the production cultivar can be easily obtained using the DNA from musts, especially during the early stages of fermentation, but not always at the end of the process or after oenological operations.

A common point to all the methods applied up to now is that they have been developed on controlled conditions and processes: laboratory, small production scale and almost always monovarietal productions. They have never been applied to a real large scale and in uncontrolled conditions.

What is the effectiveness of these methods if applied to real, large uncontrolled productions?

Can they be applied with the same good and reproducible results?

On the other hand, the transition from controlled vinification to industrial uncontrolled vinification can reduce the applicability of these methods?

For all these reasons, in the present study, it was decided to follow a real production wine chain, to consider every stage of the vinification from the moment the grapes are conferred along the fermentation process, considering different oenological practices leading to obtaining a stabilized wine ready to bottle.

Analysed samples were taken from the 2017 harvest onwards, in a large cooperative production facility “Terre d’Oltrepò”, located in Oltrepò Pavese area, in the south-west of Lombardy (Italy) that is included in the ten biggest Italian cooperative wineries. Oltrepò Pavese produces 62% of Lombardy wines, that corresponds to 13,550 hectares of vineyards. Terre d'Oltrepò is the biggest cooperative winery in Lombardy.

The cooperative has roughly 700 members and vinifies around 50,000 tons of grapes which result in 4 million bottles produced.

Due to the characteristics mentioned above, we could collect grape samples truly representative of the whole Oltrepò Pavese territory. As Terre d'Oltrepò Pavese being

a cooperative winery, it does not own vineyards. Instead, it processes grapes conferred by cooperative members. Therefore, the cooperative production facility vinifies and bottles the grapes conferred by the cooperative members (Corsi et al., 2019).

For this study, we focused on two products, one monovarietal and one polyvarietal (made by mixing five different varieties in unknown percentages but following the directives present in the product specification) from the delivered grape to the pre-bottled and bottled wine. Different samples were taken along the entire wine production process from the conferring of grape to bottle packaging.

The work focused on different topics: 1) the feasibility of DNA analysis for the determination of the identity, but not the quantity, of the variety during the entire production process; 2) the possibility to recover analytic grade DNA from raw and processed matrices; 3) the possibility to trace the production from grape to wine; 4) the influence of uncommon processes, as thermal treatments, on analyzable DNA for traceability.

To this aim standard DNA extraction procedure and standard SSR based genetic analysis, similar to methods retrievable from the scientific literature, have been applied on grape, must and wine samples obtained in large, real and uncontrolled conditions.

2. Materials and methods

2.1 Sample collection

Samples, both for grapes, musts, and wines, were collected to follow two wines, one monovarietal and one polyvarietal, of the area Oltrepò Pavese.

As monovarietal wine we considered Pinot noir PDO ("Pinot Nero dell'Oltrepò Pavese" DPR 06.08.1970 G.U.27.10.1970, https://www.politicheagricole.it) while, as polyvarietal, we considered Rosso Oltrepò TGI. Rosso Oltrepò is made by mixing the varieties: Barbera, Croatina, Uva rara, Vespolina and Cabernet Sauvignon.

The admitted cultivars are reported in the regulation (IGT "Provincia di Pavia", G.U. 285-06.12.1995, https://www.politicheagricole.it) but the relative percentages are not defined.

Finally, a third must be considered, but only for the thermovinification process ("Sangue di Giuda dell'Oltrepò Pavese o Sangue di Giuda", DPR 06.08.1970 G.U.27.10.1970, https://www.politicheagricole.it).

The sample-set for this experiment consisted of:

1) Thirty-two samples of grapes that were taken from the wagons at the time of delivery (mechanically harvested), except for Uva rara and Vespolina that were directly collected in vineyards. Of these samples, 18 belong to the Pinot noir variety, 3 to the Barbera variety, 6 to the Croatina variety, 3 to the Cabernet Sauvignon variety. Concerning Uva rara and Vespolina, considering that their cultivation is limited to small surfaces, sampling was performed directly in the vineyard and 1 sample of Uva rara and 1 sample of Vespolina were collected. The sample details are reported in Table 1. For the different varieties, the number of grape samples was based on the extension of their cultivation in the reference area: 6 samples for Croatina, which is the most cultivated, and 1 sample for Uva rara and Vespolina which are the least cultivated. Samples were frozen at -20°C to block any degradation and oxidation process that might affect DNA quantity and quality, until further analysis.
2) Fourteen must samples pulled out at different times: 24, 48, and 72 hours, after crushing and at the end of alcoholic fermentation. Must sample be immediately frozen at -20°C until further analysis. Must sample 19 was taken at the end of a thermovinification process (at an average temperature of 60 °C for 30 minutes; Termovinificatore Reda, model per Termoflash), must sample 20 after racking (without the solid part), must sample 21 after racking and tangential filtration (Tangential filter Flavy x wine 10, Bucher, pore size 0.50 μm), and, sample 22, after the most common oenological operations (racking, filtration, tangential filtration and centrifugation), details are reported in Table 2.
3) Four so-called "pre-bottled" samples of the year 2017 collected directly from tanks in which they are stored and consisting of wines without the addition of any stabilizers or oenological additives, as reported in Table 3. Samples 3V and 5V correspond to Pinot noir PDO while samples 4V and 6V correspond to Rosso Oltrepò TGI. After collection, these samples were immediately frozen at -20°C until further analysis.
4) Two bottled samples, corresponding to Pinot noir PDO (sample 1V) and Rosso Oltrepò TGI (sample 2V), taken from the shelf as reported in Table 3; these samples were stored at room temperature.

Three reference standards (Pinot Noir, Barbera, and Malvasia di Candia aromatica), belonging to the *Vitis vinifera* germplasm collection held at Università Cattolica del Sacro Cuore, Piacenza, were included in all PCR analysis to calibrate allele size of the selected SSR markers; this allowed allele profile comparison between our samples and allele profiles of the Vitis International Variety Catalogue (VIVC database, http://www.vivc.de).

All samples were provided by the largest and most representative cooperative winery in the Oltrepò Pavese, Lombardy, (Italy) during the 2017 harvest.

Table 1. *List of samples with their identification number (the first digit indicates the sample number, while the decimal digit indicates the replication), variety declared at delivery, sampling date, and type of harvest.*

Sample	Variety	Date	Harvest
1.1/ 1.2/1.3	PINOT N. (9279)	18/08/2017	Mechanical
3.1/ 3.2/ 3.3	PINOT N. (9279)	18/08/2017	Mechanical
4.1/ 4.2/ 4.3	PINOT N. (9279)	18/08/2017	Mechanical
5.1/ 5.2/ 5.3	PINOT N. (9279)	18/08/2017	Mechanical
6.1/ 6.2/ 6.3	PINOT N. (9279)	18/08/2017	Mechanical
7.1/ 7.2/ 7.3	PINOT N. (9279)	21/08/2017	Mechanical
8.1/ 8.2/ 8.3	BARBERA (974)	12/09/2017	Mechanical
9.1/ 9.2/ 9.3	CROATINA (3251)	12/09/2017	Mechanical
10.1/10.2/ 10.3	CROATINA (3251)	12/09/2017	Mechanical
11.1/ 11.2/ 11.3	CABERNET S. (1929)	13/09/2017	Mechanical
12.1/12.2/12.3	UVA RARA (12830)	13/09/2017	Manual
13.1/13.2/13.3	VESPOLINA (13018)	13/09/2017	Manual

* the number in brackets, following the name of each variety, is the univocal code of each genotype in the VIVC database.

Table 2. *List of wort samples with their identification number (the first digit indicates the sample number, the second the hours after pressing, EF indicates the end of fermentation), the varieties, and the sampling dates.*

Must varietal composition	***Sample***	***Phase: sampling date***
PINOT N. (9279)	16	24: 22/08/17 48: 24/08/17 72: 26/08/17 EF: 28/08/17
BARBERA (974)	17	24: 14/09/17 48: 15/09/17 72: 16/09/17 EF: 18/09/17
CROATINA (3251)		
UVA RARA (12830)		
VESPOLINA (13018)		
CABERNET S. (1929)		
BARBERA (974)	18	24: 19/09/17 48: 20/09/17 72: 21/09/17 EF: 23/09/17
CROATINA (3251)		
UVA RARA (12830)		
VESPOLINA (13018)		
CABERNET S. (1929)		
BARBERA (974)	19	24/09/17
CROATINA (3251)		
UVA RARA (12830)		
PINOT N. (9279)	20	01/09/17
PINOT N. (9279)	21	26/09/17
BARBERA (974)	22	30/09/17
CROATINA (3251)		
UVA RARA (12830)		
VESPOLINA (13018)		
CABERNET S. (1929)		

Table 3. *List of wines with their identification number, the varieties and types considered. The wines taken into consideration are all DOC.*

Sample	**Variety**	**Type**
1V	Rosso Oltrepò	Packaged wine
2V	Pinot Noir	Packaged wine
3V	Pinot Noir	Pre-bottling
4V	Rosso Oltrepò	Pre-bottling
5V	Pinot Noir	Pre-bottling
6V	Rosso Oltrepò	Pre-bottling

2.2 Sample preparation and DNA extraction

1) Grape samples: frozen berries were cleaned from seeds, shredded with the help of a scalpel, and placed in 2 ml sterile tube with two sterile glass beads (5 mm diameters) to improve shredding. Samples were ground using a mechanical crusher (TissueLyser LT, QIAGEN) to obtain a homogeneous puree.
2) Must and racking must: 20 ml of must were mixed to one volume of isopropanol and 0.1 volumes of sodium acetate 3M pH 5.2. After several inversion, the mixture was frozen overnight at -20°C. Samples were then centrifuged for a minimum of 2 hours at 4°C at 8,500 rpm. The supernatant was removed, the pellet was resuspended in 500 µl sterile double-distilled water and transferred to a 1.5 ml sterile tube and centrifuged at 4°C and 14,000 rpm for 20 minutes. The supernatant was removed, and the pellet was used for DNA extraction.
3) Wine: 240 ml of wine were mixed to one volume of isopropanol and 0.3 volumes of sodium acetate 3M pH 5.2. After several inversion, the mixture was divided into 50 ml aliquots in sterile tubes, stored at -20°C for at least 2 days. Samples were then centrifuged for a minimum of 3 hours at 4°C at 8,500 rpm. The supernatant was removed, the pellet was resuspended in 500 µl isopropanol and transferred to a 1.5 ml sterile tube and further frozen at -20°C for 2 days to further aid DNA precipitation. Samples were then centrifuged at 4°C and 14.000 rpm for 1 hour, the supernatant was removed, and the pellet was used for DNA extraction.

DNA extraction was carried out according to the GenElute DNA Miniprep Kit (SIGMA-Aldrich) instructions with some modifications as the addition of 4% w/v Polyvinylpyrrolidone (PVP) during the lysis step (Soffritti et al., 2016), PVP is a polymer that helps the removal of phenols and aromatic compounds that inhibit downstream application of nucleic acids; the increasing of the duration of the different steps, in particular of the incubation at 65°C; centrifugation at 4 °C and the use of a minor quantity of elution solution trying to increase DNA concentration.

For standard varieties, DNA was extracted from young leaves according to the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) as previously described.

2.3 DNA quantity, quality, and amplifiability evaluation

The quantity and quality of DNA were evaluated according to absorbance ratios (A_{260}/A_{280}, A_{260}/A_{230}) measured with a nanophotometer (NanoPhotometer® NP80, IMPLEN) according to the manufacturer's instructions.

The amplifiability of the extracted DNA was evaluated by using two pairs of universal primer, classically used in plant DNA barcoding, developed on the plastidial gene for RUBISCO large subunit (RbcL-F, RbcL-R) and on the ITS1-5.8S-ITS2 region of the nuclear genes for large ribosomal RNAs (ITS4, ITS-S2F) (Galimberti et al., 2013; Fazekas et al., 2012). The amplification test was carried out following Soffritti et al. (2016).

PCR reactions were performed in a total volume of 25 µl consisting of 1 µl of extracted DNA; 1 X PCR buffer; 0.3 mM dNTPs; 1 µmol of each primer; 1 U of Taq polymerase; 2% PVP; H_2O up to a final volume. PVP was added to improve PCR amplification.

Amplification cycles were characterized by an initial denaturation at 94°C for 5 minutes; 35 cycles composed of denaturation at 94°C for 30 seconds; annealing for 40 seconds at the temperature of 52°C for RbcL and 58 °C for ITS; extension a 72°C for 1.5 minutes and final extension step at 72°C for 10 minutes. PCR products were visualized by agarose gel electrophoresis.

2.4 Selection of SSR primers set, DNA amplification and analysis

Microsatellite analyses were carried out using nine SSR markers (VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, and VrZAG79) (Sefc et al., 1999; Bowers et al., 1996; Thomas and Scott, 1993) selected among those reported by the main databases for grape varietal comparison (VIVC; Maul et al., 2021).

PCR mixtures were set up as previously described starting from 1 µl of template, amplification was performed as follows: initial denaturation at 94°C for 5 minutes; 35 cycles composed of denaturation at 94°C for 30 seconds; primer annealing according to temperatures reported in the corresponding scientific literature and on the Italian Vitis Database (https://vitisdb.it/) for 30 seconds; extension at 72°C for 1 minute and a final extension step at 72°C for 10 minutes.

For fragment analysis 1 µl of the PCR product was loaded onto a 96-well plate along with the GeneScan™ 500 LIZ™ dye Size Standard (Applied Biosystems). After a denaturation step at 94°C for 3 minutes, the fragments were run on ABI PRISM 3100 Genetic Analyzer (AppliedBiosystem) according to the manufacturer's instructions.

3. Results and discussion

3.1 DNA extraction and evaluation

DNA was extracted from different oenological matrices, considered representative of the wine production process. The goal of the present research was to demonstrate the feasibility of wine traceability, not in the laboratory but in the field conditions under the uncontrolled conditions of a big cooperative winery. Concerning the monovarietal Pinot noir PDO, at the time of delivery to the cooperative winery, grape samples were collected from wagons conferred by different farmers to confirm the genetic identity of the different delivered Pinot noir grapes. Concerning the polyvarietal Rosso Oltrepò TGI, the five expected varieties used for its production were sampled: either at the delivery or directly in the field for the least cultivated. For the grapes, it was sufficient to adequately crush the berry with the help of a scalpel and to eliminate seeds.

Musts and wines were not prepared through micro-vinification in a laboratory, or through artificially mixing defined percentages of varieties, but they were recovered directly from the tanks from the stage of must fermentation, through the different oenological operations, until the pre-bottle stage along the whole wine production chain. This is an important point to verify if methods developed in laboratory can be effectively transferred to industrial vinification conditions, with all the negative related aspects such as: impossibility to have the controlled on the delivered raw material and the impossibility to control the entire winemaking process reducing the possibility to trace productions. In complex matrices like grapes, must and wine, the presence of secondary metabolites, both primary and secondary, polysaccharides and a range of phenolics (including tannins) can interfere with DNA extraction and enzymatic reactions and even degrade the DNA during the extraction itself (Demeke and Adams, 1992; Do and Adams, 1991). Considering this, the starting amount of material on which to carry out the DNA extraction, it is extremely important, in particular concerning musts and wines. For must, being a fresh material still rich in solid

particles, it was sufficient to use a small starting volume (20 ml) to obtain an amount of pellet sufficient to extract DNA for subsequent analyses. Finally, in wines, because of the expected reduced amount of genetic material present, in consequence of fermentation and oenological operations, a larger starting volume (240 ml) was used to obtain a pellet on which to carry out the extraction of the genetic material. In liquid matrices such as musts and wines, precipitation was performed by chemical and physical means by coupling isopropanol and sodium acetate with low temperatures (-20°C) for long periods and centrifugations at high speed. Similar protocols, using isopropanol, sodium acetate, and low-temperature precipitation are reported in the literature by other authors (Boccacci et al., 2012; Pereira et al., 2012).

Quantification and evaluation of the purity of the genetic material were carried out by spectrophotometric analyses. The results are reported in Table 4. As already observed by previously mentioned authors in similar matrices, except for Agrimonti and Marmiroli (2018), the quality parameters of the extracted DNAs were very low. Both the two classical absorbance ratios were markedly below the desired threshold of 1.7 for A_{260}/A_{280} and 2 for A_{260}/A_{230}, highlighting the presence of a low-quality DNA.

The worst situation was for the A_{260}/A_{230} ratio in which the results indicate the presence of high contamination of polyphenols and carbohydrates that are extremely rich in grapes and their derivatives. Similar results, without significant differences, were obtained for both grapes, must and wine. Regarding DNA quantification, the obtained values were comprised between a minimum of approximately 0,5 ng/µl in sample 9.1 and a maximum of 22 ng/µl in sample 20 (Table 4). It must be noted that, in presence of high levels of contaminants, as stated by the absorbance ratios, the estimated DNA quantities could not be considered as reliable witnesses of the real amount of genetic material present in the extraction product. To avoid problems of unreliable DNA readings, for the subsequent PCR analysis it was decided to use always 1 µl of extracted DNA for grapes and musts and 2 µl of extracted DNA for wines.To try to increase DNA purity, or, at least, to make DNA suitable for the subsequent analyses, PVP was added during the DNA extraction (Soffritti et al., 2016), in the lysis step, and, subsequently, in the PCR reactions (Stagnati et al., 2017), to help the removal of phenols and aromatic compounds inhibiting the downstream application of nucleic acids. Inhibitors have extraction conditions similar to those of DNA, so they are difficult to remove in the extraction process and, as a result, the anomalous absorbance ratios can be justified.

Table 4. *Spectrophotometer quantification of analysed samples and purity ratios.*

Sample	ng/µl	A260/ A280	A260/ A230	Sample	ng/µl	A260/ A280	A260/ A230
1.1	11.85	1.388	0.045	**11.1**	5.90	1.234	0.172
1.2	8.20	1.314	0.074	**11.2**	6.79	1.196	0.137
1.3	4.00	1.350	0.042	**11.3**	5.98	1.247	0.118
3.1	12.90	1.446	0.072	**12.1**	14.85	1.137	0.306
3.2	7.10	1.428	0.032	**12.2**	5.15	1.271	0.131
3.3	7.70	1.461	0.021	**12.3**	18.30	1.313	0.094
4.1	7.20	1.413	0.043	**13.1**	10.45	1.488	0.042
4.2	5.65	1.471	0.028	**13.2**	12.20	1.514	0.090
4.3	5.00	1.195	0.149	**13.3**	5.70	1.450	0.043
5.1	7.65	1.380	0.035	**16.24**	8.90	1.413	0.195
5.2	3.60	1.161	0.151	**16.48**	8.55	1.069	0.207
5.3	4.45	1.195	0.087	**16.72**	10.95	1.304	0.105
6.1	11.05	1.290	0.029	**16.FF**	18.45	1.304	0.064
6.2	10.55	1.279	0.041	**17.24**	11.75	1.205	0.088
6.3	10.86	1.359	0.094	**17.48**	15.50	1.442	0.337
7.1	6.00	1.387	0.030	**17.72**	13.55	1.278	0.187
7.2	7.25	1.385	0.025	**18.24**	9.95	1.185	0.195
7.3	6.55	1.508	0.024	**18.48**	20.00	1.176	0.214
8.1	11.80	1.062	0.494	**18.FF**	16.80	1.175	0.105
8.2	12.36	1.330	0.581	**19**	10.55	1.302	0.074
8.3	11.69	1.458	0.312	**20**	21.95	1.302	0.074
9.1	0.45	0.928	0.117	**21**	14.75	1.528	0.361
9.2	9.45	1.071	0.122	**22**	12.50	1.210	0.221
9.3	10.95	1.048	0.085	**1V**	3.87	1.449	0.199
10.1	17.00	1.103	0.125	**2V**	12.2	0.917	0.113
10.2	13.00	1.148	0.142	**3V**	4.55	0.883	0.118
10.3	7.20	1.291	0.083	**4V**	4.00	1.270	0.203
				5V	3.98	1.283	0.149
				6V	5.46	1.389	0.208

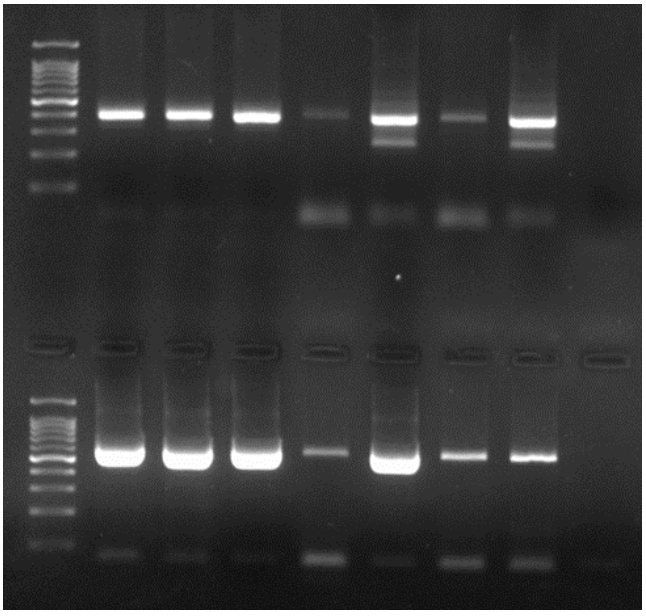
3.2 Amplifiability evaluation

The amplifiability of the extracted DNA was evaluated using two pairs of universal primers developed for DNA barcoding: one for the plastidial gene for RuBisCO and one for the nuclear ribosomal RNA region including the internal transcribed spacer 2 (ITS2) and a partial sequence of the nuclear gene for ribosomal large subunits (Galimberti et al., 2013). This step, carried out by using both nuclear and organellar DNA, is important to verify that the extracted DNA can be used in further and finer genetic analysis by using nuclear SSR markers.

The ITS primers are universal for eukaryotic DNA, so the possibility of amplifying also yeast DNA is not to be excluded but: the ITS4 primer is reported to be plant specific (Cheng et al., 2015) and the expected size of the amplicon in grapevine is, more or less, 400 bps (Figure 1).

On the contrary, the expected size of the same region in yeasts is reported, in literature, to be included between 500 and 800 bps. In conclusion, the amplicon obtained is likely the result of the amplification of grapevine DNA. Amplicons of the expected size were obtained for all samples examined, as reported in Figure 1.

Figure 1. *PCR amplicons obtained from grapes (samples 1, 2, and 3 after the 100 bp size ladder), musts (4 and 5), and wines (6 and 7) by using ITS (upper panel) and RbcL (lower panel) markers. Sample number 8 corresponds to negative control. The expected size is approximately 400 hundred bases for ITS and 600 hundred bases for RbcL.*



Despite the absorbance ratios were very low, the addition of PVP was enough to remove the main inhibitors making possible the DNA analysis. Similar results were obtained also with other difficult matrices as reported in Stagnati et al. (2020 and 2017). A good amplification signal is visible in the majority of samples with the exception of samples 4 (DNA from must) and 6 (DNA from wine) for which only a weak signal was obtained. This was particularly evident for ITS amplification. This result can be the consequence of a possible lower amount of amplifiable DNA; the presence of PCR inhibitors (polyphenols, anthocyanins, and carbohydrates) that were not completely removed by the PVP addition; the greater degradation of nuclear DNA, ITS is a nuclear marker, with respect to plastidial DNA.

It was possible to observe that similar amplification signals, concerning the intensity, were obtained from musts and wines for both the primer pairs. The presence of samples providing amplicons with a reduced intensity could be a consequence of the larger amount of inhibitors present.

Despite this was not a quantitative analysis, we could not exclude it was also a consequence of a lower amount of DNA present in must and wine extracts independently of the amount estimated with spectrophotometric analyses. The possibility to obtain amplification from wine DNA, in particular from nuclear DNA, is of particular interest since the identification of vine varieties relies mainly on nuclear DNA, which is more informative than plastidial DNA.

Savazzini and Martinelli (2006) reported that for must and wine reliable DNA extraction methods needed to be developed. Despite several publications on this topic, a fast, robust, and reliable extraction method from wine is still missing. DNA extraction is a well-established procedure for plant and animal samples, raw materials, or processed foods (Stagnati et al., 2020; Soffritti et al., 2016; Scarano and Rao, 2014). On the contrary, for must and, in particular, for wine, one of the main concerns remains the possible interference of polyphenols, tannins, and polysaccharides present in the matrix, as well as the high DNA degradation due to fermentation and oenological treatments (Işçi et al., 2014; Savazzini and Martinelli, 2006). Similar results were also obtained by other authors working on wine and similar matrices as pear juice or soybean derived drinks where DNA is highly degraded, only residual and, in many cases, not amplifiable (Savazzini and Martinelli, 2006).

3.3 Microsatellite analysis

3.3.1 Grapes

Grapes (numbered from 1 to 13, Table 1) were sampled at the time of delivery to the cooperative winery of single farmers or directly in the field. For each farmer, 3 different subsamples were taken from different positions in the wagon or the vineyard (Uva rara and Vespolina). According to the detected allelic profiles, perfect correspondence between the different subsamples was found. This indicates that, for those lots or fields, a single genotype was present. In presence of more varieties, DNA analyses could be useful to detect them, as demonstrated in a recent paper, where allele profiling of raw or processed food matrices allowed the detection of multi-genotypes cocoa batches (Stagnati et al., 2020). For all varieties, the grape profiles corresponded perfectly to the reference profile of the declared variety; genotype attribution was assessed according to the VIVIC database. The genetic profiles for the different grape samples are reported in Table 5. These results confirmed the possibility to apply DNA techniques for traceability purposes of grapes at conferring with the aim to identify varietal lots avoiding accidental misidentification or deliberate frauds (Scarano and Rao, 2014).

Table 5. *Grapes: Results of microsatellite analysis. The results obtained from the microsatellite analysis match perfectly with the profiles reported on the VIVC database*

Samp.	VVS2		VVMD5		VVMD7		VVMD25		VVMD27		VVMD28		VVMD32		VrZAG62		VrZAG79		Corresp. VIVC
1.1	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
1.2	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
1.3	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
3.1	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
3.2	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
3.3	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
4.1	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
4.2	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
4.3	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
5.1	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
5.2	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
5.3	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
6.1	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
6.2	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
6.3	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
7.1	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
7.2	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**
7.3	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	**PINOT (9279)**

8.1	133	135	228	228	249	253	239	255	186	190	234	260	252	272	192	200	243	259	**BARBERA (974)**
8.2	133	135	228	228	249	253	239	255	186	190	234	260	252	272	192	200	243	259	**BARBERA (974)**
8.3	133	135	228	228	249	253	239	255	186	190	234	260	252	272	192	200	243	259	**BARBERA (974)**
9.1	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	**CROATINA (3251)**
9.2	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	**CROATINA (3251)**
9.3	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	**CROATINA (3251)**
10.1	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	**CROATINA (3251)**
10.2	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	**CROATINA (3251)**
10.3	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	**CROATINA (3251)**
11.1	139	151	234	242	239	239	239	249	176	190	234	236	240	240	188	194	247	247	**CABERNET S. (1929)**
11.2	139	151	234	242	239	239	239	249	176	190	234	236	240	240	188	194	247	247	**CABERNET S. (1929)**
11.3	139	151	234	242	239	239	239	249	176	190	234	236	240	240	188	194	247	247	**CABERNET S. (1929)**
12.1	133	143	238	242	247	249	249	255	186	190	234	258	252	272	196	200	243	245	**UVA RARA (12830)**
12.2	133	143	238	242	247	249	249	255	186	190	234	258	252	272	196	200	243	245	**UVA RARA (12830)**
12.3	133	143	238	242	247	249	249	255	186	190	234	258	252	272	196	200	243	245	**UVA RARA (12830)**
13.1	143	155	238	242	247	249	241	255	186	186	236	260	240	262	196	200	243	251	**VESPOLINA (13018)**
13.2	143	155	238	242	247	249	241	255	186	186	236	260	240	262	196	200	243	251	**VESPOLINA (13018)**
13.3	143	155	238	242	247	249	241	255	186	186	236	260	240	262	196	200	243	251	**VESPOLINA (13018)**

* the number in brackets, following the name of each variety, is the univocal code of each genotype in the VIVC database.

3.3.2 Musts

Considering the monovarietal PDO Pinot noir musts, samples were taken at the beginning, during and at the end of the fermentation process. Specifically, samples were collected at 24, 48, 72 and 120 hours, after delivery and immediate crushing, to assess the influence of vinification process on quantity and quality of DNA that can be recovered for traceability purposes (Table 2). Extracted DNAs were analysed by using nine pairs of microsatellite markers and it was successfully amplified in all the samples. The obtained allele profiles were consistent along the entire fermentation process resulting in a perfect match at the different sampling times with the expected genetic profile of Pinot noir (Table 6). In addition to fermentation, additional samples were collected after the most common oenological operations: racking (sample number 20) and tangential filtration (sample number 21); the expected genetic profile for Pinot noir was always obtained with a perfect correspondence (Table 6). At the end of these first analyses, it was observed that, although fermentation and oenological processes in general cause degradation and reduction of DNA, this does not affect the possible application of DNA analysis for traceability. Similar results, for monovarietal productions, were reported in the literature (Recupero et al., 2013; Bigliazzi et al., 2012; Boccacci et al., 2012; Siret et al., 2000). While in the regulation it is reported that *"up to a maximum of the 5% of different varieties can be present"* ("Pinot Nero dell'Oltrepò Pavese" DPR 06.08.1970 G.U.27.10.1970, https://www.politicheagricole.it), neither clear alleles nor very small signals different from Pinot noir were detected supporting the presence of other varieties. Recupero et al. (2013) reported that, in presence of mixtures 90% - 10% of two cultivars, the detection is possible and small peaks of the second variety can be detected. In the same study, the authors reported that in mixtures 95% - 5% just small and unclear signals of the minor cultivar can be detected. The result obtained in our work can be a consequence of the high abundance of Pinot noir DNA that can offset the possible presence of other minor varieties. Being other varieties possibly present in very small percentages, we cannot exclude that the absence of a corresponding signal can be a consequence of the DNA degradation during the fermentation or the impossibility to recover enough amplifiable DNA during the extraction.

These results suggest: 1) the percentage of Pinot noir is higher than the 95%, as expected from the regulation, but it is not possible to exclude that 2) other varieties

can be present with global percentage higher than what expected from the regulation but, if the single different variety is present in amount lower than the threshold detection limit (5% as reported in Recupero et al., 2013) it cannot be detected with this method. This is a clear consequence of working under uncontrolled conditions where it is not possible to have the exact knowledge of what enters the wine production chain.
Considering the production of TGI Rosso Oltrepò, two tanks (samples 17 and 18 respectively) were followed during the same fermentation stages and after the same oenological operations mentioned before for the monovarietal musts (Table 2).
We considered more than a single tank because, being the TGI a polyvarietal production, we could expect a higher variability in the obtained results and a lower capacity to recognize the different varieties present in the sample. Also in this case, as already reported for the monovarietal production, it was not possible to have the exact knowledge of the varieties, and of the relative percentages, entering the production. Clearly, with a wine that is declared as polyvarietal by the specification, the impossibility to have the exact knowledge of the variety delivered and the corresponding relative percentages make the possibility to trace more complicated. As expected, for each microsatellite, more than two alleles were usually detected being the sample mixtures of different varieties. In Table 6, for polyvarietal samples, the sum of the different genetic profiles obtained for each SSR marker is reported. No amplification differences were evidenced between the samples obtained from the two tanks supporting a good reproducibility of the DNA extraction and analysis from different samples of musts.
Starting from the raw genetic data reported in Table 6, the subsequent step was the reconstruction of the genetic profiles of the five expected varieties (Table 7).

Table 6. *Musts: results of microsatellite analysis. For the monovarietal Pinot noir (samples 16, 20, and 21) the expected diploid genetic profile and the corresponding VIVC variety and code are reported. For the polyvarietal TGI Rosso Oltrepò (samples 17, 18, and 22) and the thermovinified (sample 19) the sum of the genetic profiles obtained from the analysis at the Genetic Analyzer is reported. As expected more than two alleles were usually detected being these two productions made by mixing more varieties. The corresponding VIVC variety and code are not attributable.*

Samp.[a]	VVS2		VVMD5		VVMD7		VVMD25		VVMD27		VVMD28		VVMD32		VrZAG62		VrZAG79		Corresp. VIVC[b]
16.24	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	PINOT (9279)
16.48	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	PINOT (9279)
16.72	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	PINOT (9279)
16.EF	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	PINOT (9279)
20	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	PINOT (9279)
21	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245	PINOT (9279)
17.24	133, 135, 139, 143, 151		228, 238		239, 247, 249, 253		239, 249 255		186, 190, 195		234, 236, 258, 260		240, 252, 272		186, 192, 196, 200		243, 245, 247, 251, 259		/
17.48	133, 135, 139, 143, 151		228, 238		239, 247, 249, 253		239, 249 255		186, 190, 195		234, 236, 258, 260		240, 252, 272		186, 192, 196, 200		243, 245, 247, 251, 259		/
17.72	133, 135, 139, 143, 151		228, 238		239, 247, 249, 253		239, 249 255		186, 190, 195		234, 236, 258, 260		240, 252, 272		186, 192, 196, 200		243, 245, 247, 251, 259		/
17.EF	133, 135, 139, 143, 151		228, 238		239, 247, 249, 253		239, 249 255		186, 190, 195		234, 236, 258, 260		240, 252, 272		186, 192, 196, 200		243, 245, 247, 251, 259		/
18.24	133, 135, 139, 143, 151		228, 238		239, 247, 249, 253		239, 249 255		186, 190, 195		234, 236, 258, 260		240, 252, 272		186, 192, 196, 200		243, 245, 247, 251, 259		/
18.48	133, 135, 139, 143, 151		228, 238		239, 247, 249, 253		239, 249 255		186, 190, 195		234, 236, 258, 260		240, 252, 272		186, 192, 196, 200		243, 245, 247, 251, 259		/
18.72	133, 135, 139, 143, 151		228, 238		239, 247, 249, 253		239, 249 255		186, 190, 195		234, 236, 258, 260		240, 252, 272		186, 192, 196, 200		243, 245, 247, 251, 259		/

18.EF	133, 135, 139, 143, 151	228, 238	239, 247, 249, 253	239, 249 255	186, 190, 195	234, 236, 258, 260	240, 252, 272	186, 192, 196, 200	243, 245, 247, 251, 259	/
22	133, 135, 139, 143, 151	228, 238	239, 247, 249, 253	239, 249 255	186, 190, 195	234, 236, 258, 260	240, 252, 272	186, 192, 196, 200	243, 245, 247, 251, 259	/
19	135, 143	228, 238	247, 249	239, 249, 255	186, 190, 195	234, 258	252, 272		245	/

a) Samples are ordered primarily according to the monovarietal and polyvarietal origin and secondly according to the sampling date. The last sample is the thermovinified one.

b) The corresponding VIVC variety and code have been attributed just for the monovarietal samples and not for the others being the genetic profile the sum of different genetic profiles.

* the number in brackets, following the name of each variety, is the univocal code of each genotype in the VIVC database.

Table 7. *Genetic profiles of the expected varieties present in the polyvarietal musts, reconstructed by considering the results of the microsatellite analysis reported in Table 1. The corresponding VIVC code is reported. The alleles of the different varieties, expected but not amplified by the microsatellite markers, are underlined and reported in bold character.*

Samp.	VVS2		VVMD5		VVMD7		VVMD25		VVMD27		VVMD28		VVMD32		VrZAG62		VrZAG79		Corresp. VIVC
	133	135	228	228	249	253	239	255	186	190	234	260	252	272	192	200	243	259	BARBERA (974)
	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	CROATINA (3251)
17.24	133	143	238	**242**	247	249	249	255	186	190	234	258	252	272	196	200	243	245	UVA RARA (12830)
	143	155	238	**242**	247	249	249	255	186	186	236	260	240	**262**	196	200	243	251	VESPOLINA (13018)
	139	151	234	**242**	239	239	239	249	**176**	190	234	236	240	240	**188**	**194**	247	247	CABERNET S. (1929)
	133	135	228	228	249	253	239	255	186	190	234	260	252	272	192	200	243	259	BARBERA (974)
	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	CROATINA (3251)
17.48	133	143	238	**242**	247	249	249	255	186	190	234	258	252	272	196	200	243	245	UVA RARA (12830)
	143	**155**	238	**242**	247	249	249	255	186	186	236	260	240	**262**	196	200	243	251	VESPOLINA (13018)
	139	151	234	**242**	239	239	239	249	**176**	190	234	236	240	240	**188**	**194**	247	247	CABERNET S. (1929)
	133	135	228	228	249	253	239	255	186	190	234	260	252	272	192	200	243	259	BARBERA (974)
	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	CROATINA (3251)
17.72	133	143	238	**242**	247	249	249	255	186	190	234	258	252	272	196	200	243	245	UVA RARA (12830)
	143	**155**	238	**242**	247	249	249	255	186	186	236	260	240	**262**	196	200	243	251	VESPOLINA (13018)
	139	151	234	**242**	239	239	239	249	**176**	190	234	236	240	240	**188**	**194**	247	247	CABERNET S. (1929)
	133	135	228	228	249	253	239	255	186	190	234	260	252	272	192	200	243	259	BARBERA (974)
	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	CROATINA (3251)
18.24	133	143	238	**242**	247	249	249	255	186	190	234	258	252	272	196	200	243	245	UVA RARA (12830)
	143	**155**	238	**242**	247	249	249	255	186	186	236	260	240	**262**	196	200	243	251	VESPOLINA (13018)
	139	151	234	**242**	239	239	239	249	**176**	190	234	236	240	240	**188**	**194**	247	247	CABERNET S. (1929)
	133	135	228	228	249	253	239	255	186	190	234	260	252	272	192	200	243	259	BARBERA (974)
	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	CROATINA (3251)
18.48	133	143	238	**242**	247	249	249	255	186	190	234	258	252	272	196	200	243	245	UVA RARA (12830)

	143	**155**	238	**242**	247	249	249	255	186	186	236	260	240	**262**	196	200	243	251	VESPOLINA (13018)
	139	151	234	**242**	239	239	239	249	**176**	190	234	236	240	240	**188**	**194**	247	247	CABERNET S. (1929)
18.EF	133	135	228	228	249	253	239	255	186	190	234	260	252	272	192	200	243	259	BARBERA (974)
	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	CROATINA (3251)
	133	143	238	**242**	247	249	249	255	186	190	234	258	252	272	196	200	243	245	UVA RARA (12830)
	143	**155**	238	**242**	247	249	249	255	186	186	236	260	240	**262**	196	200	243	251	VESPOLINA (13018)
	139	151	234	**242**	239	239	239	249	**176**	190	234	236	240	240	**188**	**194**	247	247	CABERNET S. (1929)
22	133	135	228	228	249	253	239	255	186	190	234	260	252	272	192	200	243	259	BARBERA (974)
	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245	CROATINA (3251)
	133	143	238	**242**	247	249	249	255	186	190	234	258	252	272	196	200	243	245	UVA RARA (12830)
	143	**155**	238	**242**	247	249	249	255	186	186	236	260	240	**262**	196	200	243	251	VESPOLINA (13018)
	139	151	234	**242**	239	239	239	249	**176**	190	234	236	240	240	**188**	**194**	247	247	CABERNET S. (1929)
19	133	135	228	228	249	253	239	255	186	190	234	**260**	252	272	**192**	**200**	243	**259**	BARBERA (974)
	139	**151**	238	238	247	249	239	255	190	195	234	**236**	252	272	**186**	**196**	245	245	CROATINA (3251)
	133	143	238	238	247	249	249	255	186	190	234	258	252	272	**196**	**200**	243	245	UVA RARA (12830)

* the number in brackets, following the name of each variety, is the univocal code of each genotype in the VIVC database.

The complete genetic profiles for Barbera and Croatina were obtained, independently of the fermentation stage or the oenological operation. Despite the percentages in the TGI production are not defined in the specification, these results suggest that Barbera and Croatina are present in high percentages making possible the obtaining of the complete profile. This hypothesis can be supported by cultivation data, being these two varieties commonly present in high percentage in the Oltrepò Pavese area. Concerning the other three varieties, some alleles were missing, and it was not possible to obtain the complete genetic profile. A single allele, out of eighteen, was absent in Uva rara, three alleles were missing in Vespolina, and five alleles were missing in Cabernet Sauvignon. In Uva rara and Vespolina there are not SSRs completely failing the amplification but there is the presence of just one of the expected alleles and this, in our opinion, does not hamper the possibility to recognise them. The matter is different for Cabernet where two SSRs completely failed the amplification and no one of the expected alleles are present. This situation can make more difficult the recognising of this variety. These results can be compatible with the presence of Uva rara and Vespolina in percentages close the detection limit, that we consider equal to the 5% (as reported in Recupero et al., 2013) while Cabernet is likely present in lower amount. These three varieties are cultivated in lower amount than Croatina and Barbera in the Oltrepò area and this is compatible with the results of the SSR analysis. The absent alleles can be a consequence of: 1) nucleic acid degradation that is expected to influence more the DNA of the minor cultivars; 2) the impossibility to recover sufficient amounts of amplifiable DNA during the extraction; 3) the high abundance of Croatina and Barbera DNA that can offset the presence of the other varieties; 4) amplification problems associated to the presence of more alleles that each single SSR have to amplify.

It must be stated that the presence of common alleles between the most and the least abundant varieties could have influenced and made easier the inference of the different genetic profiles. As an example, with the marker VVMD7, the two alleles of 186 and 190 bp typical of Barbera were also present in the minor varieties Vespolina and Cabernet. It is not to be excluded that, in this and similar cases, what we detected were only the alleles of Barbera and not the ones of the other two cultivars. It could be interesting to follow a polyvarietal must made by mixing varieties with completely different genetic profiles to detect a minimum threshold of detection associated with the percentage of presence in the must. In previous studies (Faria et al., 2000) it was

found that, in artificial mixtures of up to five different varieties in known percentages, the level of detection of the less present varieties was the 10% in unfermented musts and the 30% in fermented musts. The case reported in this work is slightly different, in fact the mixture was not artificially made in the lab, but really produced in the winery and the percentages of the different varieties were unknown (even if, by experience, Barbera and Croatina are the most abundant while Cabernet and Vespolina the least present).

These results underline that also a polyvarietal must be followed with DNA analysis in a real case scenario, during the wine production process, as reported also in experimental conditions by Faria et al. (2000), with artificial blends obtained by mixing known percentages of up to five different varieties and by Garcia-Beneytez et al. (2002) with the same approach but considering only two varieties. As reported for the monovarietal production, also in this case it is not possible to exclude the presence of other varieties but, if present, they are in very low percentages below the detection limit.

Finally, in order to verify if thermal treatments can affect the quality of the recoverable DNA and its use for traceability, it was necessary collect different samples following the needs of the cooperative winery. This is not a commonly adopted procedure and both the monovarietal and the polyvarietal ones, previously reported, were not meant to be subjected to this treatment.

The Sangue di Giuda PDO is made by mixing three varieties (Barbera, Croatina, and Uva rara). After thermovinification, DNA was successfully extracted and amplified. The allele profiles were successfully interpreted giving correspondence to Barbera, Croatina, and Uva rara as shown in Table 6. The application of thermal and enzymatic treatments usually impairs DNA analysis (Piskata et al., 2019). However, in our case, the genetic material was still suitable for molecular traceability purposes. For the most common oenological operations, thermal treatment determines a lower amplifiability of the extracted DNA the complete genetic profile was never obtained but, at the same time, it was possible to amplify several markers sufficient to provide the correct cultivar attribution. Bigliazzi et al. (2012) reported that, because of the high degradation of DNA in wine, to positively associate a wine to the corresponding cultivars it is necessary to have a minimum of six informative loci (12 alleles out of 18), while nine markers (18 alleles out of 18) are confirmed for musts. We think that the DNA degradation determined by the thermal treatment can be similar to the

degradation present in a wine. Considering this, it is believed that 12 out of 18 alleles can be enough to positively determinate the correct cultivars.

3.3.3 Wines

Wine is the final product of a long production chain, that starts years prior in the vineyard, continues in the vinery and finishes in the cellar. Generally, wine is the most valuable product obtained from grapes and requires protection against misidentification and fraud.

In this work, wines were analysed at two important phases of their life: before bottling (the so-called pre-bottled samples), on the product without any oenological additives and on packaged products ready to be shelved. Four pre-bottled samples were considered corresponding to two samples of monovarietal PDO Pinot Noir and two samples of polyvarietal TGI Rosso Oltrepò. These wine samples correspond to the final products of the grapes and musts previously reported. Pre-bottled samples were collected after 4 months of storage in refrigerated tanks (15 – 18 °C), while bottled samples were collected 2 months after bottling.

In the first case, wines were previously clarified and filtered, using a tangential filter, to make it as clean as possible as well as microbiologically stable. After these steps, before bottling, there is the addiction of oenological additives, whenever necessary, to ensure proper maintenance of the product during storage.

The combination of several approaches to increase DNA recovery, like the use of 2-propanol, Na-acetate, long time cold-precipitation, and the addition of PVP (Soffritti et al., 2016; Savazzini and Martinelli, 2006) were applied to increase the possibilities to obtain a PCR grade DNA.

In wine, DNA is further degraded, as compared to the previous stages, because of degradation processes can continue during storage, reducing in this way the quantity and quality of DNA that can be recovered and amplified (Pereira et al., 2017; Savazzini and Martinelli, 2006; Garcia-Beneytez et al., 2002). For this reason, and the consequent greater difficulty in analysing wine DNA, the number of SSRs adequate to recognize grape varieties starting from wines is lowered from nine (as reported in the main databases) to six (Bigliazzi et al., 2012).

At both sampling points, DNA was more degraded as compared to grapes and must, as reported in the literature (Savazzini and Martinelli, 2006; Işçi et al., 2014) and as

evidence obtained from SSR profiles. Out of nine SSR markers tested on wines, markers VVMD25 and VVMD32 always failed to produce a legible profile, and sporadic amplifications were found also for VVMD5 and VrZAG79. In the remaining cases, the number of amplicons was higher but, while some alleles of the expected varieties were found, the results are not enough to make possible a certain and reproducible determination of the varieties (Table 8).

In this study, better results were obtained with bottled samples than with pre-bottled samples. In this study, better results were obtained with bottled samples than with pre-bottled samples. Both phases are strongly related to the high DNA degradation as a consequence of the prolonged storage time of wines.

It must be underlined that pre-bottled wines were sampled from tanks in which it was not possible to shake the content to uniform the product making. Therefore, it is very likely that the four samples were not fully representative of the real situation. These results suggest that storage represents a critical step in traceability and that it can be of interest to follow the degradation of DNA in the storage conditions by sampling wines at a different time during storage.

Table 8. *Wines: results of microsatellite analysis*

Samp.	VVS2	VVMD5	VVMD7	VVMD25	VVMD27	VVMD28	VVMD32	VrZAG62	VrZAG79	Corresp. VIVC
	139		247 249			234		196		**CROATINA (3251)**
	133 135		249		186	234			243	**BARBERA (974)**
1V	133 143		247 249		186	234 258		196	243	**UVA RARA (12830)**
	143		247 249		186 186			196	243	**VESPOLINA (13018)**
										CABERNET S. (1929)
2V		230			186			188 194		**PINOT (9279)**
3V		230	239 243		186			188 194		**PINOT (9279)**
	133 135					234				**BARBERA (974)**
	133 143		247			234 258		196		**UVA RARA (12830)**
4V	139		247			234		196		**CROATINA (3251)**
										VESPOLINA (13018)
		230						188 194		**CABERNET S. (1929)**
5V		230	239		186			194		**PINOT (9279)**
					195	234				**CROATINA (3251)**
	133 135				186	234				**BARBERA (974)**
6V	133 143				186	234 258				**UVA RARA (12830)**
										CABERNET S. (1929)
	143				186 186					**VESPOLINA (13018)**

4 Conclusions

The possibility to apply DNA analysis for wine traceability has been investigated in the last 20 years by several authors with interesting results. A possible limit to the application of these methods to real large-scale situation is that the majority of the papers retrievable from the scientific literature reported studies carried out in controlled conditions (laboratory scale by using wines obtained through micro vinification, by artificially mixing a known number of specific varieties and so on). Just few papers considered real cases but focused on medium-small productive realities. In the present paper, we wanted to test the applicability of DNA analysis on the real case of a very large productive cooperative winery (with nearly 700 hundreds of different cooperative member, associated farmers, and almost 4 million bottles made) where the possibility to control the whole wine chain production is extremely low or absent. Two productions, one monovarietal and one polyvarietal, were followed from the grape to the bottled wine in the season 2017-2018. Microsatellite analysis and cultivar identification were successfully carried out for the monovarietal product, from grape to the wines at the end of the most common oenological operations, even if the possible presence of other unknown varieties under the detection limit cannot be excluded. The situation is more complicated for the polyvarietal production, especially if the percentages of the varieties entering the production are unknown. Despite of this and despite the absence of some alleles, the expected varieties could be recognised without big difficulties. As for the monovarietal production, the presence of varieties in lowest amount cannot be detected. It is important to state that the analysis we carried out is not quantitative and quantification was not a goal of the study.

Classical oenological processes did not interfere with the possibility to recognize the cultivars, but the application of not common processes as thermovinification could increase DNA degradation, thereby influencing cultivar identification.

The situation is different concerning wines: after four months of storage and two months of bottling, the obtained genetic profile was clearly incomplete. This is likely the consequence of DNA degradation during the storage in the tanks or in the bottles. Therefore, the capacity of recognizing production cultivars was strongly reduced. Future studies can be carried out to survey the possibility to easily recognise the production cultivars in correlation with the storage time.

At the end, methods developed in controlled conditions can be transferred to large, uncontrolled, realities with good results. More difficulties can be present because of the impossibility to have the control over the grape in entrance and the production process.

Better results, also in the most critical steps, can be obtained, likely, by improving the DNA extraction methods available. Other possibilities are related to the development of markers specific for the single varieties. When the identification of the cultivars is carried out by using a multilocus genetic profile, it is more likely to observe the absence of amplification (especially in the case of wine) with respect to the use of one or two cultivar specific markers. Finally, because of DNA degradation, it can be expected that moving from SSR to SNP markers can increase traceability efforts.

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Chapter 2

Published article 2: *Effect of storage time on wine DNA assessed by SSR analysis*

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Effect of storage time on wine DNA assessed by SSR analysis

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ABSTRACT

Wine traceability based on DNA analysis has been reported in several previous studies but no one until now had monitored how far molecular traceability was possible during wine storage, before and after bottling. The present study tries to fill this gap of knowledge by following, in the real case of a large wine cooperative, the possibility to trace wine production through DNA analysis during storage period. Two monovarietal productions: red sparkling Bonarda PDO and white Pinot gris PDO, were followed starting from the end of oenological practices until 1 year after bottling. During the pre-bottling period, samples were collected every 10 days during four consecutive months, after bottling samples were collected at day 1 and after 2, 8 and 12 months. DNA analysis evidences that for both wines, traceability by applying SSR analysis on the extracted DNA is possible, at least, until month 8, after that DNA degradation increases hindering, mainly for the red wine, the possibility to a correct varietal identification.

Abstract

Wine traceability based on DNA analysis has been reported in several previous studies but no one until now had monitored how far molecular traceability was possible during wine storage, before and after bottling. The present study tries to fill this gap of knowledge by following, in the real case of a large wine cooperative, the possibility to trace wine production through DNA analysis during storage period. Two monovarietal PDO productions: red sparkling Bonarda and white Pinot gris, were followed starting from the end of oenological practices: samples were collected every 10 days during four consecutive pre-bottling months and at day 1 and after 2, 8 and 12 months after bottling.

DNA analysis evidence that for both wines, traceability by applying SSR analysis on the extracted DNA is possible, at least, until month 8, after that DNA degradation increases hindering, mainly for red wine, the possibility to a correct varietal identification.

Highlights

1. Evaluation of storage time on DNA traceability in wine production
2. DNA traceability is feasible until 8 months after bottling
3. DNA analysis on red wine is more challenging compared with white wine
4. Storage condition can influence quality and quantity of extracted DNA

Keywords: DNA traceability, wine shelf life, SSR, wine, storage condition, grape variety

1. Introduction

Wine quality is strongly influenced by several factors as the vinification process, the geographical origin of grapes and the varietal composition of the must. This last point is extremely important in the case of wines protected by Denomination of Origin (DO), where only a limited number of cultivars are allowed, and even more for monovarietal wines for which a major cultivar must be present in minimum percentages as regulated by the product specifications. In the case of wine, as well as for all food and beverage products of high value, the availability of analytical tools capable to assess the quality and to find any eventual adulterations is important. In DO wines, one of the most frequent frauds is represented by the use of cultivars different from those admitted in the specification (Pereira et al., 2012; Recupero et al., 2013; Scali et al., 2014).
Among the different analytical techniques, the possibility to trace wine productions by analysing DNA extracted from wines is a topic that has been addressed in many studies over nearly twenty years, from early reports (Faria et al., 2000; Woolfe and Primrose, 2004; Baleiras-Couto and Eiras-Dias, 2006) up to the most recent ones (Boccacci et al., 2020; Zambianchi et al., 2021).

Analysis of DNA extracted from wine and food matrices can be useful in establishing and enforcing labels, especially to protect designation and the final consumer from frauds (Busconi et al., 2003; Pafundo et al., 2007; Consolandi et al., 2008). The interest in DNA analyses is mainly because only DNA can give reliable information concerning the identity of the production cultivars, being DNA independent from environmental conditions. All the other approaches, being influenced by the environment, can be extremely useful in determining the geographic origin but not for the identification of the different varieties (Scarano et al., 2011).

DNA extraction is the most critical step for cultivar fingerprinting starting from wine In fact, to apply successfully DNA analysis through molecular markers, a good amount of high-quality DNA is extremely important. DNA from processed matrices, as wine, is usually highly degraded and the recovered amount is usually very low. In addition, during the storage period, several factors as light, temperature, and oxygen concentration might affect, other than wine quality as colour and scents, DNA yield (Scarano and Rao, 2014).

Further, polysaccharides, tannins, and polyphenols that are very abundant in grapes (Demeke and Adams,1992; Cabanis et al., 1999), fermentation itself and all the most common oenological processes (as fining and filtration) are all factors that can interfere with the quantity and quality of the extracted DNA. Despite this, even if in low concentration and highly degraded, DNA can be isolated from musts or wines and subsequently analysed to identify the variety, or varieties, used for wine production (Savazzini and Martinelli, 2006; Zambianchi et al., 2021). Other crucial phases for varietal identification from wines are storage conditions and aging time. In previous research, we reported that following the wine chain production from grape to the final bottled wine, microsatellite analysis and cultivar identification were successfully in particular for monovarietal products starting from grape delivery to the winery, through fermentation and during the most common oenological practises (racking, decanting, filtration) to the pre-bottling stage (Zambianchi et al., 2021). As reported in many other studies, DNA analysis of wine is more difficult and usually not obtainable or reliable (Garciaa-Beneytez et al., 2002; Siret et al., 2002; Catalano et al., 2016). In extra virgin olive oil, it was demonstrated that storage time could affect DNA quality and quantity and consequently the possibility to use nucleic acids for traceability purposes (Pafundo et al., 2010; Galimberti et al., 2013).

It is likely that storage time can similarly affect DNA parameters in wine. Considering this, the aims of this work were to evaluate the amplifiability and the utility of DNA extracted during wine storage in pre-bottling phase and in subsequent labelled bottle ready to be sold.

For traceability purposes, it is very important to evaluate how storage conditions, time and temperature, can impact on DNA quality and quantity in order to clarify how long it is possible to perform a varietal recognition based on DNA extraction and analysis from wine. This indication is of primary importance for control bodies involved in fraud prevention.

In wine literature, there are no similar DNA based works, but they are mainly focused on the optimization of other approaches (chemical, metabolic, biochemical) for traceability along the wine production chain against frauds.

The majority of available studies on the same topic, retrievable from scientific literature, was based on samples of wines obtained by micro or nano-vinification (Barriasa et al., 2019; Pereira et al., 2017; Pereira et al., 2012) eliminating various oenological practices (repeated racking, filtration, clarification, heat treatment) that may affect quantity and quality of DNA present in samples. Only few studies consider small or medium-size real production wineries (Catalano et al., 2016; di Rienzo et al., 2017; Boccacci et al., 2020).

Regarding this, it is our opinion that in order to validated and propose a method for traceability, is fundamental to verify its applicability in real operative conditions that are certainly less controlled, controllable and reproducible than at laboratory scale but because of this more significant.

Similarly, we think that focusing on a large production winery compared to small and medium-size realities can be more informative because in bigger wineries there is less capacity to control the whole production chain, particularly at grapes delivery.

In this research, as in our previous study (Zambianchi et al., 2021), samples were obtained from a large real case production winery, “Terre d’Oltrepò” (Oltrepò Pavese, Lombardy, Italy), focusing on two very important monovarietal wines for the landscape production of Lombardy: the white wine PDO Pinot gris and the red wine PDO sparkling Bonarda.

In order to evaluate the effects of storage conditions on the possibility to use wine DNA for traceability purposes, wine samples were collected every 10 days in pre-bottling stage during four consecutive months, at day one after bottling and after 2, 8

and 12 months from bottling. Wine DNA was analysed with SSR molecular markers and the obtained genetic profiles used to recognize the production cultivars.

2. Materials and methods

2.1Sample collection

Wine samples from vintage 2020 were collected at Terre d'Oltrepò winery to follow two important monovarietal designations in the Oltrepò Pavese area: Pinot gris PDO and sparking Bonarda PDO. Pinot gris PDO is made by Pinot gris variety, in the minimum of 85% while the other 15% could be covered by Pinot noir or other non-aromatic varieties allowed in Lombardy region as declared in corresponding specification ("Oltrepò Pavese Pinot gris" DPR 6.08.1970 G.U.27.10.1970, https://www.politicheagricole.it).

Sparkling Bonarda dell'Oltrepò Pavese PDO (from here Bonarda is made by Croatina variety, in the minimum of 85% and the resting 15% could covered by Barbera and/or Vespolina and/or Uva rara varieties as reported in corresponding specification ("Bonarda dell'Oltrepò Pavese" DPR 6.08.1970 G.U.27.10.1970, https://www.politicheagricole.it).

For this study, the same batches of Pinot gris PDO and Bonarda PDO were followed from filtration to bottled wines which were sampled at the wine shop of Terre d'Oltrepò winery.

The sample-set for this experiment is showed in Table 1 and consisted of:

1) Grape samples for both Pinot gris and Croatina (the grape variety used for Bonarda wine) from the same batches of origin of the wines. These two samples represent the reference samples to confirm the genetic profiles of wines.
2) Two samples of Pinot gris and two samples of Bonarda, suitable for yielding PDO Pinot gris and Bonarda, were collected after the most common oenological operations: racking (samples 1A and 1B) and tangential filtration (samples 2A and 2B).

3) Ten samples of PDO Pinot gris and ten sample of PDO Bonarda were collected during the storage period, before bottling, starting from 17th of November 2020 until the end of February 2021 with a sampling rate of 10 days.
4) Four samples of packaged wine at different time of packaging: a) one day (A13 for Bonarda PDO and B13 for Pinot gris PDO), b) 2 months (samples A14 for Bonarda PDO and B14 for Pinot gris PDO); c) 8 months (A15 for Bonarda PDO and B15 for Pinot gris PDO) and d) 1 year after bottling (A16 for Bonarda PDO and B15 for Pinot gris PDO). Bottles wines were kept on winery wine shop shelves.

In the case of PDO Bonarda the storage took place at room temperature (RT) in a cement tank. At the time of the first Bonarda sampling, in stocking period, wine was performing malolactic fermentation.
In the case of PDO Pinot gris, storage was in a refrigerated steel tank at 10°C. After collection, samples were always stored at -20°C until DNA extraction.
Three further reference standards (Pinot noir, Barbera, and Malvasia di Candia aromatica), belonging to the *Vitis vinifera* germplasm collection held at Università Cattolica del Sacro Cuore, Piacenza, were included in all PCR analyses to calibrate allele size of the selected SSR markers.
This allowed allele profile comparison between our samples and profiles of the Vitis International Variety Catalogue (VIVC database, (http://www.vivc.de).

Table 1. *Sampling scheme. Samples are marked with two different letters highlighting the different varieties considered: A identified Croatina variety for the production of PDO sparkling Bonarda while B identified Pinot gris variety for the homonym PDO.*

Sample	Data	Production phase	Variety	Wine	Sample	Data	Production phase	Variety	Wine
A0	15/09/2020	grape	Croatina	PDO Bonarda	**B0**	05/09/2020	grape	Pinot gris	PDO Pinot gris
A1	02/10/2020	racking	Croatina	PDO Bonarda	**B1**	18/09/2020	racking	Pinot gris	PDO Pinot gris
A2	12/10/2020	tangential filtration	Croatina	PDO Bonarda	**B2**	29/09/2020	tangential filtration	Pinot gris	PDO Pinot gris
A3	17/11/2020	storage	Croatina	PDO Bonarda	**B3**	17/11/2020	storage	Pinot gris	PDO Pinot gris
A4	27/11/2020	storage	Croatina	PDO Bonarda	**B4**	27/11/2020	storage	Pinot gris	PDO Pinot gris
A5	07/12/2020	storage	Croatina	PDO Bonarda	**B5**	07/12/2020	storage	Pinot gris	PDO Pinot gris
A6	14/12/2020	storage	Croatina	PDO Bonarda	**B6**	14/12/2020	storage	Pinot gris	PDO Pinot gris
A7	24/12/2020	storage	Croatina	PDO Bonarda	**B7**	24/12/2020	storage	Pinot gris	PDO Pinot gris
A8	03/01/2021	storage	Croatina	PDO Bonarda	**B8**	03/01/2021	storage	Pinot gris	PDO Pinot gris
A9	13/01/2021	storage	Croatina	PDO Bonarda	**B9**	13/01/2021	storage	Pinot gris	PDO Pinot gris
A10	23/01/2021	storage	Croatina	PDO Bonarda	**B10**	23/01/2021	storage	Pinot gris	PDO Pinot gris
A11	02/02/2021	storage	Croatina	PDO Bonarda	**B11**	02/02/2021	storage	Pinot gris	PDO Pinot gris
A12	12/02/2021	storage	Croatina	PDO Bonarda	**B12**	12/02/2021	storage	Pinot gris	PDO Pinot gris
A13	24/02/2021	bottled wine day 1	Croatina	PDO Bonarda	**B13**	24/02/2021	bottled wine day 1	Pinot gris	PDO Pinot gris
A14	26/04/2021	bottled wine month 2	Croatina	PDO Bonarda	**B14**	26/04/2021	bottled wine month 2	Pinot gris	PDO Pinot gris
A15	28/10/2021	bottled wine month 8	Croatina	PDO Bonarda	**B15**	28/10/2021	bottled wine month 8	Pinot gris	PDO Pinot gris
A16	22/02/2022	bottled wine year 1	Croatina	PDO Bonarda	**B16**	22/02/2022	bottled wine year 1	Pinot gris	PDO Pinot gris

2.2 Sample preparation and DNA extraction

Wine: 240 ml of wine were mixed to one volume of isopropanol and 0.3 volumes of sodium acetate 3M pH 5.2. After several inversion, the mixture was divided into 50 ml aliquots in sterile tubes, stored at -20°C for at least 2 days.

Samples were then centrifuged for a minimum of 3 hours at 4°C at 8,500 rpm. The supernatant was removed, the pellet was resuspended in 500 µl isopropanol and transferred to a 1.5 ml sterile tube and further frozen at -20°C for 2 days to further aid DNA precipitation.

Samples were then centrifuged at 4°C and 14000 rpm for 1 hour, the supernatant was removed, and the pellet was used for DNA extraction.

DNA extraction from wines, at different times of storage, was carried out according to Zambianchi et al. (2021), following the indications of GenElute DNA Miniprep Kit (SIGMA-Aldrich). This protocol was adopted also for the extraction of standard varieties.

2.3 Evaluation of wine DNA and SSR analysis

Data about DNA quantity and quality, reported in Table 2, were evaluated by measuring absorbance ratios (A260/A280, A260/A230) with a nanophotometer (NanoPhotometer® NP80, IMPLEN) according to the manufacturer's instructions.

Microsatellite analyses were carried out by using the following nine markers (VVS2, VVMD5, VVMD7, VVMD25, VVMD27, VVMD28, VVMD32, VrZAG62, and VrZAG79) (Sefc et al., 1999; Thomas and Scott, 1993; Bowers et al., 1996) selected among those reported by the main database for grape varietal comparison (VIVC http://www.vivc.de/).

PCR mixtures were set up starting from 2 µl of template, PCR reactions were carried out by following the protocol adopted by Zambianchi et al. (2021).

Amplified products from selective amplifications were loaded and run on the automatic sequencers ABI PRISM 3130 Genetic Analyzer (Applied Biosystem) according to manufacturer’s instructions and manually scored.

Table 2. *Spectrophotometer quantification of analysed samples and purity ratios.*

Sample	ng/µl	A260/A280	A260/A230	Sample	ng/µl	A260/A280	A260/A230
A0	27,14	1,283	0,35	**B0**	25,2	1,285	0,284
A1	11,87	1,348	0,046	**B1**	5,64	1,375	0,029
A2	8,21	1,314	0,064	**B2**	5	1,195	0,149
A3	4,5	1,371	0,047	**B3**	7,55	1,378	0,033
A4	5,9	1,543	0,034	**B4**	3,62	1,163	0,157
A5	7,3	1,628	0,035	**B5**	4,46	1,198	0,088
A6	2,25	1,569	0,052	**B6**	11,25	1,291	0,039
A7	11,98	1,476	0,079	**B7**	10,51	1,274	0,241
A8	7,12	1,448	0,052	**B8**	10,83	1,349	0,194
A9	7,73	1,431	0,028	**B9**	6,05	1,347	0,037
A10	7,21	1,423	0,033	**B10**	7,22	1,355	0,021
A11	7,12	1,428	0,065	**B11**	5,26	1,125	0,129
A12	6,21	1,523	0,079	**B12**	8,23	1,214	0,132
A13	6,71	1,539	0,024	**B13**	9,21	1,372	0,252
A14	5,16	1,343	0,084	**B14**	9,23	1,278	0,131
A15	7,21	1,344	0,034	**B15**	6,63	1,212	0,457
A16	3,25	1,239	0,012	**B16**	5,51	1,324	0,341

3. Results and discussion

Wine is the most valuable product obtained from grapes and requires protection against misidentification and fraud.

The possibility to apply DNA analysis for wine traceability has been investigated in the last 20 years by several authors with interesting results (Siret et al., 2002; Catalano et al., 2016; Agrimonti et al., 2018; Boccacci et al., 2020; Zambianchi et al., 2021). The majority of the scientific literature reports studies carried out in controlled conditions (laboratory scale by using wines obtained through micro vinification, by artificially mixing of known number of specific varieties and so on). Just a few papers considered real cases focusing on medium-small productive realities. In our previous work (Zambianchi et al., 2021), we demonstrated, in the real case of a large winery (with nearly 700 associated cooperative farmers conferring more than 50,000 tons of grapes and producing more than 4 million bottles), that wine traceability during vinification is feasible at least until the end of fermentation process independently of the applied oenological practices. On the contrary, no clear indications were obtained

concerning the last phases of the production chain corresponding to the pre- and after-bottling phases. In these phases, PCR amplification was frequently unsuccessful, with several markers failing the amplification, and not reliable, being amplification occasionally present in different DNA extractions from the same matrix. These results were in agreement with what previously reported in scientific literature (Garcia-Beneytez et al., 2002, Catalano et al., 2016). At the end of that work, it was also possible to see that, as expected, traceability for monovarietal productions, in presence of a major variety, was far easier than for polyvarietal productions. Trying to gain more information concerning the possibility to trace wine DNA also at the end of the production process, both before and after the bottling phase, we planned the present experiment. The main idea was to investigate the effect of storage time, in the standard storage conditions of the winery, on the possibility to recover wine DNA for traceability purposes. Two monovarietal PDO productions a white wine (Pinot gris PDO) and a red wine (Bonarda PDO) were chosen. Based on the specification of the two wines the major varieties must be present with a minimum percentage of 85%. For this experiment, in agreement with the winery staff, we were able to follow two batches made by the two varieties of interest: Pinot gris and Croatina. To see the effect of storage time on wine DNA, samples were taken, starting after the main oenological practices, every 10 days in the pre-bottling, at day one after bottling and finally, after 2, 8 and 12 months from bottling. After bottling, wines were stored at the RT conditions of the storeroom and of the wine shop of the winery. The adopted sampling scheme is represented in Table 1.

Extracted DNA evaluation is reported in Table 2. As expected, the purity of the genetic material, evaluated by spectrophotometric analyses, was very low with both the absorbance ratios far below the optimal range, in complete agreement with what reported in previous papers (Siret et al., 2002; Catalano et al., 2016; Zambianchi et al., 2021).

Because of these low purity levels, quantification could not be considered reliable and the estimated DNA quantities could not be considered as reliable witnesses of the real amount of genetic material present in the extraction product. As confirm of this observation, the presence of DNA in the extraction products was not visible on agarose gel electrophoresis suggesting the presence of very low amount of highly degraded DNA.

Degradation could be a consequence of fermentation, oenological practices and storage. Beside DNA concentration and degradation, in the case of wine, a major problem is represented by the possible interference of polyphenols, tannins, and polysaccharides present in the matrix on the possibility to use wine DNA in PCR reactions (Savazzini and Martinelli, 2006; Işçi et al., 2014).

Comparing samples of Bonarda and Pinot gris (samples A and B respectively in Table 3 and 4) it was possible to see that A260/A230 for Bonarda was on average lower than for Pinot gris (average values of 0.048 for Bonarda and 0.152 for Pinot gris).

This difference could be a consequence of the different metabolic composition of the wines, being Bonarda a highly pigmented red wine while Pinot gris was obtained by white vinification. Starting with a very low-quality DNA, amplification tests could be important to evaluate a possible inhibitory effect of contaminants on DNA amplifiability through PCR reactions. In scientific literature is clearly reported that amplification is possible despite the low-quality parameters. In the present case, an amplification test was carried out successfully for all DNA extracts (data not shown).

SSR analysis was performed using the same markers used in Zambianchi et al. (2021). It is important to precise that: 1) SSR analysis is a qualitative and not a quantitative assay, they can be applied for recognising the varieties but not for making evaluation about the amount of the varieties in a wine; 2) SSR analysis cannot be used for the determination of the geographic origin being DNA independent of the environmental conditions.

Nine SSR markers were used because they corresponded to the minimum set of markers necessary for the identification of grape varieties approved in the frame of two European projects, Genres081 and GrapeGen06 (vivc.de) and recognised by OIV (International Organisation of Vine and Wine). It is important to precise that, because of the low quality of wine DNA (Savazzini and Martinelli, 2006; Garcia-Beneytez et al., 2002; Pereira et al., 2017; Piskata et al., 2019), it was suggested that the number of SSRs needed for an adequate identification of the production varieties in wine could be lowered from nine to six (Bigliazzi et al., 2012): considering that a perfect correspondence among wine and leaf DNA at the level of six SSRs is enough for cultivar attribution. The results of SSR analysis are reported in Tables 3 (Croatina) and 4 (Pinot gris).

Table 3. *Wine: results of microsatellite analysis of Bonarda PDO. The cultivar genetic profile is that reported in the VIVC database.*

	VVS2		***VVMD5***		***VVMD7***		***VVMD25***		***VVMD27***		***VVMD28***		***VVMD32***		***VrZAG62***		***VrZAG79***	
VIVC Genetic Profile	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245
A0	139	151	238	238	247	249	239	249	190	195	234	236	252	272	186	196	245	245
A1	139	151	N.A.	N.A.	247	249	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A2	139	151	N.A.	N.A.	247	249	239	249	190	195	234	236	N.A.	N.A.	N.A.	N.A.	245	245
A3	139	151	N.A.	N.A.	247	249	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A4	139	151	N.A.	N.A.	247	249	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A5	139	151	N.A.	N.A.	247	249	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A6	N.A.	N.A.	N.A.	N.A.	247	249	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A7	139	151	N.A.	N.A.	247	249	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A8	139	151	N.A.	N.A.	N.A.	N.A.	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A9	139	151	N.A.	N.A.	N.A.	N.A.	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A10	139	151	N.A.	N.A.	247	249	N.A.	N.A.	190	195	234	236	N.A.	N.A.	186	196	245	245
A11	139	151	N.A.	N.A.	247	249	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A12	139	151	N.A.	N.A.	247	249	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A13	N.A.	N.A.	N.A.	N.A.	247	249	239	249	190	195	234	236	N.A.	N.A.	186	196	245	245
A14	139	151	N.A.	N.A.	247	249	239	249	N.A.	N.A.	234	236	N.A.	N.A.	186	196	245	245
A15	139	151	N.A.	N.A.	247	249	239	N.A.	N.A.	N.A.	234	236	N.A.	N.A.	186	196	245	245
A16	139	151	N.A.	N.A.	247	249	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	186	196	N.A.	N.A.

Table 4. *Wine: results of microsatellite analysis of Pinot gris PDO. The cultivar genetic profile is that reported in the VIVC database.*

	VVS2		**VVMD5**		**VVMD7**		**VVMD25**		**VVMD27**		**VVMD28**		**VVMD32**		**VrZAG62**		**VrZAG79**	
VIVC Genetic Profile	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B0	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B1	N.A.	N.A.	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B2	137	151	N.A.	N.A.	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B3	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B4	137	151	N.A.	N.A.	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B5	N.A.	N.A.	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B6	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B7	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B8	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B9	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B10	N.A.	N.A.	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B11	137	151	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B12	N.A.	N.A.	230	240	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B13	137	151	N.A.	N.A.	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B14	137	151	N.A.	N.A.	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B15	137	151	N.A.	N.A.	239	243	239	249	186	190	218	236	240	272	188	194	239	245
B16	N.A.	N.A.	N.A.	N.A.	239	243	239	249	186	190	N.A.	N.A.	N.A.	N.A.	188	194	239	245

Before analysing wines, we carried out the analysis of two samples of grapes, one for Croatina and one for Pinot gris to confirm: 1) that all markers were working correctly and 2) that grapes matched without any doubt to the respective varietal assignation. Analysed grapes were sampled from the same batches of origin of wines.

As a general observation concerning wines, in all the cases both for Pinot gris and Croatina, only the expected alleles of the two varieties were detected confirming that only the expected varieties were present in the two batches and supporting the absence of other contaminating varieties. Considering samples, A (Table 3), corresponding to Bonarda wine, out of the 9 SSR markers microsatellites VVMD5 and VVMD32 always failed to produce a profile, with the only exception of grape DNA, in all the samples considered, from racking to 1-year bottles. This result was somehow strange considering that the same two markers successfully amplified on the majority of Pinot gris samples, both grape and wine (Table 4). Concerning this point, we can try to explain by speculating that these two markers amplify DNA regions representing hot spots of fragility of DNA double helix. Supporting this speculation, VVMD5 failed to amplify also in six samples of Pinot gris and notably in all the DNA recovered from bottled wines, so from the samples stored for the longer period. On the contrary, all the other markers amplified successfully producing, almost all the time, a genetic profile perfectly matching the expected Croatina. In the case of sample A15 (8 months after bottling), with marker VVMD25, we observed the amplification of just a single allele. In all other samples and until 2 months after bottling, the complete genetic profile with six or seven SSR markers was detected making possible cultivar attribution and traceability.

The situation was different at 1 year after bottling. At this time-point the amplification was successfully only for three SSRs: VVS2, VVMD7 and VrZAG62.

Thus, if we consider the amplification of six markers as the threshold for varietal recognition in wine, we can assume with certainty that all the samples are attributable to Croatina variety until 2 months after bottling. At month 8, 5 SSR amplified perfectly while the sixth SSR (VVMD25) amplified correctly just one of the expected alleles. Considering this, sample A15 was just slightly below the threshold of the six markers but we believed that also the genetic profile at month 8 could be enough for variety identification. After this time, the lack of amplification impairs varietal recognition in 1 year bottled wine.

To support this, and to confirm that five or six markers instead of nine can be enough for production cultivar attribution, we used the genetic profile obtained for the sample A15 to query the VIVC database, accessed in date 18/02/2022. In doing this, we obtained correspondence only with Croatina and her synonyms; no other varieties included in the database had the same genetic profile with the six amplified markers. Currently, in the database, 5716 genetic profiles corresponding to different varieties are registered.

In Pinot gris wine, all the markers provided good amplification and detectable allele profiles with the exception of markers VVS2 and VVMD5 that only occasionally failed the amplification (Table 4) and, across the entire storing time considered, the number of positively amplified markers ranged from eight to nine ensuring the possibility to correctly recognise the cultivar. The genetic profile of sample B15, based on eight SSR markers, was used to query the VIVC database obtaining perfect correspondence with Pinot gris and with other varieties of the Pinot family, including Pinot noir that is likely the founder of the Pinot varieties complex. At one year after bottling five SSR successfully amplified out of the six required for wine identification: VVMD7, VVMD25, VVMD27, VrZAG62 and VrZAG79. Although the number of positive amplifications was higher in Pinot gris wine with respect to Bonarda wine, five SSR are below the threshold of six SSRs proposed by Bigliazzi et al. (2012) so, likely, not sufficient to identify variety. Despite of this, the 5 SSR genetic profile of sample B16 was used to query the VIVC database and, again, we obtained the same correspondences previously reported for sample B15. These results indicates that DNA based traceability is surely possible until 8 months after bottling but, also after one year we cannot exclude the possibility of identification. Tentatively, we can propose, in this case, ten months as a possible time limit for cultivar identification.

In general, considering data reported in Table 2, and with differences among wines, the storage period seems not to negatively affect, at least in the short, the possibility to extract amplifiable DNA even if quality parameters are generally low. The lack of amplification of some microsatellites (Table 3 and 4), mostly in pre bottling stages, may have been due to a consequence of the high DNA degradation of wines that make the results of analyses strongly dependent on the sampling phase and by the availability of homogeneous sample. It must be underlined that stored wines were sampled from tanks of over 100,000 L of volume where shaking and mixing are almost impossible. In the case of finished bottled, wine traceability success seemed to be strongly related

to the high DNA degradation of wine and to the time lapse between bottling and analysis. For both Bonarda and Pinot gris wine varietal recognition is possible to at least 8 months after bottling. In both cases, we showed that, also in the most advanced phases of the wine production process, it was still possible to recover DNA from monovarietal wines and to carry out DNA analysis for traceability.

The critical period for DNA based traceability in wine is in the interval between 8 months and 1 year from bottling reducing more, in the case of Bonarda, or less, in the case of Pinot gris, strongly the possibilities to identify the corresponding varieties.

From these results, while traceability is possible for both wines, it appears that amplification of the DNA from a white wine can be easier and more efficient than the amplification of DNA from a red wine. A possible explanation of this difference can be correlated with the different metabolic composition of the two wines. In case of PDO sparkling Bonarda, it should be considered that Croatina grapes are very rich in tannins, phenolic compounds and anthocyanins that are then found in red wines. These components are, at the same time, peculiar characteristics of the wine but strong known inhibitors of the subsequent PCR analysis (Do and Adams, 1991). On the contrary, regarding Pinot gris, white vinification process helps to store less quantity of such molecules typically present in red wines. This can be a crucial point that can make easier and more efficient the DNA extraction from white wines than from red wines and the subsequent analysis. Another possible explanation can take in consideration the different storing conditions of the two wines before bottling. Pinot gris is stored at controlled temperature, in refrigerate tanks, to preserve taste and colour. This procedure is not applied in the case of red wine that it is usually stored at room temperature. Red wine is considered as a more robust wine respect to white wine because of the presence of antioxidants, natural compound extracted during the maceration process of red vinification. Low temperatures may have improved the preservation of DNA during storage thus making more efficient the subsequent analysis. For the future, it could be interesting to see the effects also of temperature, in addition of time, to the possibility to maintain DNA in wine for traceability purposes. Finally, in the case of sparkling wines as Bonarda PDO, where 5 g/l of carbonic dioxide are present in accordance with the current regulation, the presence or addition of carbonic dioxide could be another factor influencing the stability and/or possibility to recover DNA from wines. Further investigations may be necessary to evaluate also this aspect and its relevance on wine traceability using molecular markers.

4. Conclusions

Determining for how much time it is possible to carry out wine traceability after the vinification process can be of extreme interest for control bodies in order to prevent or to detect possible frauds. That traceability is possible until the end of vinification is known while less certainty have been obtained for the subsequent steps during the storage period, pre- and after- bottling, when the majority of papers reported a high difficulty or impossibility to successfully analyse wine DNA. We tried to add something to this topic by analysing the possibility to recover and use wine DNA for traceability purposes during the storage time. All the samples were collected from the same batches starting from the end of the oenological operations until 1 year after bottling. Two monovarietal wines were followed, Pinot gris PDO and sparkling Bonarda PDO from the harvest 2020. Obtained results demonstrated that a reliable SSR profile can be detected, in particular for the white vinified Pinot gris for which a complete genetic profile based on 8 – 9 microsatellites were generated all the times, at least until month 8 after bottling. For the red wine, usually, we had genetic profiles with 6 – 7 markers. These results suggested that white wines could be analysed easier than red wines. Possible explanations are the different metabolic composition of the wines that can interfere with DNA extraction and analysis, the different storage conditions of the two wines or the combination and interaction of both factors. In both cases, it was possible to recognise univocally the production cultivar in the majority of the considered sampling points through storage to bottles and that traceability is feasible, in monovarietal wines, at least until 8 months in standard condition preserved bottles. At the last sampling point, 1 year after bottling, the obtained alleles, despite being below a proposed unofficial threshold, were still sufficient to proceed with varietal recognition in the case of Pinot gris. Further investigations considering a higher number of monovarietal wines sampled in the frame 8-12 months after bottling may be useful to establish a time threshold of DNA traceability in wine making. As a natural prosecution, in the future, it would be interesting to repeat the same experiment using polyvarietal wines to investigate the possibility to detect the different varieties used in the blend.

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Chapter 3

Published article 3: Metagenomic bacterial diversity and metabolomics profiling of Buttafuoco wine production

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Metagenomic bacterial diversity and metabolomics profiling of Buttafuoco wine production

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Abstract

Buttafuoco dell'Oltrepo' Pavese (or Buttafuoco) is an important and renowned red wine, protected by a Denominazione di Origine Controllata (DOC) designation established in 2010, produced in the Northern Italy in the province of Pavia (Italy). The knowledge of factors as the typical microbial terroir and the metabolite composition of the wine is fundamental for producing excellent wines. In this work, two productions of Buttafuoco Storico dell'Oltrepò Pavese were followed in order to assess the microbial populations through different stages of the wine production chain and the metabolomic composition of the final wines.

Microbial terroir was investigated through a metagenomic analysis that revealed a wide microbial consortium which is, for the major taxonomic groups, affected by sampling time over location. Before the metagenomic analysis, being DNA extraction from wine a difficult task, two different approaches were compared for a precise quantification of microbial DNA (bacteria and yeast): digital and real time PCR. Obtained results clearly evidenced that digital PCR being was more sensitive than real

time PCR and likely the method of choice for quantifying DNA extracted from processed matrices.
Metabolomic profiling, focused on phenolic compounds, was able to clearly distinguish among vineyards and to highlight the presence of discriminating molecules that can be related to the different edaphic conditions.

Highlights

1. Quantification DNA fraction from yeast and bacteria by RT-qPCR and ddPCR.
2. Metagenomic analysis: interaction of microbial communities along wine production chain.
3. Metabolomic analysis: studying of metabolic profile in relation to the geographic origin.

Keywords: metagenomics, microbial terroir, metabolomics, DNA quantification, wine traceability

1. Introduction

Grapevine is one of the most widespread crops in the world, and wine production is a global billion-dollar industry. Wine characteristics are regionally distinguished, and this is particularly true in the case of rich Italian wine scenario (Bubeck et al., 2020).
Wine quality and market value are not only related to local oenological tradition, to oenologist skills or to the grapevine varieties used, but an important role is played by terroir, considered as the influence of environment, soil and climate (terroir) and as a set of microbial populations that colonize the grape, must, wine and cellar (microbial terroir). These aspects confirm the importance of studying terroir of wine to understand how it can affect the quality of wine in the finished bottle as it has also been demonstrated by several studies about ecological regional compositions and microbial community (Pinto et al., 2015; Liu et al., 2017; De Filippis et al., 2019; Bubeck et al., 2020). For this reason, gathering knowledge about, among the others, the microbial ecosystem can be useful to understand how microbiome changes along the wine chain production and how it is able to influence wine flavour, aroma and overall quality in

either a positive or negative way (Van Leeuwen and Seguin, 2006; Bokulich et al., 2014).

Due to its complexity, wine chain production system is considered as a particularly interesting model to understand interactions between different microorganisms (Liu et al., 2017): the first important exchange occurs in the vineyard where grapes grow and develop. Interactions continue and change according to the different manufacturing stages and oenological processes such as alcoholic fermentation by yeasts (Ciani et al., 2010) and malolactic fermentation by lactic acid bacteria (Alexandre et al., 2004). The metagenomics analyses of microbial community are based on the possibility to recover DNA from musts and wines (Zambianchi et al., 2021; 2022). Metagenomics can be defined as the genetic analysis of microbial genomes contained with a particular sample (Thomas et al., 2012). As demonstrated in previous research, wine matrix represents analytical criticalities related to DNA extraction phase in which it is not easily recoverable a good amount of high-quality DNA (Zambianchi et al., 2021, Scarano et al., 2011) necessary prerequisite for any future omics approaches. To carry out a metagenomics analysis, it is important to have an idea of the microbial fraction DNA present in the global extraction product. Quantification approaches could be made by following classical methods based on RT-qPCR (Whale et al., 2012; Hindson et al., 2013), or more innovative approaches based on dd-PCR (Hindson et al., 2011; Taylor et al., 2015), in order to assess DNA fraction from yeast and bacteria.

In fact, to successfully apply further analysis, as metagenomic approaches, a good amount of high-quality DNA is extremely important. Metagenomics studies have been successfully applied in different wine samples as reported in different research (Portillo et al., 2016; Marasco et al. 2018; Mezzasalma et al., 2018).

Metabolomics is defined as a comprehensive analysis of the metabolites that are present in a biological sample. In recent years, the application of metabolomics approaches has allowed focusing on the metabolome of grape, must and wine, representing, in this way, an interesting methodology for investigating the metabolic composition of wines of different origin (Lopez-Rituerto et al., 2012; Rocchetti et al., 2018). The main objective of these analyses is to obtain qualitative and semi-quantitative information and to compare changes in metabolites patterns. The obtained results, coupled with multivariate techniques of analyses are useful for the classification of wines based on the origin and composition (Roullier-Gall et al., 2014; Amargianitaki and Spyros, 2017). Among the different classes of secondary

metabolites, the phenolic classes of anthocyanin, flavonol and stilbene derivatives are present in high abundance in grape, must and wine (Flamini et al., 2013). Phenolic composition is strongly affected by several factors as the variety, the environment, the cultural practices and so on (Bavaresco et al., 2008, Lopez-Rituerto et al., 2012; Rocchetti et al., 2018) making this class of molecules attractive for geographical identification.

Buttafuoco dell'Oltrepo' Pavese (or Buttafuoco) is a very important and renowned red wine, made by a blend of four different grape varieties (mainly Croatina and Barbera, with lesser amount of Uva rara and Ughetta di Canneto) protected by a DOC designation established in 2010.

The goal of the work is to get a global idea on Buttafuoco wine production by applying different analytical approaches based on metagenomics and metabolomics. A metagenomic analysis of the microbiota of musts (at various stages of processing) and wines produced under different cultural regimes was conducted. Two productions of Buttafuoco were followed in order to assess what kind of interaction can occur between the plant, its product and the microbial populations that colonize it. This was made trying to understand dynamics of microbial communities across the entire wine production chain starting from the must to the final bottle and to reveal the biogeographic distribution of these two Buttafuoco Storico productions. These two products fall under the same designation of production but come from two subareas with different terroir that can be reflected in the metabolic composition.

To get a complete picture of the productions, a metabolomic analysis was carried out on the Buttafuoco wine samples in order to assess how the metabolic profile changed in relation to the geographic origin.

2. Materials and Methods

2.1Sample collection

Must and wine samples from vintage 2020 were collected in two important wineries pertaining to "Consorzio Club del Buttafuoco Storico": organic Vigna Pregana (Pregana) and Vigna Bricco Versira (Bricco). The sample-set for the metagenomics and metabolomic experiments is showed in (Table 1) and consisted of:

1 must samples of Pregana and Bricco pulled out at 24 hours after inoculating yeasts and after 72 hours during alcoholic fermentation. An additional sample for Bricco was collected at post fermentation (PF). Must samples were immediately frozen at -20°C until further analysis.

2 wine samples of bottled Pregana and Bricco. Both wines underwent the most common oenological practises before bottling.

In Pregana production, the maceration and vinification process took place in steel tank at controlled temperature. In the case of Bricco the phases of maceration and vinification were conducted in glass resin. After collection, samples were always stored at -20°C until DNA extraction.

Table 1. *Sampling scheme: list of samples with their name, type of matrices, date and stage of sampling.*

Sample	Date	Sampling time	Type
Pregana	13/10/2020	24 h	Must
Pregana	16/10/2020	72 h	Must
Pregana	09/12/2020	wine	Wine
Bricco	16/10/2020	24 h	Must
Bricco	19/10/2020	72 h	Must
Bricco	23/11/2020	Post fermentation	Wine
Bricco	03/12/2020	wine	Wine

2.2DNA extraction and sequencing

2.2.1 Sample preparation

Must and wine (480 ml) preparation, before DNA extraction, was carried out according to Zambianchi et al. (2021). After the procedure, the recovered pellet was removed, stored at – 20°C until the subsequent steps.

2.2.2 DNA extraction

Genomic DNA was extracted from must and wine by using GenElute DNA Miniprep Kit (SIGMA-Aldrich) with some modifications regarding manufacturer's instructions

as reported in Zambianchi et al. (2021), and an additional phase with FastPrep®-24 Instrument following the manufacturer's instructions. DNA integrity was checked by agarose gel electrophoresis and DNA quantity measured by Qubit H dsDNA fluorescence assay (Life Technologies, Carlsbad, CA, USA).

2.2.3 Real-Time PCR Analysis

Real-time PCR (RT-qPCR) analysis was performed to quantify yeast and lactic acid bacteria (LAB) DNA. Genomic DNA samples were all diluted 1:10 in nuclease-free water and amplification was carried out using 5 µL of 1:10 diluted genomic DNA. The reaction mix was prepared using 10 µl of KAPA SYBR® FAST qPCR Master Mix (2X) Kit (Kapa Biosystems, Wilmington, MA, USA), 0.8 µl of 10 µM Forward/Reverse primer mix, and DNase free-water in order to obtain a final volume of 20 µl. The analysis was performed using the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific) according to the following thermal cycle: PCR initial activation step for 5 min at 95˚C, denaturation for 30 sec at 95˚C, annealing for 60 sec at 60˚C and extension for 5 min at 72˚C (40 cycles). Each sample was analysed in triplicate. The utilized primers are reported in Table 2.

2.2.4 Digital PCR Analysis

To increase the possibility of quantifying samples with very low amount of DNA, digital PCR methodology was applied. The sensitivity of RT-QPCR technology was compared with the sensitivity of droplet digital PCR (ddPCR) by using the same primers for analysing musts and wines of Buttafuoco Storico production.

Due to the higher sensitivity of the ddPCR, DNA was diluted 1:100 and 1:1000 before amplification.

Then, water-in-oil emulsion droplets are generated using a microfluidic chip with crossing channels. The generated droplets with a diameter between 90 and 120 µm may contain zero, one, or multiple target molecules. The distribution of the targets in the partitions follows a Poisson distribution. The droplets are subsequently heated by a thermal cycler for amplification (Hindson et al., 2013).

Digital PCR amplification were performed using 5 µL of 1:100 and 1:1000 diluted genomic DNA and the reaction mix was prepared by using 11 µl of 2X QX200™ ddPCR™ EvaGreen Supermix (cat. no. 1864034; Bio-Rad Laboratories, Inc.), 0.44 µl

of 10 µM Forward/Reverse primer mix (Table 2), and DNase free-water in order to obtain a final volume of 22 µl.

The analysis was performed by using QXDx AutoDG ddPCR System, following thermal cycle: EvaGreen ddPCR-polymerase activation at 95˚C for 5 min, 40 cycles of amplification at 95˚C for 30 sec (denaturation) and 58˚C for 1 min (annealing), droplets stabilization at 90˚C for 5 min followed by an infinite hold at 4˚C. Each sample was analysed in triplicate.

Table 2. *The primers used for RT-qPCR and ddPCR*

Name	Oligonucleotide sequence (5'→3')	Target	References
U1	GTGAAATTGTTGAAAGGGAA	Yeast	*Wu et al., 2002*
U2	GACTCCTTGGTCCGTGTT	Yeast	*Wu et al., 2002*
WLAB1	TCCGGATTTATTGGGCGTAAAGCGA	Bacteria	*Lopez et al., 2003*
WLAB2	TCGAATTAAACCACATGCTCCA	Bacteria	*Lopez et al., 2003*

2.2.5 16S rRNA gene amplicon Illumina sequencing

Targeted metagenomics analysis was performed in order to profile the bacterial community in all tested samples. The V3-V4 hypervariable region of the 16S rRNA gene was amplified using the custom barcoded, bacterial primers 341F (5′CCTACGGGNGGCWGCAG 3′) and 805R (5′GACTACHVGGGTATC TAATCC 3′) as previously described (Patrone et al., 2018). Amplicons were checked by gel electrophoresis, pooled in equimolar ratio, and purified by means of the AMPure XP Reagent (Beckman Coulter Life Sciences). Illumina sequencing of pooled libraries was performed at Fasteris SA (Geneva, Switzerland) using a nano Miseq instrument (paired-reads run 2x 250 bp) with Nano V2 chemistry.

2.2.6 Sequencing Data Processing and Statistical Analysis

Illumina sequencing of 16S rRNA gene amplicons provided a total of 1,196,019 reads. Barcode sorting was performed by the facility through internal Perl scripts while paired-reads overlapping was carried out using ea-utils (version 1.1.2, revision 537)

(Aronesty, 2011). The Trimmomatic package (version 0.32) was used to remove bases that corresponded to the standard Illumina adapters or that were under quality threshold. Sequences were mapped against the SILVA database (Version SSURef_NR99_115_tax_silva_DNA.fasta) for taxonomic identification by means of the Burrows-Wheeler Alignment Tool version 0.7.5a (http://bio-bwa.sourceforge.net/). Additionally, the package SAM tools was applied to merge alignments and calculate the number of reads mapped onto each OTU (Li et al., 2009). Alpha and beta diversity analyses of wine bacterial communities were conducted using the MicrobiomeAnalyst software (Dhariwal et al., 2017; Chong et al., 2020).

2.2.7 Metabolomic analysis

In order to further investigate Buttafuoco designation, in this work an untargeted metabolomics-based analysis was carried out by coupling high resolution mass spectrometry (HRMS) based on a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) with a Vanquish ultra-high-pressure liquid chromatography (UHPLC) pump and equipped with heated electrospray ionization (HESI)-II probe (Thermo Scientific, USA). Regarding the extraction process, the wine samples were thawed at room temperature and then extracted in triplicate. Briefly, an aliquot of 850 µL of each sample was added to 850 µL of acetonitrile (LC-MS grade, Sigma-Aldrich, Madison, CA) acidified with 1% (v/v) formic acid, processed with ultrasounds for 10 min and then centrifuged (Eppendorf 5810R, Hamburg, Germany) at 12,000g for 15 min at 4°C. Next, the samples were filtered using 0.22 µm cellulose syringe filters and then transferred to amber vials until the further instrumental analysis.

Afterwards, the chromatographic separation was achieved on a water-acetonitrile (both LC-MS grade, from Sigma-Aldrich, Milan, Italy) gradient elution (from 6% to 94% acetonitrile in 35 min) as a mobile phase with 0.1% formic acid as a phase modifier, using a BEH C18 (2.1x100 mm, 1.7 µm) analytical column maintained at 35°C. The flow rate was set to 200 µL/min and the injection volume was 6 µL. Each prepared wine extract was injected twice and analysed in positive polarity (ESI+) using a Full scan MS analysis in the m/z range 100-1200 with a nominal mass resolution of 70,000 FWHM at m/z 200. Pooled QC samples (made by pooling a small aliquot of each extract) acquisition was based on a data-dependent Top N analysis (Top N = 3)

with collisional energy of 10, 20, 40 eV to induce molecular fragmentation. The electrospray ionization parameters were previously optimized by Rocchetti et al. (2021a). Raw-metabolomics based data were processed using the software MS-DIAL (version 4.80) (Tsugawa et al., 2015) for post-acquisition and data filtering operations. The detailed information regarding the MS DIAL parameter settings for untargeted metabolomics-based analysis is accurately described in previously published works (Rocchetti et al., 2021a; Rocchetti et al., 2021b). The identification step (set > 60% for the annotation) was based on accurate mass tolerance (5 ppm), isotopic pattern and spectral matching. Annotation of wine metabolites was achieved against the comprehensive database FooDB (http://foodb.ca/). Furthermore, MS Finder (Tsugawa et al., 2016) was used for in-silico prediction of unknown structures according to Lipid Maps and FooDB libraries only for compounds having an in-silico prediction score >5. Overall, according to our process, a level 2 of identification in this untargeted workflow was achieved, as reported by Salek et al. (2013). Moreover, the spectral information of QC samples was used for a further identification and / or confirmation step using the publicly available MS / MS experimental spectra available in the MS-DIAL software (e.g., Mass Bank of North America), thus increasing the level of confidence in annotation. As regarding data processing and multivariate statistical analysis, two different software were used according to the workflow previously defined by Rocchetti et al. (2018).

3. Results and Discussion

3.1 DNA analysis, microbiota composition and community diversity associated with wine

In wine, DNA is highly degraded and in very little amounts due to microbe metabolism and winemaking process, reducing in this way the quantity and quality of the recovered DNA (Garcia-Beneytez et al., 2002; Savazzini and Martinelli, 2006; Pereira et al., 2017;). In addition, the presence of secondary metabolites inhibiting the activity of polymerases can hinder the possibility of DNA analysis.

The possibility to correct estimate the extractable and amplifiable amount of fungal and bacterial DNA is a good starting point for conducting any metagenomic analysis. The results of the quantification carried out by two different analytical approaches (RT-qPCR and ddPCR) and reported in Table 3 show that 1) yeast DNA fraction is predominant than LAB fraction and 2) ddPCR is more sensitive than RT-qPCR in detecting very low amounts of DNA of interest. Better performances of ddPCR are also confirmed by several research present in literature as Hindson et al. (2011; 2013), Taylor et al. (2015) Falzone et al. (2020).

Despite these results showing a predominant fraction of yeast DNA over LAB DNA, as also reported in literature (Portillo et al., 2016; Marasco et al. 2018), the subsequent metagenomic analysis was based just on the LAB fraction because of: 1) the two wines were obtained by starter yeast inoculation and not based on the indigenous population and 2) in consideration of this aspect, we decided to focus just on LAB that are not exogenously inoculated (Marzano et al., 2016; Portillo et al., 2016; Stefanini and Cavalieri, 2018). Results of the quantification analysis of yeast and LAB DNA, expressed as number of copies of DNA molecules present in the starting 480 mL of musts and wines, are reported in Table 3. As shown, both molecular targets were effectively quantified in analysed samples when ddPCR was used with the exception of LAB in Bricco PF; on the contrary, RT-qPCR failed to detect both yeast in Pregana wine and LAB in Pregana must at 72h and wine.

In general, as far as yeast content is concerned, our results are aligned with data from a previous study (Martorell et al., 2005) being the level of yeasts DNA in Buttafuoco Storico samples in the same logarithmic range as those reported, while Hierro et al. (2006) estimated different values, higher of two logs considering must fermentation steps and at end of fermentation. Actually, there are no legal limits for the content of yeast in wine, but only recommendations by the International Organization of Vine and Wine (OIV) stating that the microbial load should be less than 10^4 to 10^5 CFU ml^{-1} for microorganisms that produce powdery sediments and less than 10^2 to 10^3 CFU ml^{-1} for microorganisms that produce flocculent sediments.

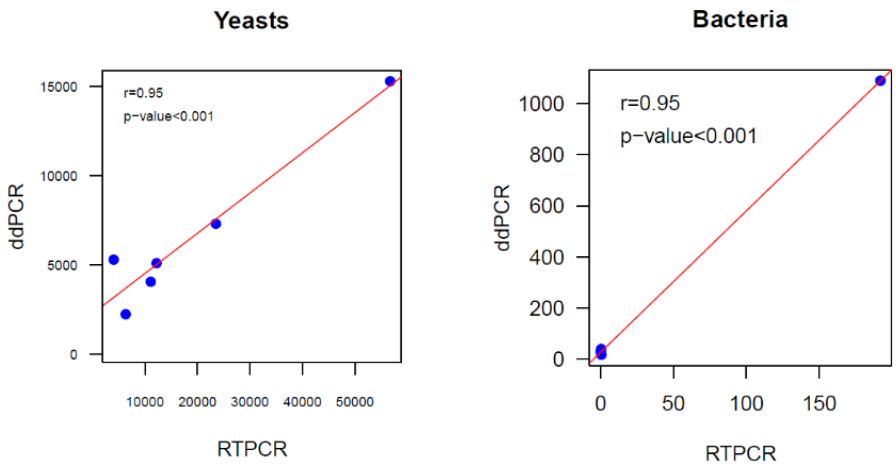
It is known that as fermentation proceeds, both the increase in the alcohol content of the product and the consequent nutritional depletion of the medium bring to the weakening of fermentation by yeasts. This explains a lower quantification of yeasts in the initial phase, 24 h after yeast inoculation, a progressive increase in the full fermentation phase corresponding to 72 h and a new reduction at the end of

fermentation. As mentioned before, since it is a process influenced by many factors, the reaction kinetics can undergo variations in relation to microbial contamination, hygienic conditions of the cellar, parameters such as temperature, pH, amount of oxygen and nutrients.

Similar considerations can be made for the quantification of LAB. It is important to stress that, according to the specification of Buttafuoco Storico, wine malolactic fermentation is performed spontaneously and not with the addition of selected starters. In general, MLF of wine starts spontaneously when the population of indigenous LAB reaches a sufficient level. In this case, as well as for yeasts, LAB densities in grapes, must and wine are influenced and depends on climatic conditions during the final days of grape maturation, and inversely correlated with must acidity.

Quantifications revealed that our study is aligned with the identification made by Nannelli et al. (2008) and Barata et al. (2012) in which the number of LAB present in samples at different winemaking stages is in the same logarithmic value range as resumed in Table 3. The generally lower concentrations of LAB in wine, consistent in our samples, have been attributed to their mostly anaerobic lifestyles, suggesting competitive advantages for yeasts and other bacterial species under the aerobic conditions (Barata et al., 2012). Overall, even if the number of observations is limited to derive a general conclusion, we have observed a good correlation between RT-qPCR and ddPCR quantification for both Yeast ($r = 0.95$, $P \leq 0.001$) and Bacteria ($r = 0.99$, $P \leq 1.86 \times 10^{-7}$), reported in Figure 1, meaning that it is possible to correct the overestimation and compare both results and methods.

Figure 1. *Yeast and Bacterial correlations*



Both RT-qPCR and ddPCR analysis suggest a decrease in the proportion of yeasts in Pregana wine along the manufacture process, with highest values recorded after 24 h fermentation; as regards Bricco, the amount of yeast DNA remained constant across all the tested samples; the same trend was observed also for LAB.
Results in Table 3 showed better ddPCR performance than RT-qPCR in detecting both yeast and bacteria.

Table 3. *Quantification analysis of yeast and LAB DNA with qRT and ddPCR analysis*

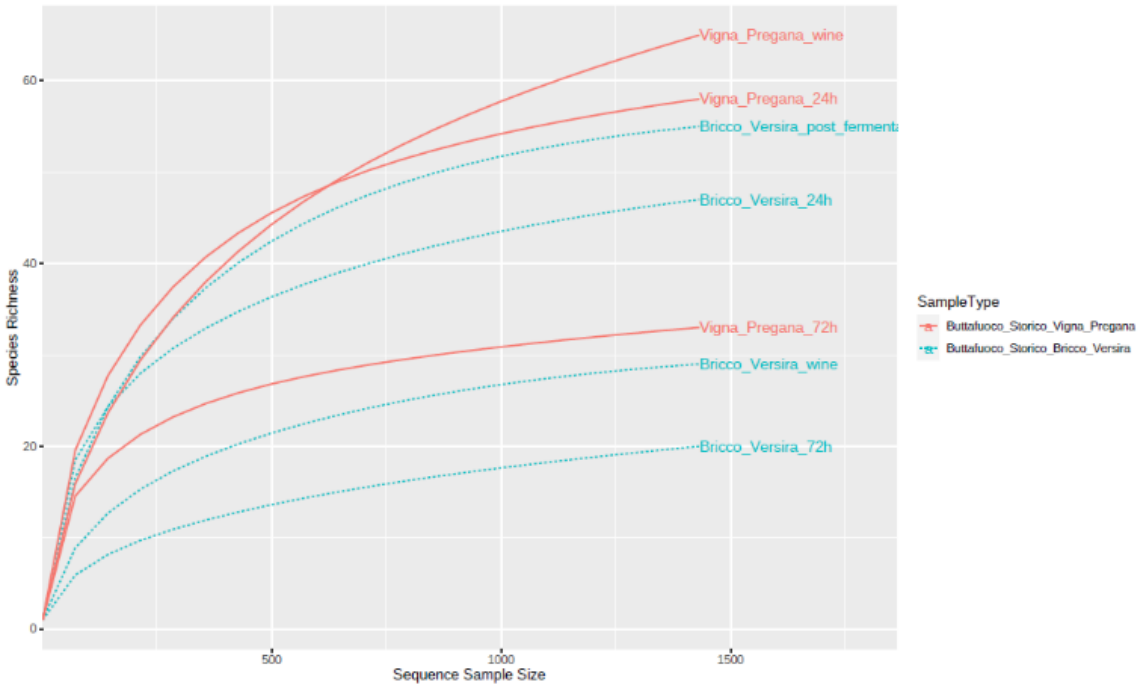
	RT-qPCR yeast	**ddPCR yeast**	**RT-qPCR LAB**	**ddPCR LAB**
Sample	**n° copies/ml**	**n° copies/ml**	**n° copies/ml**	**n° copies/ml**
Pregana_24h	5,67E+04	1,53E+04	1,92E+02	1,09E+03
Pregana_72h	4,04E+03	5,31E+03	N.A.	4,53E+01
Pregana_wine	N.A.	1,72E+02	N.A.	1,88E+01
Bricco_24h	6,33E+03	2,25E+03	4,17E-01	3,85E+01
Bricco_72h	1,22E+04	5,11E+03	5,73E-01	1,67E+01
Bricco_PF	2,35E+04	7,32E+03	7,46E-01	N.A.
Bricco_wine	1,11E+04	4,07E+03	9,15E-02	2,81E+01

These results were obtained according to serial DNA dilutions (100-1000) which probably also considerably reduces the inhibitors concentration. In fact, in the case of wine, a major problem, in addition to DNA concentration, is represented by the possible interference of polyphenols, tannins, and polysaccharides present in the matrix on the possibility to use wine DNA in subsequent PCR reactions (Savazzini and Martinelli, 2006; Işçi et al., 2014). In case of DOC Buttafuoco Storico, it should be considered that Croatina and Barbera grapes, that are the major component in blend, are very rich in tannins, phenolic compounds and anthocyanins that are then found in red wines. These components are, at the same time, peculiar characteristics of the wine but strong inhibitors of the subsequent PCR analysis (Do and Adams, 1991).
Diluting DNA can be an efficient strategy for lowering inhibitors concentrations making easier PCR amplification. Concerning this, the availability of methods that make it possible to amplify DNA also at very low concentrations can be of extreme

importance. The higher sensitivity of ddPCR allowed to correctly quantify the different targets DNAs despite the dilutions as opposed to RT-qPCR (Falzone et al., 2020). It is likely that that the better performance obtained in ddPCR, conferred by its higher analytical sensitivity (Taylor et al., 2015; Hindson et al., 2013), can be related to the lowering of PCR inhibitors due to the higher dilution range and partition of the reaction mix in 20,000 nanodroplets obtained by using oil-water emulsion (Hindson et al., 2011).

Bacterial taxonomic microbiota composition of wine was determined by 16S rRNA gene amplicon sequencing resulting in 602,325 taxonomically assigned sequences from analysed samples. Approximately 68% of the sequences were classified as either plant- or fungi-derived reads. Plant- and fungi-derived reads were removed for analysis, resulting in a dataset of 192,446 bacterial sequences. Low abundance features were filtered (minimum count 3, prevalence in sample 20%) as well a slow variance feature (inter-quantile range 10%). To address the variability in sampling depth, data were rarefied to the minimum library size (1,496 reads per sample). Rarefaction curves tended to level off for all samples but for Vigna Pregana wine (Figure 2), suggesting that the sequencing depth was suitable to describe bacterial diversity in study samples although new phylotypes could still be identified by increasing coverage.

Figure 2. *Refraction curves for Vigna Pregana and Vigna Bricco Versira*



In order to check the variations of bacterial diversity, the Chao1 and Shannon (Figure 3 and 4 respectively) indexes were calculated at the OTU level.

Figure 3. *Dynamic changes in bacterial alpha diversity of wine samples determined by Chao1 index.*

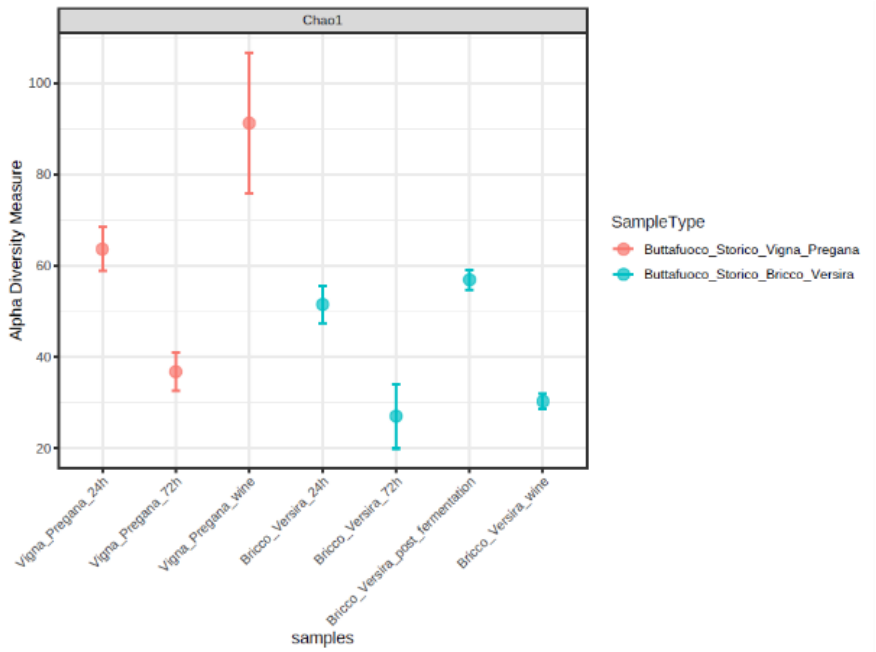
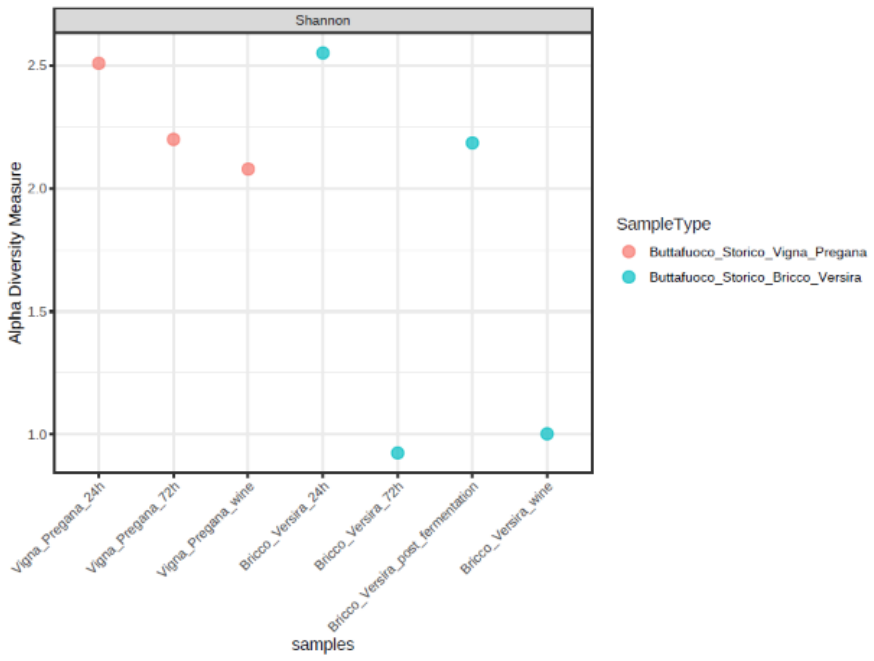


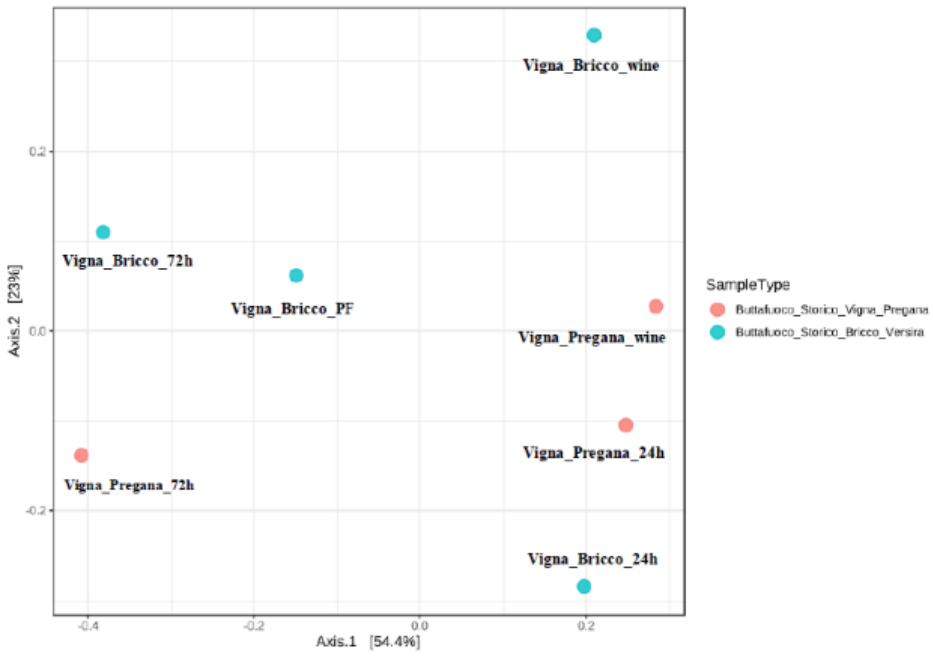
Figure 4. *Dynamic changes in bacterial alpha diversity of wine samples determined by Shannon index.*



As shown in Figure 3, the Chao1 richness estimator decreased for both wines at 72h but increased for Pregana wine while decreasing for Bricco wine in comparison to their respective 24 h samples. Shannon index (Figure 4) indicated a decrease of bacterial diversity in Pregana wine as compared to 24h sample, suggesting that more species were present in the wine but just a few of them dominated the sample. This decreasing trend is in agreement with the results of Kamilari et al. (2021). Indeed, the sample at 24h showed the highest value of alpha diversity, which corresponds, from an oenological point of view to the time when commercial starters are inoculated for alcoholic fermentation (AF). The process of fermentation is dynamic, leading to modifications in microbial diversity (Wang et al., 2021). The progression of fermentation exposes microorganisms to adverse environmental conditions that allow survival of only those strains displaying a high degree of stress resistance. This assumption justifies the decreasing trend of bacterial alpha diversity across Pregana production. Results in the final sampling step, wine, are in line with the majority of the studies according to which the stressful environment created by the high alcohol concentration leads to the elimination of many yeasts and bacteria species (Stefanini and Cavalieri, 2018). Therefore, the microbial consortia of wine are comprised of very specific microbial species (Bokulich et al., 2014; Campisano et al., 2014). Surprisingly, the bacterial community of the Bricco sample collected post fermentation showed an increase in the alpha-diversity level as compared to the previous time point. This manufacturing stage corresponds to the time when the alcoholic fermentation is finished and the wine is waiting to be processed according to the oenological objective to be pursued, so it is possible that some unwanted fermentations occurred in the meanwhile. An important aspect for the production of full-bodied red wine as Buttafuoco Storico is aging one year in oak barrels (new and old). Their porous microstructure allows the influx of small amounts of oxygen (Swaffield & Scott, 1995) and the presence of cellobiose can serve as sugar resource (Boulton et al., 1996) to support microbial proliferation. In addition, difficulty of sanitation (old barrels) is favourable to established dangerous microbial populations and promotes contamination of wine (Pollnitz et al., 2000). Another aspect that may justify an increase in bacteria alpha diversity at this sampling point is the consistent presence of acetic acid bacteria (AAB) of the genus *Gluconobacter* and *Gluconoacetobacter* as confirmed by sequencing results. They are frequently found in wine but undesirable for wine production due their ability to efficiently convert ethanol to acetate, which is

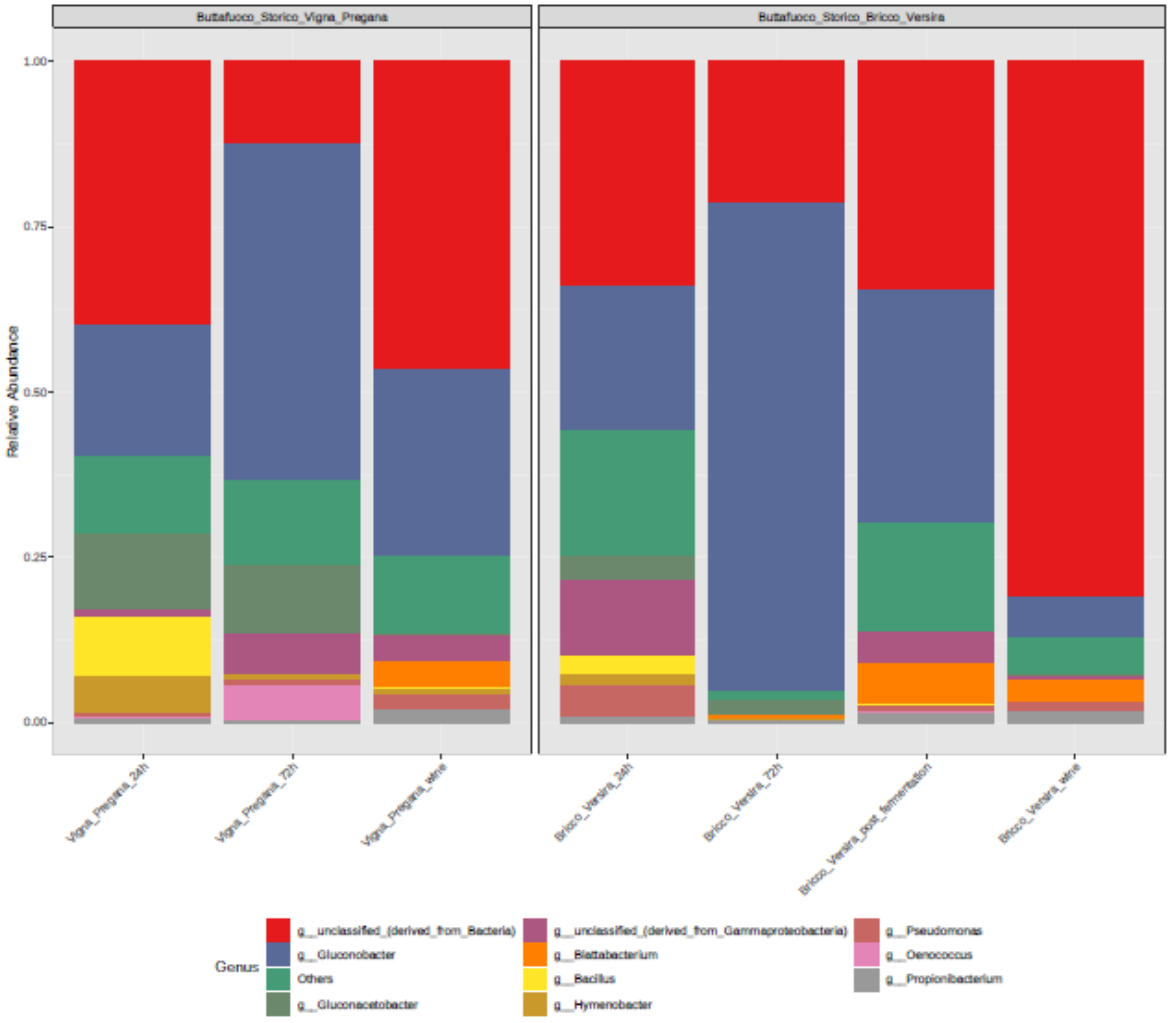
associated with spoilage, alters the wine aroma and reduces its commercial value (Bartowsky, E. J., & Henschke, P. A., 2008). Bubeck et al. (2020), reported in their work a consistent AAB presence directly on grape skin affected by *Botrytis cinerea* or *Drosophila suzukii* resulting in increased loads in final wines. AAB have long been known for their ability to negatively affect wine quality through the formation of acetic acid, which constitutes more than 90% of wine's volatile acidity. Elevated levels of acetic acid can be detrimental to wine quality as it imparts a vinegary/acetone-like aroma (Oelofse et al., 2008). Coming to the last sampling point, corresponding to the wine, the alpha diversity values show again a decrease as detected in Pregana wine. These results are not surprising, since a possible explanation could be related to the addition of SO_2 and the related oxygen availability that is ideal in wine to reduce microbiological instabilities (Romano & Suzzi, 1992). Benito et al. (2014) demonstrated that also filtration and racking, considered as common oenological practises, can reduce the development of unwanted microbial populations. These practices are recommended for wines destined to ageing in oak barrels as in the case of Buttafuoco Storico (Oelofse et al., 2008). Beta-diversity analysis was performed by calculating the Bray-Curtis dissimilarity matrix at the I level and using the Principal Coordinates Analysis (PCoA) ordination method.

Figure 5. *Principal Coordinate Analysis (PCoA) based on Bray-Curtis distances between bacterial OTU abundance profiles of different wine microbiota samples*



As shown in Figure 5, no distinct clustering of Bricco and Pregana samples was observed. Indeed, the composition of bacterial communities correlated mostly according to the processing stage rather than the specific wine. More specifically, the results suggested that 72h and post-fermentation samples separated across the first principal coordinate, indicating that the conditions established during late fermentation are the most critical for bacterial populations.

Figure 6. *The top 20 bacterial genera occurring in the two wines, Buttafuoco Storico Vigna Pregana (on the left) and Buttafuoco Storico Bricco Versira (on the right) in the different sampling points are shown.*



The most predominant taxon during winemaking (Figure 6) was the acetic acid bacterial genus *Gluconobacter*, followed by *Gluconacetobacter*. Similar results have

been reported in other studies (Bokulich et al., 2012; Lleixà et al., 2018). The abundance of these bacterial genera varied among the samples across the different fermentation stages. In both wines, the level of *Gluconobacter* increased at 72h (51% and 74% in Pregana and Bricco, respectively) as compared to their corresponding samples at 24h and decreased afterwards. As for *Gluconacetobacter*, the bacteria population decreased along fermentation being detected in lowest amounts in the final products. Studies demonstrated that the presence is related to the status of grapes indicating that *Gluconobacte*r are more abundant in healthy grapes, whereas *Gluconoacetobacter* (or even *Acetobacter*) are more present in damaged grapes (Guillamón and Mas, 2017). Considering this assumption, we can speculate that the health status of grapes was good as supported by the high abundance of *Gluconobacter* in all samples. Portillo et al. (2016), in a research based on wine fermentation, showed that AAB and LAB were more abundant during fermentation than previously thought, with a dominance of *Gluconobacter* during the mid-fermentation. The latter finding contradicts the previous idea that *Gluconobacter*, being alcohol sensitive, usually declines during the alcoholic fermentation (González et al., 2005; Lambrecht et al., 2022). Similar results have also been reported in other studies on low-sulfited or unsulfited wine fermentations (Bokulich et al., 2014). The same authors found *Acetobacter*, *Gluconobacter*, and *Gluconoacetobacter* as dominant bacteria during winemaking processes (Bokulich et al., 2012). According to Rivas et al. (2022) and Morgan et al. (2019), the reduction of *Gluconobacter* in the later stages of fermentation and wines is due to the predominant commercial high-activity *Saccharomyces* that inhibit the growth of acid-producing bacteria. This assumption can explain the drastic decrease of *Gluconobacter* observed in in the late phase of Bricco Versira production: in this wine, in fact, commercial *Saccharomyces* strains are used for winemaking unlike in Pregana wine. Musts at 24h harboured higher proportions of *Bacillus* and *Hymenobacter* as compared to the other samples. The higher presence of *Bacillus* in Pregana must is consistent with results by Mezzasalma et al. (2017). These authors found that organic must is enriched in *Bacillus* spp. which are typical microbes in manure, even if their influence on wine composition is still not clear. *Blattabacterium* and *Propionibacterium* were detected mainly in the Bricco Versira post fermentation and in both final wines. The endophytic community of *Propionibacterium* and *Bacillus* species and their structures can vary amongst grape cultivars and is also influenced by agronomic practices (Campisano et al., 2014, Perazzolli et al., 2014). This may account

for the different abundances of the species in relation to the cultural regimes adopted for the production of the different wines. Lactic acid bacteria are very important for wine composition (LAB, predominantly *Oenococcus oeni*) while in the must modulate the flavour and aroma of wine (Swiegers et al., 2005). The LAB genus *Oenococcus* represented an important proportion of the sequences in late fermentation of Pregana (5.7%), while it was scarcely detected in the rest of the samples (≤0.57%). According to Lleixà et al. (2018) the abundance of *Oenococcus oeni* could be related to the grape health status. These results are aligned with others works in which it was demonstrated that the levels of this species increase during malolactic fermentation and that, in many cases, is the dominant taxa (Marzano et al., 2016; Portillo et al., 2016). Other LAB often encountered include *Lactobacillus, Lactococcus, Leuconostoc,* and *Pediococcus* species (Bokulich et al., 2012; 2014; Pinto et al., 2015; Portillo et al., 2016). *Oenococcus oeni* seems to be rarely encountered in grape must except in one study where it was found to be dominant in fermentations of Grenache and Carignan grapes (Portillo et al., 2016). Some genera, including *Wolbachia* and *Blattabacterium*, are known to be endosymbionts of insects; our results are in line with previous studies (Mezzasalma et al., 2018) and provide further evidence that insect transportation may represent an additional route for microbial colonization of grapes (Gilbert et al., 2014). In addition to the "wine-associated" bacteria, the plant pathogens *Tatumella*, remains constant along the entire production process. The genus *Tatumella* from the family *Enterobacteriaceae* was identified as the most abundant bacterial taxon in both red and white wines in a recent study conducted in Germany (Bubeck et al., 2020). Moreover, Nisiotou et al. (2011) showed that *Enterobacteriaceae* persists in fermentation and Ruiz et al. (2010) confirmed its prevalence at beginning, mid and final stages of MLFs in different Spanish wineries. Among all these bacterial populations, lactic acid bacteria and acetic acid bacteria play a key role in wine fermentation, affecting wine aroma and overall quality (Carpena et la., 2021; Virdis et al., 2021). When comparing the two different wines for their microbial composition no statistically significant difference were found (FDR>0.05). Overall, the mean relative levels of *Oenococcus* were higher in Pregana than in Bricco. The same trend was observed for *Gluconacetobacter* and *Acetobacter* (and *Tatumella*); conversely, Bricco harboured more *Acinetobacter* than Pregana.

3.2 Untargeted screening and multivariate statistical discrimination of the different Buttafuoco wine samples by UHPLC-HRMS

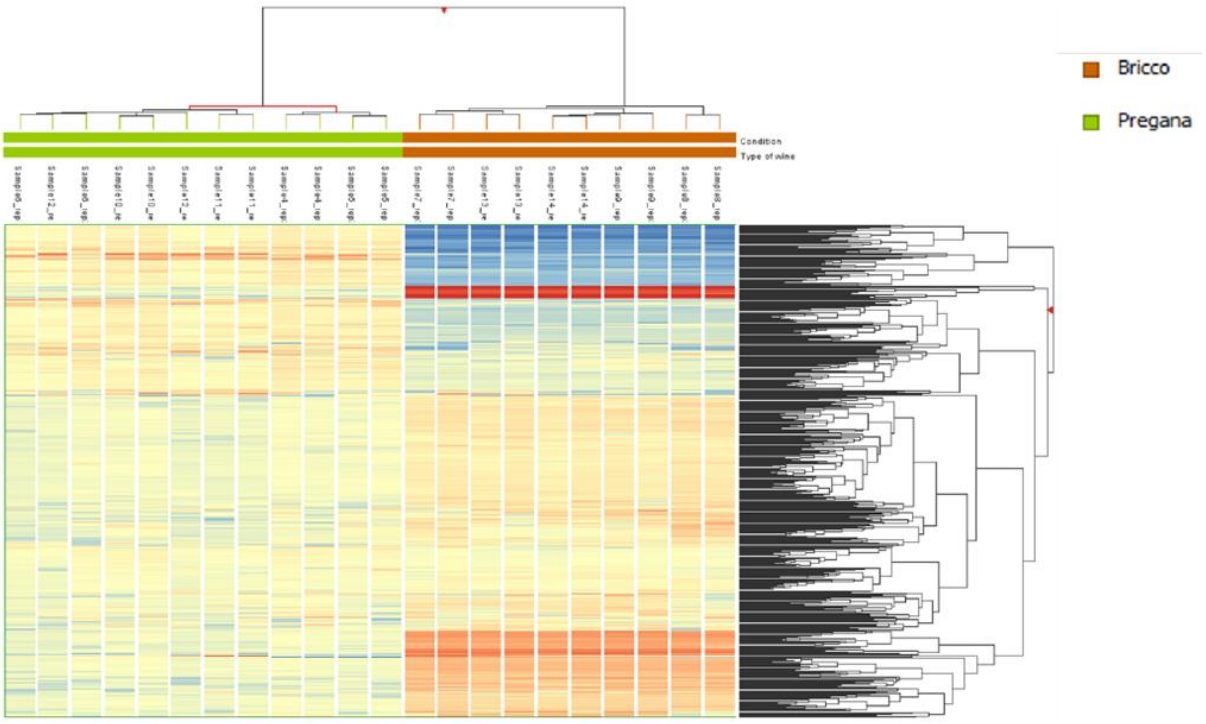
In this work, the untargeted metabolomics-based approach was carried out to comprehensively investigate those markers characterizing the different Buttafuoco wine samples.

Overall, according to our analytical workflow, 522 unique structures were annotated using a dedicated tandem-MS approach on pooled quality control (QC) samples.

As the first step, an unsupervised hierarchical cluster analysis (HCA) was performed to native group samples according to their similarities.

In figure 7 is reported the corresponding HCA analysis produced from the fold change-based heat map of metabolites abundance.

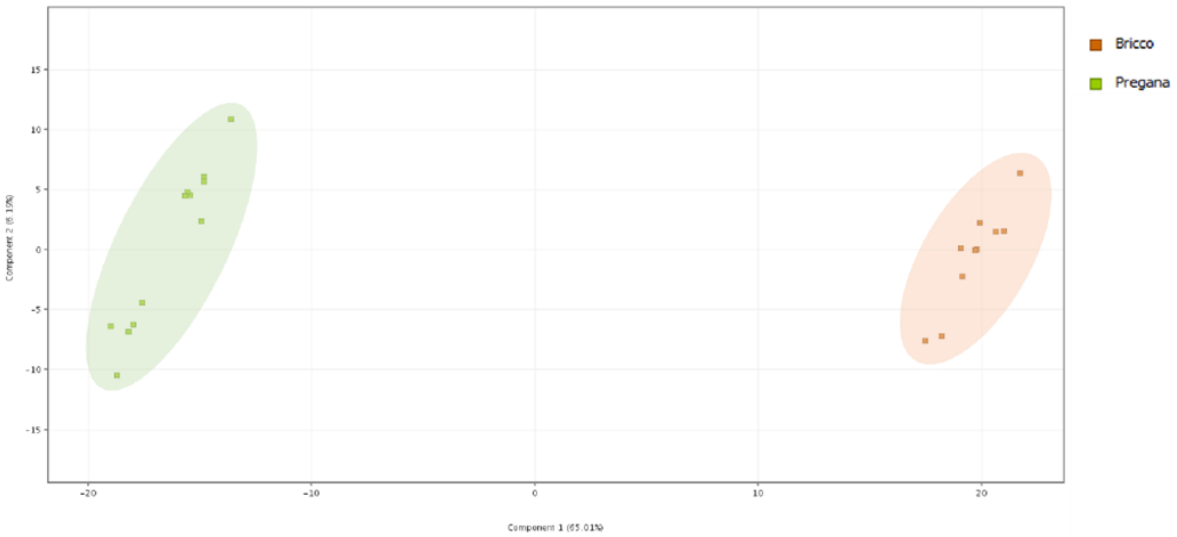
Figure 7. *Unsupervised hierarchical cluster analysis (HCA) built considering the metabolomic profile of the different Buttafuoco wine samples under investigation (i.e., Pregana vs Bricco).*



As can be observed, two main principal clusters were obtained: the first cluster hierarchically included all Pregana wine samples, whilst the second cluster consisted of the Bricco samples.

In this regard, similar results were evaluated through the analysis of the output gave by principal component analysis (PCA) that highlighted a clear separation between the two categories along the first principal component (PC1) (Figure 8).

Figure 8. *Unsupervised principal component analysis (PCA) built considering the metabolomic profile of the different Buttafuoco wine samples under investigation (i.e., Pregana vs Bricco).*

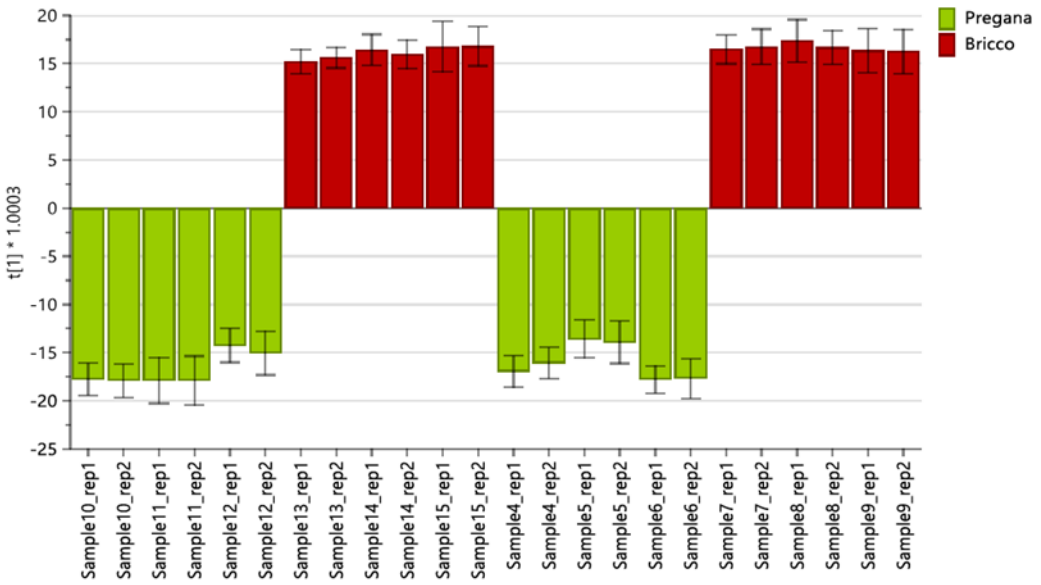


As the next evaluation, in order to investigate on the marker compounds better responsible for the discrimination of these two categories, a supervised multivariate statistical approach, namely OPLS-DA, was then carried out.

The OPLS-Da model, built considering the two different types of wines (i.e., Pregana vs Bricco) as class-discriminant information, showed a high goodness of fitting (R^2cum= 0.994) and prediction ability (Q^2cum= 0.992), with adequate permutation test cross-validation.

The corresponding OPLS-DA score plot is reported in Figure 9.

Figure 9. *The OPLS-DA score plot, built considering the two different types of wines (i.e., Pregana vs Bricco) as class-discriminant.*



As can be observed from the Figure 9, the supervised prediction model allowed confirming the outputs of unsupervised statistics with a single predictive component t1 able to discriminate the different Buttafuoco Storico wine samples.

Afterwards, the variables importance in projection of the OPLS-DA model were extrapolated using the VIP approach.

In Table 4 are reported the most important compounds highlighted by VIP analysis and organized in classes (according to the classification provided by the comprehensive database FooDB), together with their Log FC values when considering the comparison “Pregana vs Bricco”.

Looking at our findings, the VIP markers consisted mainly of amino acids, flavonoids, terpenoids, and fatty acyl derivatives. Interestingly, most of the detected amino acids were characterized by a general up-regulation, thus suggesting that different soil type had a specific incidence of the amino acid profile of the wine product.

The amino acids showing the highest VIP score were proline (VIP score = 1.35368) and phenylalanine (VIP score = 1.35747) and several their derivatives (mainly dipeptide compounds) were annotated with high VIP score values. In particular, Pereira et al. (2007) showed that the metabolomic profile of a given wine cultivar could be affected by soil type and proline has been detected as a potential biomarker of calcareous-clay-based soil.

This result is consistent because Pregana was produced following stringent specification and the vineyard enjoys a south-west exposure with a calcareous-clay subzone.

In this regard, Van Leeuwen et al. (2018) reported that clay soils were enrich in nitrogen and wine products benefits in flavour- and aroma-active compounds.

In addition, Coelho et al. (2009) reported that the soil has a particular influence on the chemical composition of red wine and clay soils produce wines with the higher concentration of varietally relevant compounds (such as monoterpenoids, sesquiterpenoids and C13 norisoprenoids) than sandy-based soils. In our experimental conditions, monoterpenoids were found to be up accumulated, associated with higher average Log FC value (Log FC = 1.305116622). In particular, cumin aldehyde was annotated with an up-accumulation trend, its presence has been found in some wines and it gives the smell of spicy notes. Also, alpha-terpineol is a naturally occurring monoterpene alcohol that has been isolated from a variety of wines fermented by *Saccharomyces cerevisiae* yeast and this metabolite has been attributed to grape-derived flavour-active precursor compounds in aromatic wines.

Also, cinnamic acid derivatives contribute directly to the flavour profile of wines and these metabolites occur extensively in plants. In particular, ethyl cinnamate is responsible for a sweet-fruity and cherry-like aroma on wine matrices and its presence in red wine has been already reported by Moio et al. (1995) its presence can enhance the fruity aromatic notes of Pregana wine.

Instead, looking to the discriminant markers proposed in Table 4, it was possible to notice that flavonoid compounds were found to be down-accumulated and several markers were annotated with higher VIP score values. The flavonoids showing the highest Log FC scores were Delphinidin (Log FC = -18.532112), Myricetin 3-galactoside (Log FC = -7.5688586), and Quercetagetin (Log FC = -6.513242).

Delphinidin is an anthocyanin present in red wine and its biological effects have been reported to exert vasculoprotective and vasorelaxing properties on human endothelial cells. Instead, different behaviour was observed for isoflavonoid compounds, that were found to be up-accumulated and very significant for the OPLS-DA model (Duluc et al., 2014). In particular, three different polyphenols were highlighted to be the most discriminant of the Pregana wines, such as Daidzein, 4',7-Dihydroxy-6-methoxyisoflavan and 4',7-Dihydroxy-3'-methoxyisoflavan. Daidzein is a plant isoflavone and it has been already detected in wine matrices.

It was noticed for its antioxidant activity but also for its sensibility in dehydration process during postharvest water stress (De Sanctis et al., 2012). Another interesting phenolic VIP marker was oleocanthal. This compound belongs to the class of organic compounds known as tyrosols.

Tyrosols are antioxidant phenolic compound present in wine and olive oil and could exert beneficial effects on human in terms of preventing lipid peroxidation and atherosclerotic processes. Specifically, oleocanthal has been detected in virgin olive oil, having anti-inflammatory properties and potential therapeutic actions (Lucas et al., 2011).

Finally, two principal stilbene compounds were particularly able to explain the differences as related to the type of Buttafuoco wine under investigation. (i.e., Pregana vs Bricco) and these compounds were Ampelopsin D and (E)-Resveratrol 3-glucoside. Ampelopsin D was up-accumulated and very significant metabolite and its presence has been reported in Italian red wine Primitivo and Rabioso Piave with accurate MS/MS screening analysis based on targeted metabolomics of grape stilbenes. Another very interesting compounds was picolinic acid, that were found to be up accumulated, characterizing Pregana wine samples.

This compound has neuroprotective and antioxidant functionalities, and it is originated by kynurenine metabolic pathway in wine fermented by *S. cerevisiae*. Yilmaz et al. (2021) reported that no changes in the content of picolinic acid during malolactic fermentation were observed in red wine. Additionally, the VIP lineolic acid compounds showed an equal distribution between the two different Buttafuoco wines under investigation (i.e., Pregana vs Bricco).

In our experimental conditions, we highlighted the presence of jasmonic acid, ethyl alpha-linolenate and alpha-linolenic acid (ALA). Liu et al. (2019) demonstrated that unsaturated fatty acids have active oenological functions on the extraction and are able to enhance wine aroma through specific processes occurring in alcoholic fermentation of Cabernet Sauvignon wine.

In particular, ALA derivatives produce higher concentrations of C6 alcohols that are directly involved in the degradation of ALA compounds trough reactions catalysed by LOX/HPL (Lipoxygenase-hydroperoxide lyase) pathway of Cabernet Sauvignon wine (Forde et al., 2011).

Table 4. *Most discriminant compounds in Buttafuoco Storico wine samples characterizing the comparison "Pregana vs Bricco". Each compound is reported with its VIP score (from the OPLS-DA model), LogFC value and its accumulation trend*

Class	Discriminant marker (OPLS-DA)	VIP score (OPLS-DA)	Log FC value (Pregana vs Bricco)	Accumulation (Pregana vs Bricco)
Alcohols and polyols	2,2,6,7-Tetramethylbicyclo[4.3.0]nona-1(9),4-diene-7,8-diol	1.099 ± 0.171	0.10	up
Amines	1-Phenylethylamine	1.341 ± 0.103	0.81	up
	2,9-Dimethyl-2,9-diazatricyclo[10.2.2.25,8]octadeca-5,7,12,14,15,17-hexaene-3,10-diol, 9CI	1.068 ± 0.318	0.81	down
			LogFC (avg) = -0.23	*down*
Amino acids, peptides, and analogues	L-Prolyl-L-phenylalanine	1.358 ± 0.030	2.02	up
	Prolyl-Valine	1.357 ± 0.034	1.48	up
	Phenylalanine	1.357 ± 0.033	1.51	up
	Isoleucyl-Glycine	1.354 ± 0.058	1.03	up
	Isoleucyl-Threonine	1.354 ± 0.057	0.78	up
	L-Lysine	1.354 ± 0.058	1.54	up
	cyclo(L-Leucyl-L-prolyl)	1.352 ± 0.080	1.21	up
	(±)-erythro-Isoleucine	1.351 ± 0.095	1.87	up
	Isoleucyl-Arginine	1.351 ± 0.090	-6.00	down
	Alanyllysine	1.351 ± 0.093	-4.81	down
	Asparaginyl-Lysine	1.350 ± 0.074	0.56	up
	Leucyl-Valine	1.349 ± 0.100	2.44	up
	D-Arginine	1.349 ± 0.080	-2.10	down
	Leucyl-Tyrosine	1.348 ± 0.091	3.00	up
	Leucyl-Alanine	1.347 ± 0.111	1.25	up
	2-Aminoisobutyric acid	1.345 ± 0.132	-1.85	down
	N(6)-(Octanoyl)lysine	1.344 ± 0.106	-2.48	down
	Leucyl-Gamma-glutamate	1.342 ± 0.077	0.71	up
	Glycyl-Methionine	1.330 ± 0.138	1.20	up
	Phenylacetylglycine	1.326 ± 0.227	0.61	up
	Valyl-Leucine	1.325 ± 0.179	-1.69	down
	N-Ethylglycine	1.323 ± 0.156	-0.42	down
	Glutamylleucine	1.299 ± 0.211	3.07	up
	Proline	1.354 ± 0.168	1.26	up
	Phenylalanyl-Glycine	1.207 ± 0.365	4.31	up
	Isoleucyl-Phenylalanine	1.205 ± 0.234	-1.11	down
	Isoleucyl-Leucine	1.191 ± 0.204	1.31	up
	N-Acetyl-L-phenylalanine	1.182 ± 0.555	0.32	up
	Serylisoleucine	1.174 ± 0.386	2.19	up
	N-Acetylornithine	1.167 ± 0.258	-0.07	down
	Tyrosyl-Valine	1.125 ± 0.417	-0.95	down
	Alanylvaline	1.114 ± 0.318	0.73	up

	Glycyl-L-leucine	1.080 ± 0.478	3.16	up
	Isoleucyl-Alanine	1.039 ± 0.574	2.13	up
	Glutaminylvaline	1.030 ± 0.355	1.07	up
	Arginine	1.026 ± 0.446	0.65	up
	Isoleucyl-Valine	1.020 ± 0.398	4.12	up
	L-Citrulline	1.356 ± 0.051	0.55	up
	Pipecolic acid	1.356 ± 0.055	1.59	up
	Ile-Val-Val	1.350 ± 0.064	2.49	up
	L,L-Cyclo(leucylprolyl)	1.164 ± 0.254	1.43	up
			LogFC (avg) = 0.73	*up*
Benzene and substituted derivatives	Anthranilic acid	1.355 ± 0.067	1.52	up
	3,4-O-Dimethylgallic acid	1.348 ± 0.091	1.18	up
	2-(3-Phenylpropyl)tetrahydrofuran	1.331 ± 0.193	3.47	up
	Benzyl salicylate	1.289 ± 0.322	0.43	up
	3-Phenylpropanal	1.285 ± 0.229	1.08	up
	2-Hydroxy-3-(4-hydroxyphenyl)propenoic acid	1.285 ± 0.285	1.46	up
	1-Phenyl-2,4-pentadiyn-1-one	1.249 ± 0.329	-0.95	down
	Enol-phenylpyruvate	1.029 ± 0.560	0.83	up
	2-Propenyl phenylacetate	1.358 ± 0.033	3.54	up
	Phenethyl phenylacetate	1.357 ± 0.045	3.17	up
	alpha-Amylcinnamyl formate	1.200 ± 0.283	-1.33	down
			LogFC (avg) = 1.31	*up*
Benzodioxoles	3,4-Methylenedioxybenzaldehyde	1.326 ± 0.206	0.94	up
Carbohydrates and carbohydrate conjugates	Benzyl 6-O-beta-D-apiofuranosyl-beta-D-glucoside	1.343 ± 0.112	-3.13	down
	Benzyl O-[arabinofuranosyl-(1->6)-glucoside]	1.344 ± 0.104	-2.38	down
			LogFC (avg) = -2.75	*down*
Carbonyl compounds	4-Octanone	1.359 ± 0.026	4.00	up
	2-Octanone	1.357 ± 0.026	3.89	up
	(2E,4Z,7Z)-2,4,7-Decatrienal	1.340 ± 0.116	2.87	up
	(2E,6E)-2,6-Nonadienal	1.358 ± 0.042	1.34	up
	3-Hydroxybenzaldehyde	1.327 ± 0.199	-1.33	down
	8-Nonen-2-one	1.181 ± 0.384	-1.60	down
			LogFC (avg) = 1.53	*up*
Cinnamic acid esters	Ethyl cinnamate	1.332 ± 0.127	1.60	up
Cinnamic acids and derivatives	Cyclohexyl cinnamate	1.358 ± 0.050	3.59	up
	Tetrahydrofurfuryl cinnamate	1.355 ± 0.059	5.33	up
	trans-o-Coumaric acid	1.126 ± 0.220	-1.91	down
	Caffeic acid ethyl ester	1.351 ± 0.078	3.22	up
			LogFC (avg) = 2.56	*up*
Cinnamyl alcohols	4-Phenyl-3-buten-2-ol	1.352 ± 0.088	3.70	up
Coumarins and derivatives	Braylin	1.348 ± 0.083	1.05	up

Diarylheptanoids	(-)-(E)-1-(4-Hydroxyphenyl)-7-phenyl-6-hepten-3-ol	1.358 ± 0.032	4.19	up
	1-(4-Hydroxy-3-methoxyphenyl)-7-phenyl-3,5-heptanedione	1.235 ± 0.332	0.22	up
	5-Methoxy-7-(4-hydroxyphenyl)-1-phenyl-3-heptanone	1.349 ± 0.091	-9.41	down
			LogFC (avg) = -1.67	*down*
Diterpenoids	Gibberellin A70	1.272 ± 0.271	-1.11	down
	Digeranyl	1.358 ± 0.032	2.52	up
	Patuletin 7-glucoside	1.300 ± 0.198	-4.44	down
	Methyl (9Z)-8'-oxo-6,8'-diapo-6-carotenoate	1.347 ± 0.086	5.30	up
			LogFC (avg) = 0.57	*up*
Fatty acid esters	3-Phenylpropyl hexanoate	1.235 ± 0.228	-0.19	down
	Ethyl hydrogen fumarate	1.198 ± 0.378	1.91	up
	3-Methylbutyl pentanoate	1.076 ± 0.454	-1.19	down
	Isobutyl angelate	1.269 ± 0.102	-1.59	down
	O-Arachidonoyl ethanolamine	1.101 ± 0.454	-0.36	down
			LogFC (avg) = -0.28	*down*
Fatty acids and conjugates	3-Oxohexadecanoic acid	1.359 ± 0.026	2.58	up
	(11R,12S,13S)-Epoxy-hydroxyoctadeca-cis-9-cis-15-dien-1-oic acid	1.345 ± 0.110	5.50	up
	4-Decenoic acid	1.298 ± 0.215	1.75	up
	Adipate semialdehyde	1.265 ± 0.204	1.32	up
	2-Hydroxy-3-methylbutyric acid	1.255 ± 0.283	2.15	up
	(2'E,4'Z,8E)-Colneleic acid	1.148 ± 0.441	4.20	up
	(2'E,4'Z,7'Z,8E)-Colnelenic acid	1.132 ± 0.281	1.01	up
	9,10,18-Trihydroxyoctadecanoic acid	1.223 ± 0.274	-0.24	down
			LogFC (avg) = 2.28	*up*
Fatty amides	Butyramide	1.347 ± 0.090	-2.35	down
Flavonoids	Honyucitrin	1.353 ± 0.076	2.83	up
	Delphinidin	1.351 ± 0.082	-18.53	down
	Myricetin 3-galactoside	1.340 ± 0.122	-7.57	down
	Limocitrin	1.332 ± 0.159	-1.70	down
	5,6,7,8-Tetrahydroxy-3',4'-dimethoxyflavone	1.324 ± 0.161	0.07	up
	Isoliquiritin	1.311 ± 0.243	-2.34	down
	7-Hydroxy-3,3',4',5,6,8-hexamethoxyflavone	1.309 ± 0.214	-1.96	down
	Isorhamnetin	1.305 ± 0.196	-1.50	down
	7,4'-Dihydroxyflavone	1.301 ± 0.282	4.13	up
	Garbanzol	1.250 ± 0.328	-1.29	down
	Natsudaidain 3-glucoside	1.248 ± 0.216	-2.02	down
	Quercetin	1.203 ± 0.307	-0.29	down
	Taxifolin	1.124 ± 0.401	-0.35	down
	Taxifolin 3-rhamnoside	1.111 ± 0.395	-0.33	down
	Genistein	1.101 ± 0.634	0.04	up

	Quercetagetin	1.335 ± 0.154	-6.51	down
	Morin	1.318 ± 0.187	-2.41	down
	3',4',5',7,8-Pentamethoxyflavan	1.314 ± 0.141	-2.02	down
	3,3',4',5,6,8-Hexamethoxyflavone	1.301 ± 0.188	-2.35	down
	(-)-Epicatechin	1.206 ± 0.472	-2.51	down
	Procyanidin B1	1.199 ± 0.381	-2.23	down
	Apigenin 7-arabinoside	1.052 ± 0.263	-1.61	down
			LogFC (avg) = -2.29	*down*
Gamma-keto acids and derivatives	Prephenate	1.087 ± 0.423	-0.13	down
Glycerolipids	Glycerol tripropanoate	1.349 ± 0.085	3.03	up
Glycerophospholipids	LPA(18:2(9Z,12Z)/0:0)	1.358 ± 0.026	2.44	up
Indoles and derivatives	Tryptophol	1.343 ± 0.115	-2.03	down
	Mahanimbinol	1.339 ± 0.123	0.13	up
	N-Acetylserotonin	1.336 ± 0.146	0.75	up
	Indolelactic acid	1.319 ± 0.177	-1.58	down
	Melatonin	1.290 ± 0.190	0.66	up
	Serotonin	1.208 ± 0.308	3.21	up
	2-Amino-a-carboline	1.358 ± 0.050	3.14	up
	5-Methoxyindoleacetate	1.119 ± 0.113	-0.44	down
			LogFC (avg) = 0.48	*up*
Isoflavonoids	4',7-Dihydroxy-6-methoxyisoflavan	1.352 ± 0.047	3.04	up
	Daidzein	1.295 ± 0.114	2.67	up
	Glyceollin III	1.294 ± 0.197	0.03	up
	4',7-Dihydroxy-3'-methoxyisoflavan	1.357 ± 0.042	4.67	up
	Kanzonol R	1.291 ± 0.192	1.26	up
			LogFC (avg) = 2.33	*up*
Keto acids and derivatives	Ethyl levulinate	1.091 ± 0.298	1.15	up
Lignans, neolignans and related compounds	Cubebin	1.350 ± 0.071	-3.15	down
Lineolic acids and derivatives	Ethyl alpha-linolenate	1.356 ± 0.052	2.72	up
	Dihydrojasmonic acid	1.351 ± 0.076	0.81	up
	Jasmonic acid	1.347 ± 0.105	-3.42	down
	alpha-Linolenic acid	1.266 ± 0.197	-0.21	down
	Steridonic acid	1.118 ± 0.539	-0.23	down
	Methyl dihydrojasmonate	1.331 ± 0.092	0.04	up
			LogFC (avg) = -0.05	*down*
Medium-chain hydroxy acids and derivatives	Gulonic acid	1.336 ± 0.119	0.28	up
Monoterpenoids	Linalyl benzoate	1.357 ± 0.041	3.69	up
	Cuminaldehyde	1.355 ± 0.053	3.87	up
	Isobornyl formate	1.349 ± 0.104	1.20	up
	trans-Carvyl formate	1.345 ± 0.084	0.54	up

	Genipin	1.315 ± 0.172	0.05	up
	Valdiate	1.314 ± 0.105	-1.60	down
	p-Menth-3-en-9-al	1.188 ± 0.505	0.73	up
	9'-Carboxy-gamma-tocotrienol	1.107 ± 0.235	1.43	up
	(R)-alpha-Terpineol	1.061 ± 0.248	0.76	up
	Dendrolasin	1.354 ± 0.049	2.82	up
	O-Geranylvanillin	1.346 ± 0.126	0.87	up
			LogFC (avg) = 1.31	*up*
Nucleosides, nucleotides, and analogues	5'-Deoxyadenosine	1.338 ± 0.078	0.56	up
Organic carbonic acids and derivatives	Diethyl dicarbonate	1.356 ± 0.060	1.49	up
Phenols	4-Methylphenyl dodecanoate	1.354 ± 0.062	2.73	up
	p-Tolyl phenylacetate	1.234 ± 0.287	-0.36	down
	Oleocanthal	1.354 ± 0.051	4.65	up
	Coniferyl aldehyde	1.197 ± 0.335	-1.92	down
			LogFC (avg) = 1.27	*up*
Purine nucleosides	isopentenyl adenosine	1.346 ± 0.105	-4.12	down
Pyridines and derivatives	1-Methylnicotinamide	1.358 ± 0.045	4.31	up
	3-Hexylpyridine	1.333 ± 0.143	-1.11	down
	5-Ethyl-2-methylpyridine	1.140 ± 0.467	-0.08	down
	Picolinic acid	1.353 ± 0.074	0.42	up
			LogFC (avg) = 0.88	*up*
Quinolines and derivatives	Kynurenic acid	1.300 ± 0.201	1.25	up
Resorcinols	2-Methyl-5-(8,11-pentadecadienyl)-1,3-benzenediol	1.352 ± 0.083	-5.24	down
Sesquiterpenoids	beta-Atlantone	1.346 ± 0.109	0.62	up
	Furanoeremophilane	1.314 ± 0.137	-2.07	down
	(S)-Curzeone	1.318 ± 0.184	-1.53	down
			LogFC (avg) = -1.00	*down*
Stilbenes	Ampelopsin D	1.342 ± 0.106	1.37	up
	(E)-Resveratrol 3-glucoside	1.078 ± 0.561	-0.13	down
	ε-Viniferin	1.344 ± 0.109	1.42	up
	Hopeaphenol	1.342 ± 0.102	1.62	up
			LogFC (avg) = 1.07	*up*

4. Conclusions

Today, the protection of productions is not only aimed at verifying the authenticity of the product but is also concerned with evaluating all the characteristics that make a bottle unique and sought after in the eyes of the consumer.

The theme of sustainability and respect for different "microbial terroir" have now become pillars of modern agriculture, which seeks to be ever more at the forefront of environmental respect and a trump card for obtaining products that are identifiable with the terroir of production. For this reason, we had investigated the microbiota composition of Buttafuoco Storico wine, following different analytical approaches and sampling steps.

An initial metagenomic analysis depicted a wide variability and microbial complexity despite that the two vineyards are included in the same restrict production area called "Sperone di Stradella". Overall, the main groups of microorganisms are not significantly affected by the location but seems to be more affected by sampling time during wine production. Microbial differences between locations exist if secondary species are considered.

The quantification of bacterial and yeast DNA fraction was performed by using RT-qPCR and ddPCR resulting in a good correlation between both evaluation method even if the sensitivity of ddPCR is higher than RT-qPCR. To complete the picture of the analysis, a metabolomics-based approach was carried out to comprehensively investigate characteristics of the two different Buttafuoco Storico wines.

Metabolomic analysis showed that is possible to distinguish the different vineyards according to the different metabolic profile. Moreover, VIP markers were linked to the geographical terroir consistently to previous researches.

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Conclusions

In conclusion, it can be said that all the goals set in the doctoral project were largely achieved. First, an efficient extraction system from all the matrices examined was optimized at the expense of that largely claimed in the literature.

Amplifiable DNA was obtained throughout the production chain, although, as was predictable, of varying quality.

On the other hand, as far as the actual microsatellite analysis was concerned, it was possible to obtain very precise data down to the must, allowing the identification of grapes and pure products in general with excellent reliability. Excellent results were also obtained in the blends allowing the identification of profiles of different grape varieties even if not in a certain and unambiguous way.

This was possible both in artificial blends consisting of fresh grapes and in musts taken in the different steps of processing the matter highlighting the potential of the method even on particularly processed matrices and therefore containing degraded genetic material.

As expected, wine, among the matrices considered, proved to be the most difficult to extract and analyse, although good quality amplifications were obtained compared to what has been reported in the literature.

For that reason, based on the results obtained in the present paper, the subsequent step was focused on optimizing the DNA extraction phase and on SSR wine analysis.

In fact, in an effort to monitor and protect wine supply chain, special attention was paid to the storage, a step that had not yet been considered in literature but to be remarkably sensitive. Determining how long wine traceability is possible after the winemaking process can be of extreme interest to control-bodies in order to prevent or detect possible fraud. That traceability is possible up to the end of winemaking is well known, while less certainty has been obtained for later stages during the storage period, before and after bottling, when most work has reported a high degree of difficulty or impossibility in successfully analysing wine DNA.

An attempt was made to bring a practical contribution to this topic by analysing the possibility of recovering and using wine DNA for traceability purposes during the storage period. All samples were collected from the same lots from the end of winemaking operations until 1 year after bottling. The results obtained showed that a

reliable SSR profile can be detected, at least until the 8th month after bottling, with some differences related to the type of wine, white or red. The results obtained suggest that white wines can be analysed more easily than red wines. Possible explanations are the different metabolic composition of the wines that may interfere with DNA extraction and analysis, the different storage conditions of the two wines, or the combination and interaction of both factors. In both cases, it was possible to uniquely recognize the production cultivar at most of the sampling points considered through bottle storage, and traceability is feasible in single-varietal wines at least up to 8 months in bottles stored under standard conditions.

Thus, it can be said that it is possible to develop a traceability system based on microsatellites that allows the entire production chain of wines to be followed, from vineyard to bottle, overcoming the limitations dictated by the processing and treatments normally provided by universally adopted oenological protocols.

In addition, as mentioned in the introduction, today, the protection of productions is not only aimed at verifying the authenticity of the product but is also concerned with evaluating all the characteristics that make a bottle unique and sought after in the eyes of the consumer. For this reason, we had investigated the microbiota composition of Buttafuoco Storico wine, following different sampling steps and analytical approaches as metagenomic analysis, quantification of bacterial and yeast DNA fraction by RT-qPCR and ddPCR and Metabolomic analysis.

Overall, it is possible to distinguish the different groups of microorganisms especially affected by sampling time during wine production and not from location. Quantification of bacterial and yeast DNA fraction resulting in a better performance of ddPCR and to complete the picture metabolomics analysis showed that is possible to distinguish the different vineyards according to the different metabolic profile.

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Tutti pensano che arrivati a questo punto la parte più complicata si sia conclusa, ma non è così! Fisso il foglio bianco e, allo stesso tempo, ripercorro velocemente tutti i momenti che hanno contraddistinto questa avventura, fermoimmagine di un'esperienza che custodirò, per sempre, nel mio cuore.

Quando partii con il progetto "DNA del VINO" non fu per nessun motivo facile, incontrai grosse difficoltà e diffidenza nel trasmettere la bontà di questo progetto, che agli occhi dei più risultava qualcosa di estremamente sconosciuto e pertanto da evitare.

Ed è stato in questi momenti, anche di sconforto e delusione, che l'innata perseveranza e tenacia del mio carattere mi hanno spinto a continuare sulla strada che avevo abbozzato.

E proprio come in un lungo trekking alpino, ricco di insidie, fatica e difficoltà, non c'è cosa più bella dell'arrivo alla cima, che apre davanti a sé un panorama mozzafiato che guarisce e nutre le fatiche del percorso, facendo dimenticare tutto quello che ha generato turbamento nel durante.

Ed ora mi trovo proprio lì, su quella rupe panoramica ad ammirare, con grande soddisfazione il risultato di questi tre anni, con l'orgoglio e la consapevolezza di aver aggiunto un piccolo tassello alla parete della conoscenza in questo settore.

Sinceramente non so se questi tre anni mi permetteranno di poter raggiungere posizioni più prestigiose rispetto a quelle che avrei potuto ottenere con, la si fa per dire semplice, Laurea Magistrale.

Una cosa è certa, non per forza ogni percorso, per essere considerato utile, necessita di un risvolto economico. Esiste qualcosa di più sottile e "sostanzioso", che va al di là della mera remunerazione dell'individuo, qualcosa che sia in grado di arricchire anime e menti.

Quella fiammella di conoscenza, che brilla di una luce fioca, va protetta ed incentivata, in un mondo impoverito di sentimenti sinceri, oramai perso verso una deriva così materialista.

Questo cammino mi ha aiutato a guardare la realtà con occhi diversi, e grazie a ciò anche a scoprire nuove dimensioni di me stessa, ad affrontare paure e timori, ma soprattutto mi ha permesso di investire culturalmente sulla mia persona, sulla mia mente.

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Loro si sono fatti carico di me in tutti questi anni e, per questo motivo, mi sento di ringraziarli con tutte le mie forze per lo spazio e la comprensione che hanno avuto. Non tutte le persone hanno la fortuna di avere genitori così disponibili e comprensivi come i miei, e non sarà sufficiente una vita intera per poterli ripagare di tutto il sacrificio, l'amore e l'appoggio che mi hanno donato.
Non posso sicuramente dimenticare un'altra importante persona che mi ha accompagnato, camminando accanto a me su questo sconosciuto sentiero, e sei tu Pietro.
Complementare a me proprio come l'Adenina con la Timina, rispettoso delle mie scelte e sempre impegnato a disegnarmi il sorriso sul volto.
Loro sono la mia famiglia, e sono loro che hanno goduto sia le mie gioie che i miei problemi, che hanno sopportato i lati peggiori del mio carattere senza ostacolarmi o girarmi le spalle.
È a loro che voglio dire GRAZIE DI CUORE!