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Genomic variations in *Saccharomyces cerevisiae* populations: diffusion in vineyards and effect on vinification processes

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Abstract

"Genomic variations in *Saccharomyces cerevisiae* populations: diffusion in vineyards and effect on vinification processes"

The project of this doctoral thesis arisen from a previous work of vineyard yeast isolation, collection and characterization, which was held at the Wine Microbiology laboratory of the University of Padova in Conegliano (CIRVE). Some genomic variations have been uncovered thanks to the genome sequencing of four *Saccharomyces cerevisiae* yeast strains. The relationship between the yeast genotype and phenotype is one of the most debated topics in wine microbiology. Aimed to trying deepening the knowledge regarding the genomic variations effects on the oenological performances of wine yeasts, this thesis is focused on the diffusion of some genomic variations in vineyard yeasts populations and on their implications on the strains technological phenotype.

The oenological characterization of Brazilian *Saccharomyces cerevisiae* yeast strains has been performed, paying attention to the link between the local agricultural practices and the strains biodiversity in vineyard. Results showed that the heavy use of copper in plant protection contributed to develop a strong copper tolerance in the autochthonous yeast population. This effect did not affect the genotype biodiversity of the yeast strains in the vineyards, which were confirmed as great reservoir for wine yeast strains isolation and selection.

The strains copper and sulphites tolerance has been studied on the Italian and the Brazilian *S. cerevisiae* yeast collections. The studied topics were: the relationships between the *CUP1* copy-number variation and the copper tolerance and the relationship between the presence of two chromosomal translocations and the sulphites tolerance. Results evidenced an association between genetic traits and tolerant phenotypes at

vineyard population level, in particular for copper tolerance and the number of *CUP1* copies. Moreover, hints of an association between Cu and SO_2 tolerance are discussed.

The fermentable carbon sources uptake in four *S. cerevisiae* strains has then been investigated. The expression of the hexose transporters genes has been analysed by Real Time PCR during the stationary phase of synthetic must fermentation. The study involved the *FSY1* gene, found in EC1118 and encoding for a high affinity fructose/H^+ symporter. *FSY1* was present in the 25% of the vineyard strains. Gene expression analysis evidenced deep differences in the sugar transporters utilization, in particular for the fructose transporter gene: in oenological conditions, the differential expression of *FSY1* enhanced the carbon sources utilization ability of the yeasts strains. This work can contribute in improving the wine yeasts characterization by giving a tool for their distinction for fitness in the winemaking environment, at transcriptional level.

Lastly, the *S. cerevisiae* strains EC1118 and QA23have been studied under the Martinotti's method for sparkling wine production. The yeast cells viability during the *pied-de-cuve* preparation, the pressure evolution in autoclave and the cells response to the wine chilling at the end of the second fermentation were taken into account. During the wine chilling, cells have been recovered for the total RNA extraction to be used in transcriptomic analysis. Preliminary results show that EC1118 has been characterized by lower cells viability than QA23 since the ethanol adaptation procedure and all along the fermentative process. This difference reflected to the pressure evolution kinetic. Data of the total RNA extraction, quantification, integrity and quality check are also presented.

Riassunto

"Variazioni genomiche in popolazioni di *Saccharomyces cerevisiae*: diffusione in vigneto ed implicazioni nei processi di vinificazione"

Il progetto di questa tesi di dottorato nasce da un precedente lavoro di isolamento, raccolta e caratterizzazione di lieviti da vigneto, svoltosi presso il laboratorio di Microbiologia Enologica dell'Università di Padova a Conegliano (CIRVE). Alcune variazioni genomiche sono state scoperte grazie al sequenziamento del genoma di quattro ceppi di lievito *Saccharomyces cerevisiae*. La relazione tra genotipo e fenotipo nel lievito è uno degli argomenti più dibattuti nel campo della vinificazione. Con l'obiettivo di approfondire la conoscenza riguardo alle possibili conseguenze delle variazioni genomiche sulle prestazioni enologiche dei lieviti, questa tesi di dottorato si concentra sulla diffusione di alcune variazioni genomiche nelle popolazioni di lieviti di vigneto e sulle loro implicazioni sul fenotipo tecnologico del lievito.

È stata caratterizzata una popolazione di ceppi brasiliani della specie *Saccharomyces cerevisiae*, prestando attenzione al legame tra le pratiche agricole locali e la biodiversità dei ceppi in vigneto. I risultati hanno mostrato che l'importante uso del rame nella protezione della vite ha contribuito a sviluppare una forte tolleranza a questo metallo nella popolazione di lieviti autoctona. Questo effetto non ha influenzato la biodiversità genotipica dei ceppi di lievito nei vigneti interessati, che si sono stati confermati un'importante riserva per l'isolamento e la selezione dei ceppi di lievito enologici.

La tolleranza verso il rame e i solfiti è stata studiata tra i ceppi *S. cerevisiae* delle collezioni di lieviti italiani e brasiliani. Gli argomenti trattati hanno riguardato il rapporto tra la variabilità del numero di copie del gene *CUP1* e il rapporto tra la tolleranza al rame e la presenza di due traslocazioni cromosomiche e la tolleranza ai solfiti. I risultati hanno evidenziato un'associazione tra i tratti genetici e i fenotipi tolleranti a livello di

popolazione di vigneto, in particolare tra la tolleranza al rame e il numero di copie del gene *CUP1*. È inoltre discussa la possibile associazione tra la tolleranza al rame e quella ai solfiti.

È stato studiato l'assorbimento delle fonti di carbonio in quattro ceppi *S. cerevisiae*. L'espressione dei geni dei trasportatori degli esosi è stata analizzata in fermentazione durante la fase stazionaria. Lo studio ha coinvolto il gene *FSY1* codificante per un trasportatore a simporto fruttosio/H^+ ad alta affinità. L'analisi dell'espressione genica ha evidenziato profonde differenze nell'utilizzo dei trasportatori degli zuccheri esosi, in particolare per i geni per il trasporto di fruttosio: in condizioni enologiche la diversa espressione genica di *FSY1* migliora la capacità di utilizzo delle fonti di carbonio dei ceppi di lievito. Questo lavoro può contribuire a migliorare la caratterizzazione dei lieviti del vino dando uno strumento per la loro distinzione per il fitness nell'ambiente di vinificazione, a livello trascrizionale.

Infine, due ceppi di *S. cerevisiae*, EC1118 e QA23, sono stati studiati in spumantizzazione secondo il metodo Martinotti, utilizzato nella produzione del Prosecco DOC Spumante (Conegliano-Valdobbiadene). Sono state tenute in considerazione la vitalità delle cellule di lievito durante la preparazione del *pied-de-cuve*, l'evoluzione della pressione in autoclave e la risposta delle cellule al raffreddamento del vino alla fine della seconda fermentazione. Durante il raffreddamento forzato del vino le cellule sono state campionate per l'estrazione del RNA totale, da utilizzare nella successiva analisi del trascrittoma. I risultati preliminari mostrano che il lievito EC1118 è stato caratterizzato da una vitalità cellulare sempre inferiore rispetto a QA23, dalla procedura di adattamento all'etanolo e per tutto il processo fermentativo. Questa differenza si riflette sulla cinetica di evoluzione della pressione. Sono inoltre presentati e discussi i dati riguardanti l'estrazione, quantificazione, integrità e verifica della qualità del RNA totale.

1. Introduction

Yeasts are a very large group of microorganisms belonging to the Fungi reign. Yeasts involve, with different roles and impacts, almost all human activities. Yeast is also one of the most important model organisms for studies on eukaryotes and it was proposed for such role in the mid-1930s, by Hershel Roman (Roman, 1981). The advantages deriving by the adoption of this unicellular organism as model organism are a lot, from its ability to easily grow in laboratory conditions, to the small size of its genome with respect to other eukaryote organisms, and other but no less important reasons.

The *Saccharomyces* genus represents the most of the oenological yeasts, such as the *Saccharomyces cerevisiae* and the *Saccharomyces bayanus* species. *S. cerevisiae* is generally associated to grape juices fermentation but it is very ubiquitous yeast: it can be found in several environments such as the surface of fruits or the tree trunks or the soils, but also in more anthropic ones such as food and beverages industries. When talking about "yeast" the first species on mind is *S. cerevisiae*, even if the microbial group surrounds thousands of species.

Considering the oenological environment, *S. cerevisiae* is very common in vineyards and cellars, where it is used as grape musts fermentation starter (the technological process which leads to wine) and in the sparkling wine production protocols. In the production of sparkling wines, in both Champenoise and Martinotti (also known as Charmat) methods, a second fermentation is carried out on a so-called base wine: basically, the method consists on putting a sweetened wine in a pressure-tight vessel (a bottle in the Champenoise method or an autoclave in the Martinotti method), then adding nutrients and yeasts and let fermenting. This receipt allows obtaining a second fermentation (also

known as refermentation), which is carried out for two main purposes: to give bubbles to the wine (literally, sparkling the wine) and to enrich the wine aromas *bouquet*.

The *Saccharomyces cerevisiae* species represent the greatest part of the yeasts used in oenology. The fermenting aptitude and the other essential characters for winemaking can change in intensity among the strains belonging to this species, but almost all of them are able to perform grape must fermentation.

However, since the microbiologists understood that the *Saccharomyces* yeasts were the microbial agents of the grape juices transformations into wines, the winemakers are keeping looking for the best yeast strains for their wines. It has been extensively proven, in fact, that different yeast strains can give highly different outlines to the obtained wines, from the olfactory to the tactile and gustative profiles (Álvarez-Pérez et al., 2012; Cortés et al., 2011; Callejon et al., 2010; Pickering et al., 2008; Torrens et al., 2008; Romano et al., 2003; Egli et al., 2002; Lilly et al., 2000). Such consciousness leaded to a wide, and still increasing, interest on the oenological properties of the *S. cerevisiae* yeast strains.

The technological progresses allowed isolating (mostly from fermenting musts and wines) yeast strains with excellent fermentation properties and obtaining marketable industrial yeast-based products, so that winemakers can use them all around the world, being sure that their fermentations will always lead to good wines. This represented a very important progress in the wine industry area: thanks to these advances the quality of the wines strongly increased, together with the food safety. Indeed, before the use of industrial yeasts, able to ensure rapid and complete fermentations, the vinification was initially led by the indigenous microflora, and only in a second moment the indigenous *Saccharomyces* yeasts could overcome the others. The major risk derives from this time gap, in which the other microbial species could give positive, but also negative

characteristics to the future wine. Moreover, the fermenting strains often were not able to complete the sugars conversion into ethanol, so that a sugar residue remains into the obtained wine. In this context the bacterial contaminants can find an ideal environment where start growing and this eventuality can have negative effects on the final product.

To date, the *S. cerevisiae* yeasts strains used for the wine and sparkling wine production which are available on the market come from selection campaigns mostly performed in cellars but also in vineyards.

The consumers' choice criteria are still changing and winemakers do lots of efforts to meet their preferences: the selection of novel yeast strains, able to give unique characteristics to the finale wines, is one of the most effective tools to reach such goal.

1.1 Wine yeast strains selection from vineyards

The grape berries represent the best starting point for novel wine yeast strains isolation. They are, indeed, the natural habitat of numerous microorganisms, and among them there is the *S. cerevisiae* species. After the isolation, the strains must be submitted for oenological characterization and, if the results will be promising, they can go further in the selection program. This process is named "clonal selection" and provides strains clones which can meet the desired traits for winemaking in different extent, but it allows the constitution of a biodiversity background maintaining.

The selection is generally focused on the *Saccharomyces* genus and isolation starts from grape juices or wines. The main reason why the isolation from berries is that the cells of such yeasts are numerically very scars and the other species would generate an excessive background noise, so an enrichment procedure is needed (Constantí et al., 1997; Versavaud et al., 1995). The method requires a lab-scale fermentation thanks to which the *Saccharomyces* strains can first adapt to the media and growth until becoming the most

present species. In this way the oenological yeasts isolation become easier and more effective. The counterpart of such a compromise is that not all the *Saccharomyces* yeast cells originally present on the grapes will be found at the moment of the isolation: only those which were able to overcome the initial stress, due to the growth media or to the other microorganisms competition, and which were able to handle the worsening of the fermentation conditions (carbon and nitrogen depletion, ethanol concentration increase) will be at a sufficient number to be detected by the isolation. Moreover, this enrichment procedure produces a high clonal duplication background, so after isolation a genetic screening will be needed to identify the isolates deriving from the same cell (clones).

1.2 Selection strategies for new strains of oenological interest

The selection and the genetic improvements targets specific traits of the organisms. In wine microbiology some basic traits have to compulsory be take into account during yeast strains selection and represent the biggest limit, so the selection programs can concentrates on others traits, varying on the basis of the wines to be obtained.

The main oenological yeasts characters are here reported:

Fermentation properties: rapid adaptation to the fermenting medium, high fermentation performances, rates, ethanol tolerance, high osmotolerance, low optimal temperature of fermentation, moderate biomass production;

Technological properties: high genetic stability, sulphites tolerance, low sulphites binding activity, low foam formation, flocculation properties, copper tolerance, resistance di dehydration, killer factor, proteolytic activity, low nitrogen demand;

Flavour characteristics: low volatile acidity production, moderate higher alcohols production, low sulphites/DMS/thiols formation, liberation of glycosylated flavour precursors, high glycerol production, modified esterase activity, enhanced autolysis, hydrolytic activity;

Metabolic properties with health implications: low sulphites formation, low biogenic amine formation, low ethyl carbamate (urea) potential.

The first selection programs were developed to obtaining strains that were essentially able to achieve a better than 98% conversion of grape sugar to alcohol and carbon dioxide, at a controlled rate and without the development of off flavours. The modern selection programs, instead, pay attention also the others quality traits.

1.3 Phenotypic characterization of yeasts

As previously reported, after isolation the cells have to be characterized for their phenotype and genotype. At the beginning of microbiology the taxonomists classified the yeast species using morphological and physiological criteria, meaning that the first classifications were based on phenotypic differences between yeasts: cell shape, size, spore formation, cultivation demand, fermentation ability, carbon and nitrogen source assimilation, growth survival factors, and resistance to cycloheximide. Since then, many rapid, effective and easy diagnostic kits have been developed to determine yeast response to different physiological tests (Ribéreau-Gayon et al., 2006).

During a selection program the most used phenotype-based tests for *Saccharomyces* species distinguishing from others are based on the selective growth media and phenotypic evaluation of the colony colour and morphology (i.e. on WL nutrient agar). On the counterpart, *S. cerevisiae* yeasts form colonies which can only slightly differs

between the strains of the same species. Moreover, on this kind of media the morphological characteristics can be unstable under several multiplications. Thus, this approach cannot be considered decisive, since possible variations at strain level could lead to erroneous attributions. It is therefore currently accepted that phenotypic analyses are not sufficient to reach a complete identification (Kurtzman and Robnett, 2003).

1.4 Selection of autochthonous yeast strains

In the last few years several studies were performed aimed to improve the selection criteria of wine yeasts, in order to enhance the winemaker's tools for wine quality improving. As a result, there has been an increasing use of new local selected yeasts for controlled must fermentation in long-standing winemaking tradition countries.

Local yeasts are supposed to be more competitive, as they should be better acclimated to the specific environment, and so they can better dominate the fermentation becoming the biological agent responsible for the vinification. Selection of local yeasts contributes allowing the maintenance of the typical sensory properties of the wines produced in a given region (*terroir*).

It is believed *S. cerevisiae* autochthonous that strains from specific vineyards and/or wineries tend to show high homozygous levels for most of the genes. The process supposed to be responsible for that is known as "genome renewal" (Mortimer et al. 2000). This process seems eliminate the recessive lethal, or deleterious, genes that potentially compromise the yeast fitness (e.g. slow growth and fermentation rates, reduced spore viability …). Genome renewal seems also be responsible for the population substitution of the original heterozygous strains by the derivate homozygous diploids, which bear new recessive alleles that potentially increase the fitness. Although dramatic improvements in most characteristics is almost impossible, the intra-strain selection has been used for

decades to obtain improved wine yeast strains and is still, up to date, one of the most utilized selection strategies (Pretorius, 1999).

1.5 Genetic Characteristics

S. cerevisiae strains in natural condition are generally diploid. They are characterized by vegetative reproduction, through multi-polar budding (from here the appellative of "budding yeast"). Under specific nutritional conditions the cells may sporulate forming four haploid spores of different mating types (a or α). Wine strains are mostly homotallic, and descendants of haploid spores mate with their own progeny to form a diploid. Homotallism is frequent in wine yeast: around the 70% of the strains are known to be homotallic (Mortimer et al., 2000). Upon sporulation and the self-mating of homothallic spores, homozygous diploids are generated. This process allows eliminating recessives mutation deleterious for the strains, or allowing the expression of recessive mutation which can increase the strains fitness. Genome renewal is therefore likely to be involved in the yeasts adaptation to stressful wine environment. Poor knowledge there is about the sexual activity of yeasts in wine environments. The frequency of yeasts sporulation and mating in such environment is unknown. If most strains are diploid, some were found to be polyploid or aneuploid (Bakalinsky and Snow, 1990).

S. cerevisiae has a small (75 kb), circular mitochondrial DNA genome devolved to encode a small set of proteins. Such proteins are mostly involved under respiration metabolism.

Mitochondrial DNA is not essential for yeast survival, but it has been observed that ethanol tolerance can depend on it: the ethanol tolerance of a laboratory strain could be enhanced by flor yeast mitochondria introduction (Ibeas and Jimenez, 1997).

1.7 Next generation sequencing technology

In the recent past scientists did great development of high-throughput and low-cost sequencing platforms, which rapidly increased the number of sequenced genomes and the transcriptional profiling of the organisms, but also studying the chromatin structures and non-coding RNAs.

Such technologies, known under the name of Next Generation Sequencing (NGS) technologies, have a great impact on research opportunities, by increasing data production and costs lowing. The application field can be divided into three main arguments:

- genomic tasks (genome assembly, SNPs and structural variations);
- transcriptome analysis (gene prediction and annotation, alternative splicing discovering);
- epigenenome investigations.

GS FLX by 454 Life Sciences/Roche diagnostics, Genome Analyzer, HiSeq, MiSeq and NextSeq by Illumina, Inc., SOLiD by ABI, Ion Torrent by Life Technologies, and some others represent the second generation sequencing technologies. These high-throughput sequencing systems, using new sequencing chemistries, replaced the Sanger's technology avoiding electrophoresis and individual amplification of the templates. They are based on the parallelization of the sequencing process to produce thousands of sequences at once and lower costs and time required for DNA sequencing (Zhou et al., 2000). Evolution in sequencing technology is running fast, and there are already present platforms of for the third generation sequencing: Helicos™ Genetic Analysis System by SeqLL, LLC, SMRT Sequencing by Pacific Biosciences, Nanopore sequencing by Oxford Nanopore's, Complete Genomics by Beijing Genomics Institute and GnuBIO by BioRad, and others (Ambardar et al., 2016).

Thanks to these powerful technologies is possible to sequence lots of genomes, getting several information comparing them. As previously said, the sequencing of wine yeast strains can be a powerful approach to identify the still unknown genes involved in fermentation, but also those ones involved in the aromas production. Moreover, the transcriptional profile of a strain can be used to identify the differentially expressed genes with respect to other strains, and to highlight those genomic differences mirrored in the gene expression and in the phenotype.

1.6 From genotype to phenotype

The association between different oenological phenotypes and specific molecular patterns can be an effective tool to simplify the characterization of the indigenous yeast populations during wine yeast selection.

Recently, a close correlation between molecular polymorphism and specific phenotypic traits was reported in non-*Saccharomyces* wild yeast strains (Rodríguez-Navarro et al., 2004). However, the results obtained from genotype–phenotype relationships studies in wild *S. cerevisiae* populations leaded to unclear conclusions (Nadal et al., 1996; Comi et al., 2000). The correlation was estimated taking into account the total number of isolates as a whole: when this statistical method is applied, very low correlation coefficients are obtained. The use of more powerful statistical tools (i.e. the Generalized Procrustes Analysis (GPA) for the simultaneous analysis of molecular and physiological traits – Gower, 1975), allows to weigh the relationships for each isolate, denoting a better degree of agreement between molecular and physiological data. Application of the GPA in on the genetic and/or phenotypic variability studies in microbiology allows quantify the

relationship between molecular and phenotypic characteristics in wine yeasts (Lopes et al., 2006).

The NCBI Genome Project Database reports 46 genome sequencing projects of *S. cerevisiae* strains, but only the S288c genome is completed. The sequenced strains include isolates from various origins.

Most of the sequencing projects leaded to the comparison of the genomes to genomic traits of specific phenotypes, inferring phylogenetic relationships and evolutionary histories. The analysis of closely related strains has been performed too: for example, the genomes of six industrial strains of *S. cerevisiae* used in wine fermentation and brewing were compared to find characteristics typical of these industrial classes of yeast (Borneman et al., 2011).

Regularly updated information concerning the genomic and functional analysis of yeasts is available on several databased, such as the Génolevures project web site (Souciet, 2011), the Stanford Genome Database (SGD), the Munich information Centre for Protein Sequences Comprehensive yeast Genome database (MIPS CYGD) and the Yeast Proteome Database (YPD).

Genome-wide transcriptional profiling has important applications in evolutionary biology, for example allowing studying the heterozygosity extent for alleles showing quantitative variation in gene expression in natural populations. The perspectives opened thanks to such studies stimulated renewed interest on the interactions between the metabolic pathways and the metabolic flux control by the cells.

The genetic variability of the wine yeasts has been demonstrated by several analysis molecular tools (Schuller *et* al., 2004). The major differences between *S. cerevisiae* laboratory strains have been found in sub-telomeric regions (Winzeler *et* al., 2003).

Moreover, the *S. cerevisiae* wine strains show a gene Copy Number Variation (CNV) that differentiates them from the laboratory and clinical origin strains. The main differences were found in genes related to the fermentative process, such as membrane transporters, ethanol metabolism and metal resistance (Dunn *et* al., 2005; Carreto *et* al., 2008).

1.8 Phylogenetic relationship

The *S. cerevisiae* genome has been influenced by the association of this species with the human activities: this resulting consequence is due to the combined effect of multiple independent cycles of wild yeast domestication and thousands generations of artificial selection.

The resulting genomes are similar for some technological traits, as they were influenced by the desirable yeast properties. Such multitude of conscious as unconscious selection schemes lead to highly specialized *S. cerevisiae* strains, suitable for specific industrial applications (Querol *et* al., 2003; Fay and Benavides, 2005).

During genome characterization studies on different industrial *S. cerevisiae* strains uncovered several interesting differences, which included Single Nucleotide Polymorphisms (SNPs), strain-specific ORFs and Copy Number Variations (CNV).

However, the complete understanding of the origin, the role and the effect on phenotype of such genomic variations is still limited by the available technology (e.g. CGH arrays relying on the laboratory strain as a ‘‘reference’’ genome), or by the economic resources required for the production of high quality genomic assemblies: this setback limited the number of whole-genome sequences available for comparison, for example.

Moreover, with the aim of limiting the genome complexity at understandable level, the most of the whole-genome sequencing studies on industrial strains used haploid representations of diploid. To furthermore understand the extreme simplification that the such studies did, is to take on mind that the haploid derived from heterozygous diploids of both commercial and environmental strains (Liti *et* al., 2009; Borneman *et* al., 2008; Doniger *et* al., 2008; Argueso *et* al., 2009).

1.9 Project outline

The project of this work arisen from a previous work of vineyard yeast isolation, collection and characterization which was held at the Wine Microbiology laboratory of the University of Padova in Conegliano (CIRVE). Some genomic variations have been uncovered thanks to the genome sequencing of four *Saccharomyces cerevisiae* yeast strains: P283, P301, R008 and R103 (Treu *et* al., 2014).

As the relationship between the yeast genome on its phenotype is one of the most debated topics in wine microbiology, this work was aimed to trying deepening the knowledge regarding the genomic variations effects on the oenological performances of wine yeasts. The work has been divided into four main sections, each focused on a specific aspect of the genomic variability and phenotype relationship.

In the present study the oenological characterization of Brazilian *Saccharomyces cerevisiae* yeast strains has been performed, paying attention to the connection between the local agricultural practices and the strains biodiversity in vineyard. Brazil, one of the main new winemaking countries, is indeed an interesting potential reservoir of novel wine yeast genotypes collection, for both technological and research purposes.

The strains copper and sulphites tolerance have been studied on the Italian and Brazilian *S. cerevisiae* strains collections. The study topics were the assessment of the copper and sulphites level, together with the relationships between the *CUP1* copy-number variation and the copper tolerance, and between the presence of two chromosomal translocations and the sulphites tolerance.

A focus has then been pointed on the fermentable carbon sources uptake in *S. cerevisiae*. The expression of the hexose transporters genes has been analysed by Real Time PCR during the stationary phase of synthetic must fermentation. The study involved the *FSY1*

gene, found in EC1118 and coding for a high affinity fructose/H^+ symporter (Galeote *et* al., 2010). The *FSY1* presence in vineyards and hexose transporters genes expression under oenological conditions have been investigated.

Lastly, *S. cerevisiae* strains EC1118 and QA23 strains, both used in the production of the Prosecco DOC Sparkling wine (Conegliano-Valdobbiadene) have been studied under the Martinotti's sparkling wine production method. The yeast cells viability during the *pied-de-cuve* preparation, the pressure evolution in autoclave and the cells response to the wine chilling at the end of the second fermentation were taken into account. With the aim of better understanding how cell transcriptome changes during the wine chilling, and so trying to understand which metabolic pathways are involved in the yeast responses to this technological procedure, the cells have been recovered for the total RNA extraction to be used in the analysis and study of the transcriptional profiles.

2. Chapter I

Genetic variability and physiological traits of *Saccharomyces cerevisiae* strains isolated from "Vale dos Vinhedos" vineyards reflect agricultural practices and history of this Brazilian wet subtropical area

The contents of this chapter are based on the published paper:

Title: ***Genetic variability and physiological traits of* Saccharomyces cerevisiae *strains isolated from "Vale dos Vinhedos" vineyards reflect agricultural practices and history of this Brazilian wet subtropical area.***

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2.1 Introduction

In fermentation practices, such as winemaking, *Saccharomyces cerevisiae* plays a crucial role. Therefore, lots of efforts have been made to select, from the environment, those strains that possess the technological traits suitable for industrial purposes. Recently, grape marcs, the winemaking by-product, were demonstrated to be a good source for isolation of novel yeasts for biotechnological applications (Favaro et al., 2017). In the same way, vineyard environment is of crucial importance, as, due to the presence of the grape microbiota, can be considered a genetic reservoir of yeasts possessing both traditional and innovative technological characters. Many studies have been focused on strain presence and genetic variability in vineyards and during spontaneous fermentation in order to quantify their number and characteristics (van der Westhuizen *et* al., 2017; Viel *et* al., 2017; Fay and Benavides, 2005; Schuller *et* al., 2005; Cappello *et* al., 2004; Redžepović *et* al., 2002; Mortimer and Polsinelli, 1999; Pérez-Coello *et* al., 1999; Versavaud *et* al., 1995). Moreover S. cerevisiae abundance and genetic differences have been correlated with agricultural practices (Cordero-Bueso *et* al., 2010), grape variety, geographical distances (Schuller *et* al., 2012; Garofalo *et* al., 2016) and vineyard diffusion of commercial starters (Valero *et* al., 2005, 2007). Most of these studies are related to geographical areas and wineries from traditional winemaking countries, but a very small number explore yeast vineyards diversity in new winemaking countries such as China (Yang *et* al., 2013), India (Chavan *et* al., 2009), Russia (Kachalkin *et* al., 2015), and Brazil (Baffi *et* al., 2011; Da Silva *et* al., 2016).

Brazilian grape cultivation is present mainly in the southern states: Rio Grande do Sul, São Paulo, Pernambuco, Paraná, Bahia, Santa Catarina and Minas Gerais. The greater volume of Brazilian wine production is localized to the temperate southern region,

particularly the South East hills of Rio Grande do Sul State (Echeverrigaray *et* al., 2013). The most relevant characteristic of Brazilian viticulture is the diversity of grape varieties, the environments and the production systems (Camargo, 2011). Moreover, the wines produced in these regions can be classified into two main groups: fine and table wines, produced with traditional Vitis vinifera varieties (Merlot, Cabernet Sauvignon and Cabernet Franc), or Vitis labrusca varieties (Bordeaux and Isabel), respectively (Schuck *et* al., 2009).The greatest production of Brazilian wine comes from Rio Grande do Sul state (Ramírez‐Castrillón *et* al., 2017), accounting around 90% of the Brazilian wine production (Mello, 2014). In this way, Vale dos Vinhedos is the most important wine area in Brazil, located in the north-east of Rio Grande do Sul (Brazil) and lies on three municipalities: Bento Gonçalves, Garibaldi and Monte Belo do Sul respectively for 60%, 33% and 7%. The average altitude is 742 m above the sea level and the total extension of the area is 81,123 km2. The surface is variously covered: vineyards interest the 26% of the total, forests 43%, other farming activities 31% and human activities interest the 10% of the total.

Vale dos Vinhedos has a recent history as wine region. Although wine production started around 1620 with the arrival of Jesuit fathers, only in the early 20th century Italian emigrants were able to start winemaking using Vitis vinifera vines. Around 1970, due to several investments from foreign wine companies, industrial winemaking took place and only in the last 2 decades Brazilian wine companies started to face first the local and, very recently, the international market (Fensterseifer, 2007).

Among the technological traits that have to be considered in order to evaluate strain aptitude to winemaking, fermentation performance is the most important. Yeast copper and sulphites tolerance is also relevant, as those chemicals are strongly associated to vine

growing and winemaking practices. Copper homeostasis has been extensively studied in *S. cerevisiae* (De Freitas *et* al., 2003; Fernandes and Sa-Correia, 1999; van Bakel *et* al., 2005). This yeast species exhibits a significant variability in copper resistance and the acquisition of this trait seems to be the result of an environmental adaptation (Azenha *et* al., 2000; Romano *et* al., 2008; Shinohara *et* al., 2003). In traditional winemaking countries, since the end of the eighteenth century, the properties of sulphur dioxide (antiseptic, antioxidant and antioxidasic) made it a fundamental tool in winemaking (Ribéreau-Gayon *et* al., 2006). *Saccharomyces cerevisiae* sulphites tolerance and production are well studied (Divol *et* al., 2012; Nardi *et* al., 2010) and have been demonstrated to be strain-dependent (Treu *et* al., 2014; Nadai *et* al., 2016). Finally, hydrogen sulphide production is generally investigated as related to a well-known wine off flavour (Fleet, 2003; Mendes-Ferreira *et* al., 2009).

This study was the first attempt to investigate genetic and phenotypic variability of *Saccharomyces cerevisiae* strains isolated from Brazilian vineyards in order to evaluate strain aptitude to winemaking. Grape bunches were collected from vineyards of Vale dos Vinhedos Appellation of Origin area and yeast isolation was performed after single bunch fermentation. The isolates were genotyped and a phenotypic characterization was performed in order to evaluate strains fermentation performances, copper and sulphites tolerance together with hydrogen sulphide production.

Finally, in this paper, the possible correlations among yeast genotypes, phenotypic traits and sampling areas are discussed.

2.2 Material and methods

2.2.1 Wine making areas, grapevine varieties and sampling

Grape bunch samples of Merlot grape variety were collected in 2014 in three vineyards of the winemaking area Vale Dos Vinhedos, the most important Appellation of Origin of the Rio Grande do Sul state (Brazil). Merlot, the most relevant grape variety cultivated in this area. Samplings were performed in the vineyards of three different wineries. Grape bunch samples were collected from 3 to 5 days before harvest and samplings were organized in order to cover the whole areas where the selected grape variety was cultivated. The different number of sampled subareas was due to the diffusion in each region of the selected varieties. The geographic location of the sampled areas is reported in **Fig. 1.1**.

Sampling area 1 (SA1) is the largest, with approximately 91,000 sqm and lies at 2376 – 450 meters above sea level (masl). The Sampling area 2 (SA2) is the smallest one, with a surface of about 15,400 sqm, and lies at 650-700 masl. The Sampling area 3 (SA3) lies at 500-550 masl and covers a surface of about 21,250 sqm. SA2 is geographically separated from the others by a wide valley that it is crossed by several rivers and includes wooded areas.

The sampling and the sample processing was performed as described by Viel *et* al. (2017). Briefly, stomacher sterile bags have been used to collect about 300-400 grams of grapes (corresponding to one bunch). Each bag (from here onwards called “sample”) have been sealed, identified with a sample code (UM-progressive number) and then transferred to the laboratory.

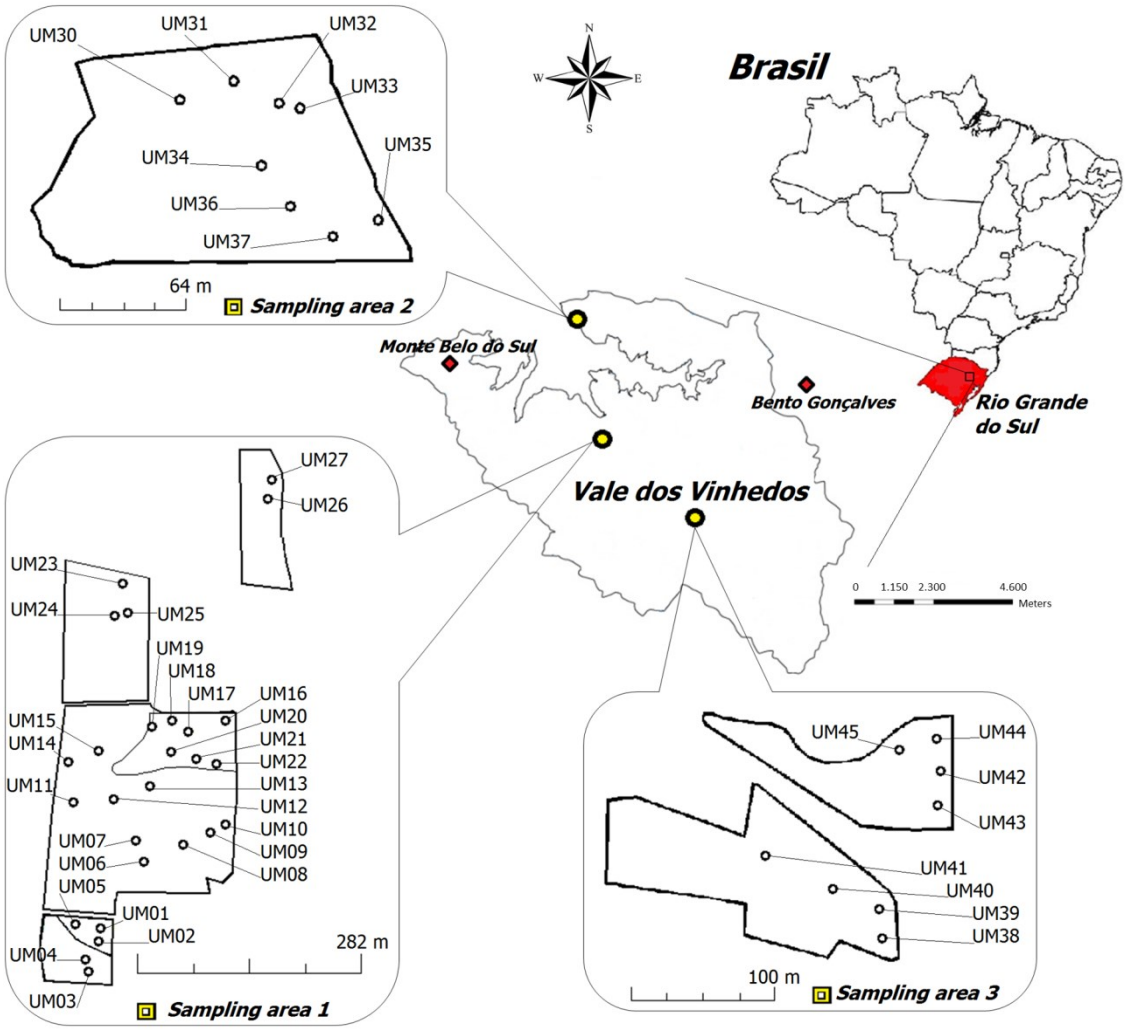


Figure 1.1

Vale dos Vinhedos Appellation of Origin area and geographic distribution of collected samples.

Then, to each sample half fructose and half glucose, corresponding to 2% of grapes weight, have been added with 500 µl of sulphur dioxide at 5% vol., in order to promote the grown of *Saccharomyces cerevisiae* yeast species. Samples have been manually pressed and let them spontaneously ferment at room temperature. The fermentation process has been monitored by daily weight loss until the stabilization of the weight, which indicates the end of the fermentation. Samples which, in the first week of fermentation, reached less than the half of the average total weight loss value (5%) were discarded.

At the end of the fermentation, samples from each bag were diluted and spread on WL plates (Oxoid). The plates were incubated for 2 days at 25°C.

For each single bunch fermentation a total of 20 colonies, randomly chosen from isolation plates from the same dilution series, was collected and stored at -80°C in a 40% vol. glycerol solution.

2.2.2 Species identification

From the isolation plates, microscope observation, Multiplex PCR (Nardi *et* al., 2006) and HRM analysis (Nadai *et* al., 2018) have been used to discriminate colonies generated by yeasts and to identify *Saccharomyces cerevisiae* species. DNA extraction and PCR conditions were carried out as reported by Bovo *et* al. (2012).

2.2.3 MtDNA RFLP analysis

In order to group and evaluate the genetic variability among the *Saccharomyces cerevisiae* isolates based on their genotype, the Querol and Ramon (1992) method has been followed. Briefly, the isolates were plated on YM agar medium and incubated for 48 h at 25°C. Then, yeast colonies were resuspended in 1 mL of sterile water, centrifuged for 3 minutes at 14,000 rpm and processed for the DNA isolation. The DNA digestion was carried out using *Hinf*I enzyme (Fermentas, Thermo Scientific, Waltham, MA, EUA). After the digestion, DNA fragments were separated by electrophoresis on agarose gel, as described by Bovo *et* al. (2011). The mtDNA restriction profiles were analysed using BioNumerics® 6.6 software (Applied Maths) and yeast isolates showing the same genotype were identified by the same code ("P" followed by a progressive number).

2.2.4 Fermentation kinetics trials and H_2S production

One isolate for each genotype has been chosen in order to test the differences among the genotypes present in the collection. Fermentations were performed in three biological replicates for each isolate at 25°C in synthetic must (SM) (Delfini and Formica, 2001). Synthetic must was composed as follows: 0.1 gL^{-1} $CaCl_2$, 0.1 gL^{-1} NaCl, 1 gL^{-1} KH_2PO_4, 0.5 gL^{-1} $MgSO_4 \cdot 7H_2O$, 3 gL^{-1} tartaric acid, 200 mgL^{-1} $NaMoO_4 \cdot 2H_2O$, 400 mgL^{-1} $ZnSO_4 \cdot 7H_2O$, 500 mgL^{-1} H_3BO_3, 40 mgL^{-1} $CuSO_4 \cdot 5H_2O$, 100 mgL^{-1} KI, 400 mgL^{-1} $MnSO_4 \cdot H_2O$, 0.4 mgL^{-1} $FeCl_3 \cdot 6H_2O$, 400 mgL^{-1} pyridoxine hydrochloride, 400 mgL^{-1} thiamine hydrochloride, 2 gL^{-1} inositol, 20 mgL^{-1} biotin, 400 mgL^{-1} calcium pantothenate, 400 mgL^{-1} nicotinamide, 200 mgL^{-1} p-aminobenzoic acid, 0.3 gL^{-1} $(NH_4)_2SO_4$, 0.3 gL^{-1} $(NH_4)_2HPO_4$, 200 gL^{-1} glucose, 0.2 gL^{-1} casein hydrolysate, 2 gL^{-1} malic acid. The final pH was adjusted to 3.2 with KOH. The test took place in 100 mL glass Erlenmeyer flasks

sealed with a silicon cap. The cap was supplied with a folded Pasteur pipette to allow the produced CO_2 to flow out. A loopful of three days cells grown on YPD agar plate (yeast extract 10 g L^{-1}, peptone 10 g L^{-1}, dextrose 20 g L^{-1}, agar 16 g L^{-1}) for each isolate has been used to inoculate 10 mL of liquid YPD broth (yeast extract 10 g L^{-1}, peptone 10 g L^{-1}, dextrose 20 g L^{-1}) and grown for 24 h at 25°C. The flasks were sterilized, filled with 100 mL of SM and inoculated with 10^6 cfu mL^{-1} cells, collected from the pre-cultures. Alcoholic fermentation was monitored by measuring the weight loss twice a day during the whole fermentation process. At the end of the fermentation, the residual sugars was determined by HPLC analysis using Waters 1525 HPLC binary pump equipped with a Rezex RPM-Monosaccharide 300×7.8 mm column. Refractive Index Detector (Waters, Milford, MA) set to 600 nm was used for the determination of glucose.

The production of hydrogen sulphide was evaluated by streaking each strain on Biggy agar (Oxoid Ltd, UK).

2.2.5 $CuSO_4$ tolerance assay

A loopful of three days plated culture for each isolate has been used to inoculate 5 mL of liquid YPD broth. After 24 hours of incubation at 30°C a stationary phase culture, with approximately 10^7-10^8 cells mL^{-1}, has been obtained. Fifty µl of the yeast culture were resuspended in 5 mL of fresh YPD broth and grown for 4 hours at 30°C to obtain an exponential phase culture. Then, 10 µl of this yeast culture (in order to obtain a starting OD_{600nm} of 0.1, approximately 10^6 cells mL^{-1}, were inoculated into 96-wells sterile microplates (Greiner Bio-One, Germany) filled with 300 µl of YNB broth (Yeast Nitrogen Base 6.7 g L^{-1} – Sigma®, glucose 20 g L^{-1}) with the addition of $CuSO_4$ at different concentrations (0.1, 0.4, 0.8 and 1 mM). Microplates were incubated at 30°C

and the cell turbidity (OD_{630nm}) was monitored at 18 and 48 hours from inoculum using a microtiter plate reader (SpectraMax® M5, Molecular Devices Corporation - USA). The assay has been performed in triplicate. The effect of copper on yeast growth was evaluated using the ratio between the OD_{630nm} values measured in present of $CuSO_4$ and in the control condition.

2.2.6 SO_2 tolerance assay

For each isolate, a loopful of three days YPD plated culture has been used to inoculate 10 mL of liquid YPD broth (yeast extract 10 g L^{-1}, peptone 10 g L^{-1}, dextrose 20 g L^{-1}) and grown for 24 h at 25°C. Fifteen mL capacity tubes have been sterilized, filled with 11.2 mL of SM (Delfini and Formica 2001) with 50 or 100 mg L^{-1} SO_2, and then inoculated with 800 µl of pre-cultures in order to obtain a starting OD_{600nm} of 0.1 (approx. 10^6 cells mL^{-1}). Control yeast cultures were prepared with the same procedure without the SO_2 addition. Cell turbidity (OD_{600nm}) was monitored at 24 and 48 hours using a spectrophotometer (UV-VIS Spectrophotometer UV-2600, Schimadzu – Japan). The effect of SO_2 on yeast growth was evaluated using the ratio between the OD_{600nm} values measured in present of SO_2 and in the control condition.

2.2.7 Principal Component Analysis

For Principal Component Analysis (PCA) the XLSTAT Software (version 7.5.2) has been used. The variables used were: fermentation vigour (g CO_2 (100 mL)$^{-1}$ produced after 48 h), the CO_2 production at 168 h (g CO_2 (100 mL)$^{-1}$), the fermentation length and the

growth decreases due to the presence of sulphites or copper sulphate in the growth medium.

Regarding the growth decreases, for each concentration and for each time point the ratio between the observed OD value in treated sample and the control (without added chemicals) has been calculated as percentage (%OD). The growth decrease (GD) for each concentration of sulphate or copper was calculated as follows: GD=100-%OD. Finally, for each strain the sum of all the GD values has been calculated (SGD_{SO2} and SGD_{Cu}) and used as variable for describing the tolerance to the relative chemicals. The assay was performed in triplicate.

2.3 Results

2.3.1 *Saccharomyces cerevisiae* isolated from vineyards

Grape bunch samples of Merlot variety were collected in three different vineyards of Vales dos Vinhedos Appellation origin, Rio Grande do Sul (**Figure 1.1**).

A total of 43 samples, each one composed of a single grape bunch, were collected: 27 from the SA1 (UM01 to UM27), 6 from the SA2 (UM30 to UM35) and 10 from the SA3 (UM36 to UM45). For each sample, single bunch fermentations were performed and the fermentation process was monitored by measuring the sample daily weight loss. Ten samples were discarded as the fermentation process was sluggish, indicating the absence of strong fermenting species such as *S. cerevisiae*. From the remaining 33 samples a total of 450 yeast isolates have been obtained. Among them, 223 were identified as *S. cerevisiae* by molecular methods. *S. cerevisiae* was present in 21 samples: 12 out of 27 collected in SA1, 5 out of 6 in SA2 and 4 out of 10 in SA3. Among them, in 5 samples 100% of the isolates were *S. cerevisiae*, while in the remaining samples the range was between 8.33% and 95.00% (**Figure 1.2**).

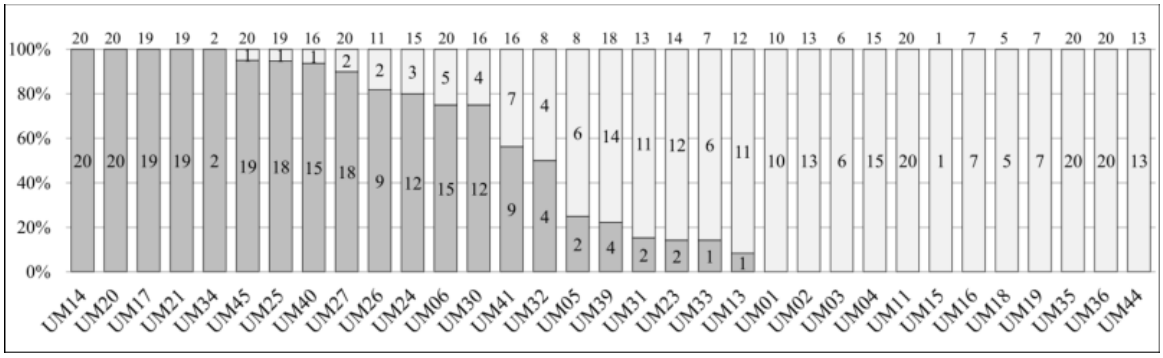


Figure 1.2

Frequency of *Saccharomyces cerevisiae* in each single bunch fermentation. Dark grey bars indicate *S. cerevisiae* percentage while light grey bars other yeasts species. On the top of the bars the total number of isolates is indicated

2.3.2 Evaluation of *Saccharomyces cerevisiae* genetic diversity by mtDNA RFLP analysis

All the collected isolates from the 33 single bunch fermentation have been analysed by mtDNA RFLP analysis, in order to discriminate the different *S. cerevisiae* genotypes (Lopez *et* al., 2001; Querol *et* al.,1 996). This, together with Delta sequences and multi-locus microsatellites analysis, is one of the most used methods for wine yeast strain identification. (Viel *et* al., 2017; Capece *et* al., 2010; Legras and Karst, 2003; Sabate *et* al., 2002; Vezinhet *et* al., 1990, 1992). MtDNA has an elevate mutation rate (Galtier *et* al., 2009; Zeyl, 2000), therefore if recent genetic differences are present they are preferentially included in the mtDNA. During single bunch fermentation clonal populations develop due to cell duplication: DNA typing by mtDNA analysis allows identifying clonal isolates and strains number.

Therefore, in the single bunch fermentation, each group of isolates that shared the same mtDNA profile has been considered as a single genotype.

A total of 63 different mtDNA genotypes has been found (P1-P63) out of the 223 *S. cerevisiae* isolates (**Figure 1.3**).

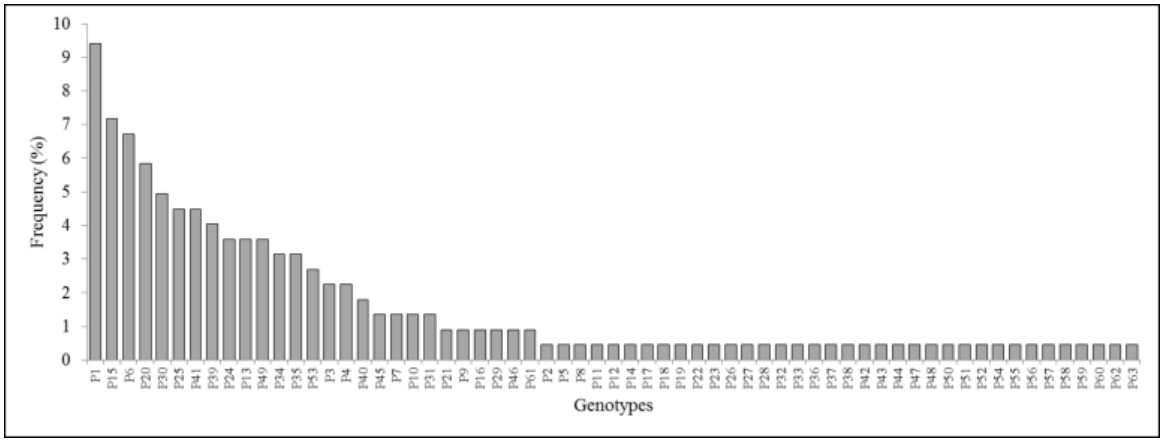


Figure 1.3

Relative frequency of *Saccharomyces cerevisiae* genotypes

The mtDNA RFLP genotypes of 8 commercial strains, which are commonly used in the wineries of this winemaking region, have been added to the dataset, and included in the cluster analysis. No overlapping between mtDNA genotypes of yeast isolates and commercial strains was evidenced, showing no commercial strain dissemination in the sampled vineyards (**Supplementary 1**). Regarding the genotype frequency, 66.6% of genotypes were found only once or twice among the 223 yeast isolates, while the genotypes which identified more than 10 yeast isolates from the same sample represented only the 8% (**Figure 1.4**). The most represented mtDNA genotypes, among the 223 yeast isolates, have been found in SA1. They were P1, P15 and P6, that identified 21, 16 and 15 *Saccharomyces cerevisiae* isolates, respectively.

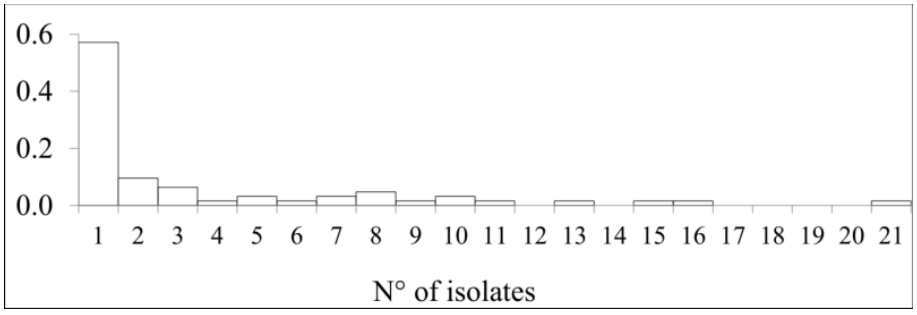


Figure 1.4

Relative frequency of genotypes among *Saccharomyces cerevisiae* isolates

High genotype variability per sample was found, as shown in **Figure 1.5**. The highest numbers of genotypes per sample were present in UM21 (SA1) and UM45 (SA3) where 8 and 7 genotypes were identified, respectively. Four samples (UM23, UM34, UM13 and UM33) showed only one genotype, even when more than one *S. cerevisiae* isolate was collected. In the case of ten genotypes, each one was isolated from two samples (P13, P15, P21, P25, P30, P34, P39, P45, P49 and P53). Only 4 of them (P13, P30, P45, P49) were identified in samples collected from neighbouring vine plants.

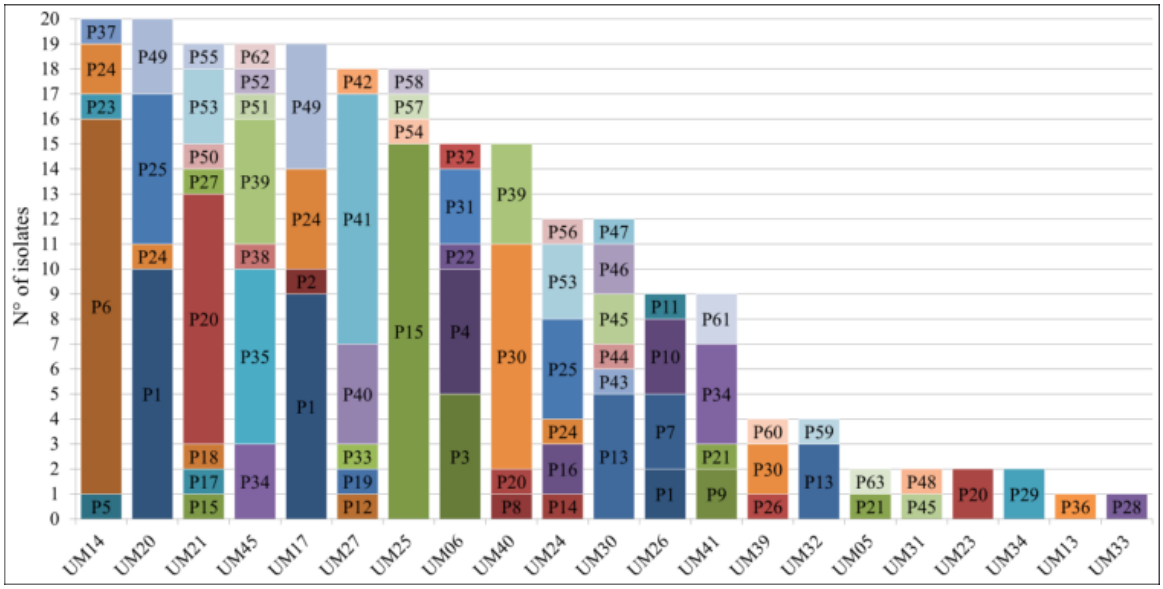


Figure 1.5

Genotype abundance in single bunch fermentations

The most spread genotypes in the vineyards were P24, P20 and P1. P24 was found in 4 samples (UM14, UM20, UM17 and UM24) all collected from SA1; both P1 and P20 have been fund in 3 different samples. All P1 (UM20, UM17, UM26) samples were collected from the SA1, while P20 samples (UM21, UM23, UM40) were collected from SA1 and SA3.

MtDNA cluster analysis (**Figure 1.6**) showed a genetic variability which ranged from 45.40 to 96.30 % of similarity value. Three major clusters were identified using a similarity value threshold of 50.00 %. The genotype distribution between the sampling areas is reported in **Fig. 6**. The first cluster (I) contains 21 genotypes: 16 from the SA1, 2 from SA2 and 3 from the SA3; the second cluster (II) contains 20 genotypes: 7 from SA1, 8 in SA2 and 5 in SA3; finally, the third cluster (III) contains 22 genotypes: 15 genotypes from SA1, 5 genotypes from SA3 and 2 genotypes (P20 and P21) found in both SA1 and SA3. In the third cluster no genotypes have been isolated from SA2 (see table in **Figure 1.6**).

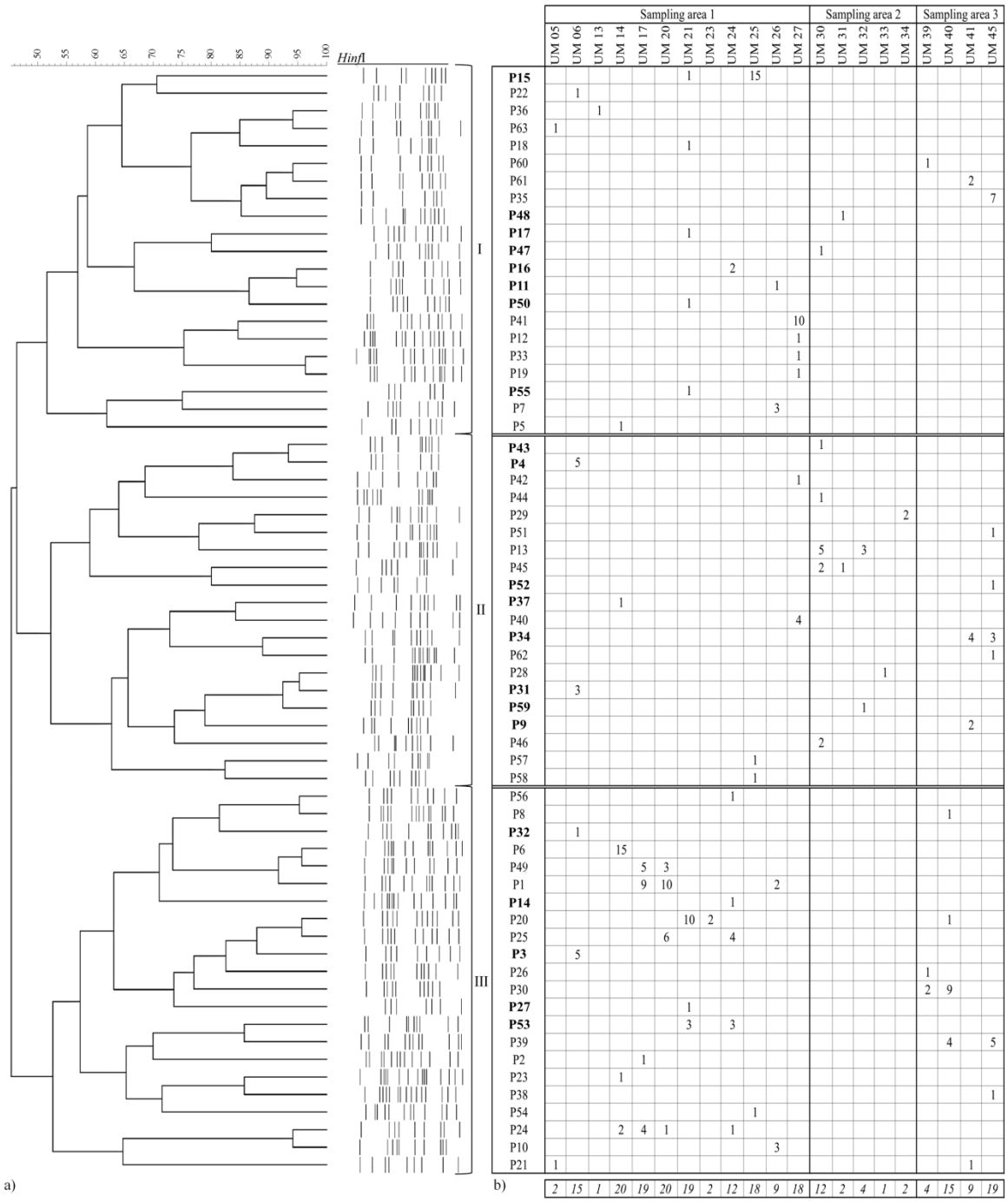


Figure 1.6

MtDNA RFLP cluster analysis using Dice's similarity coefficient and setting as optimization and tolerance the values recommended by the software (optimization 0.0%; tolerance 1.55%). Dendrogram was generated by the unweighted pair group method using arithmetic average (UPGMA) clustering algorithm, calculated by BioNumerics® software 6.6 (Applied Maths) (a). For each genotype, the sample name, the sampling area and the abundance are indicated in the table (b)

2.3.3 Fermentation performances

One isolate for each of the 63 genotypes has been selected to evaluate the fermentation performances. Fermentations were carried out in standard synthetic must (Delfini and Formica, 2001) at 25°C. The CO_2 production was monitored during fermentation process. To assess strain fermentation performances, the fermentation vigour, in terms of CO_2 production after 48 h of incubation, was considered in order to evaluate the adaptation ability to the must conditions. CO_2 production after 168 h was considered, as at this fermentation step the widest range of CO_2 production was found between strains. The daily increment of CO_2 production after168 h decreased very rapidly indicating that the strains reached the stationary phase. Therefore after 168 h yeast cells can be considered in the mid/late-stationary phase. The fermentation time (days of fermentation) were reported, as well (**Supplementary 2**). Box plot analysis (**Figure 1.7**) revealed that the variability of the values related to CO_2 production and fermentation time was narrow, indicating similar fermentation behaviour among the strains.

Nevertheless, P9, P14 and P62 showed the highest CO_2 production at 48h (ranging from 3.03 to 3.30 g $(100\ mL)^{-1}$), while P7, P21, P24, P33 and P36 the lowest (from 0.39 to 1.79 g $(100\ mL)^{-1}$). When CO_2 production at 168 h was considered, the fast fermenting P9, P14 and P62 confirmed the high fermentation rate producing 8.67, 8.79 and 8.51 g $(100\ mL)^{-1}$, respectively. Strains showing the genotypes P7, P21, P24, P33 and P36 at 168 h produced 6.34, 6.50, 6.72, 5.90, 1.69 g $(100\ mL)^{-1}$ of CO_2 confirming the slow fermenting rate during the exponential and early-stationary phase.

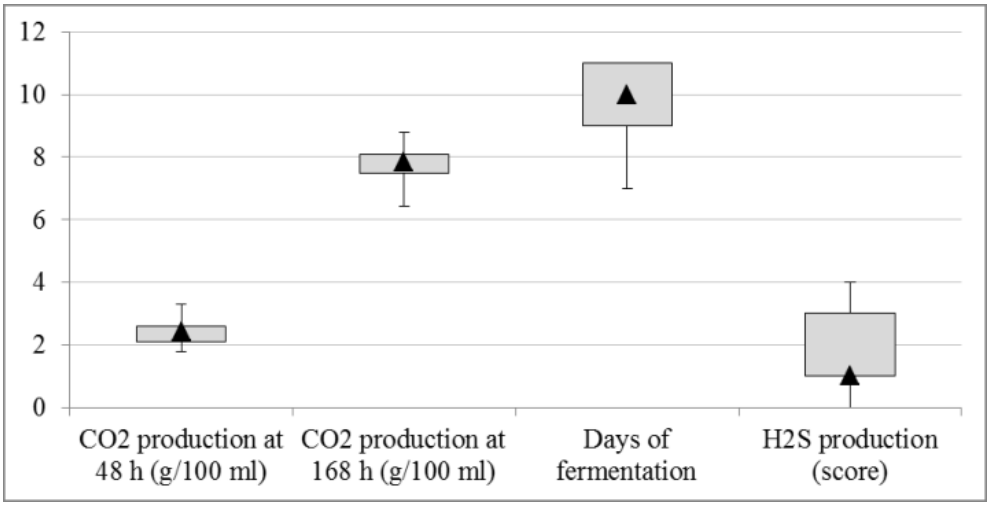


Figure 1.7

Strains fermentation performances in synthetic must and H_2S production. The production of hydrogen sulphide was determined on BIGGY agar (DIFCO), scoring the browning degree of yeast streak according to the following scale: 0 = white; 1 = light brown; 2 = brown; 3 = dark brown; 4 = black

Fermentation time ranged between 7 and 16 days (only P36 completed the fermentation after 19 days). Regarding fast fermenting strains fermentation time values were consistent with those of CO_2 production, as P14 completed the fermentation after 7 days and P9 and P62 after 9 days. These results confirm their good fermentation performances. Regarding the slow fermenting strains P7, P21, P24, P33 and P36, the fermentation time ranged between 11 and 13 days (except for P36 that needed 19 days to complete the fermentation). These strains seem to speed up the fermentation during the late stationary phase, showing intermediate fermentation performance. P51, P40 P54, although showed intermediate fermentation vigour and CO_2 production values at 168h, completed the fermentation after 16 days and can be considered slow fermenting strains. The slowest fermenting strain was P36 that showed the lowest values for all considered parameters. Regarding residual sugars at the end of the fermentation, most of the strains transformed

all the glucose (glucose concentration lower than 1 g L^{-1}). In the case of P5, P12, P19 and P50 the sugar concentration at the end of the fermentation process was less than 3.5 g L^{-1}; P33 and P35 residual sugars concentrations were 4.15 and 7.12, respectively. These results indicate that P33 and P35, notwithstanding their fermentation performances, revealed some difficulties to transform all the sugar present in the synthetic must. The H_2S production was evaluated by plate assay method. Among the strains tested, 27 showed very low or low H_2S production level, while 16 an intermediate level and 20 produced high H_2S level (**Supplementary 2**).

2.3.4 Copper tolerance

In order to investigate strains copper tolerance, a growth test in YNB minimal medium was performed at different copper concentrations in a range comprised between 0.0 and 1.0 mM. The growth was measured after 18 and 48 h yeast inoculum (**Figure 8**). To determine the copper concentration range some preliminary trials have been carried out with two strains: S288c, a laboratory strain that has been investigated to understand the mechanism of copper toxicity (Yasokawa *et* al., 2008) and EC1118, a well-studied industrial wine starter (data not shown). To discriminate copper tolerant from sensitive strains the values 0.5 of the ratio between the OD_{630nm} measured in presence of $CuSO_4$ and in the control condition was considered as threshold. This means that sensitive strains in presence of $CuSO_4$ at least halve the cell concentration measured in control condition. At 18 h EC1118 showed an OD_{630nm} ratio lower than 0.5 at $CuSO_4$ concentration 0.8 and 1 mM, while at 48 h only at 1 mM. This indicated 0.8 mM $CuSO_4$ a critical concentration for this strain in these experimental conditions. S288c revealed to be more tolerant, as after 48 at 1 mM the OD ratio was higher than 0.5. The vineyard strains revealed to be generally tolerant. At 18 h, at $CuSO_4$ concentration 0.8 mM and 1 mM, about 8% and

27% showed an OD_{630nm} ratio lower than 0.5, respectively; at 48 h this percentage dropped to 3% at 1mM.

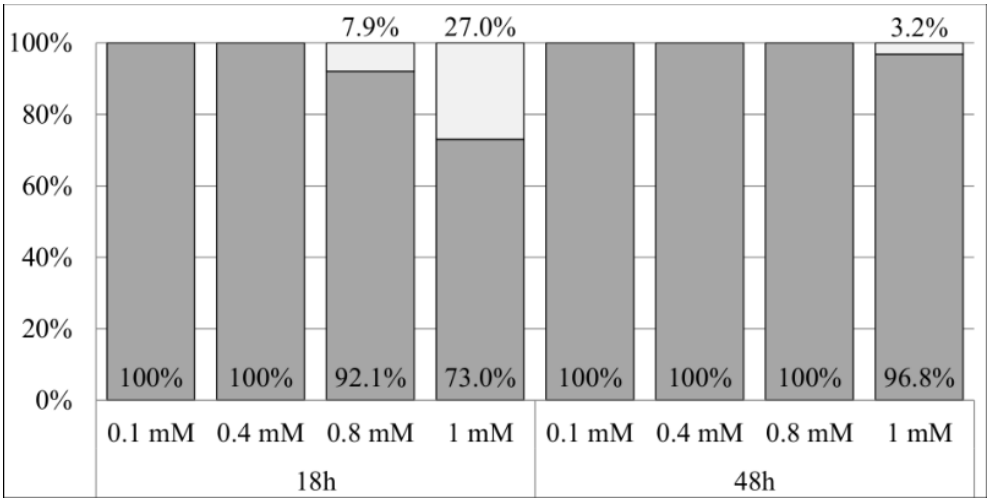


Figure 1.8

Effect of different copper concentrations on yeast growth at 18 and 48 h. The dark grey bars represent the percentages of tolerant strains and the light grey the sensitive ones

2.3.5 Sulphites tolerance

Sulphite tolerance has been studied mimicking oenological condition where this chemical is added before the fermentation. The strains have been grown in synthetic must (Delfini and Formica, 2001), with the addition of SO_2 at the concentration 50 and 100 mg L^{-1}, that are consistent with those generally used in winemaking (Divol *et* al., 2012; Henick-Kling *e*t al., 1998; Ribereau-Gayon *et* al., 2006). During the fermentation the OD_{600nm} value after 24 and 48 h was measured. To discriminate sulphite tolerant from sensitive strains the value 0.5 of the ratio between the OD_{600nm} measured in presence of SO_2 and in the control condition was considered as threshold. Strains S288c and EC1118 have been used as control. In this condition, at both 24 and 48 h, EC1118 showed an OD_{600nm} ratio lower

than 0.5 at SO_2 concentration 100 mg L^{-1}, whereas at 50 mg L^{-1} was higher than 0.5. This indicated that 100 mg L^{-1} was a critical concentration of SO_2 for this strain. S288c revealed to be less tolerant than EC1118, as after 24 h at 50 mg L^{-1} the OD ratio was lower than 0.5. The vineyard strains revealed to be generally tolerant at 50 mg L^{-1} SO_2, while at 100 mg L^{-1} the percentage of tolerant strains showed a strong decrease. At 50 mg L^{-1} SO_2 after 24 h more than 95% of the strains showed an OD_{600nm} ratio higher than 0.5, whereas at 48 h this percentage rose to 100%. At 100 mg L^{-1} SO_2 the tolerant strains dropped to 52.4% and 73.0% at 24 and 48 h, respectively (**Figure 9**).

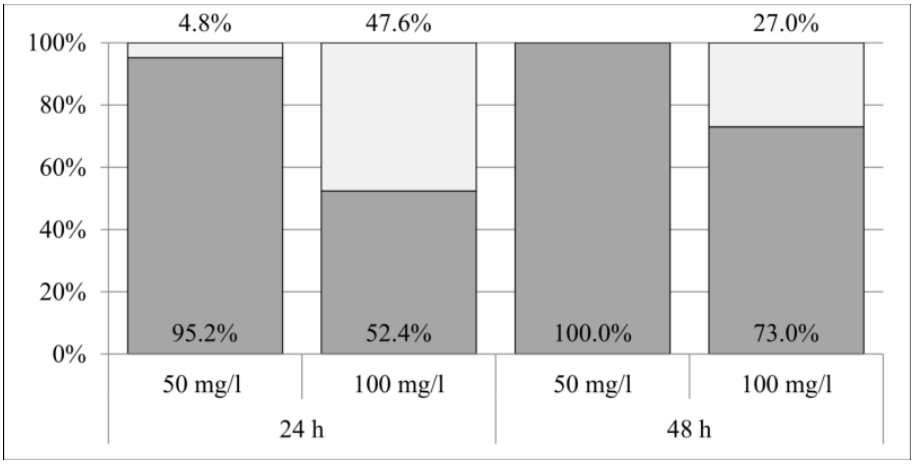


Figure 1.9

Effect of different SO 2 concentrations on yeast growth at 24 and 48 h. The dark grey bars represents the percentage of tolerant strains and the light grey the sensitive ones

2.3.6 Genotypes, phenotypic traits and geographical origin

In order to evaluate the relationship among the phenotypic traits tested, the genotypes and the geographical origin (SA1, SA2, SA3) a principal component analysis has been performed (**Figure 10**).

In this analysis, all phenotypical traits investigated have been included: the $CuSO_4$ and SO_2 tolerance (SGD_{SO2} and SGD_{Cu}), the CO_2 produced after 48 h (CO_2-48 h) corresponding to the fermentation vigour (Delfini and Formica 2001), the CO_2 produced after 168 h (CO_2-168h) and the days of fermentation (FD).

The resulting PCA plot explained 71.86% of the total variance for the first two principal components. The first principal component (PC1, 47.73% of the variance) was positively correlated to the CO_2 production (CO_2-48 h, CO_2-168h) and negatively correlated to the fermentation length (FD). The second principal component (PC2, 23.93% of the variance) was positively correlated to the growth reduction due to SO_2 and Cu (SGD_{SO2} and SGD_{Cu}).

In the 4^{th} quadrant are located the genotypes possessing the highest Cu and SO_2 tolerance level and the best fermentation performances in terms of fermentation vigour, amount of CO_2 produced after 168 h and fermentation time. The 4^{th} quadrant includes 21 genotypes (33.3% of the total) equally present in the three major clusters obtained by mtDNA analysis (**Fig. 6**, bold fonts in the table). About their area of origin, 35.0% (14/40) have been found in SA1, the 40.0% (4/10) in SA2 and the 21.4% (3/14) in SA3.

As expected, P36 genotype is strongly separated from the others. This is mainly due for its poor fermentation performance as fermentation variables (CO_2-48 h, CO_2-168h and FD) give the highest contribution to the PC1.

Regarding isolate origin, genotypes collected from SA2 showed similar phenotypic traits as reveal by their positions in the PCA plot. On the contrary, genotypes collected from

SA1 and SA3 showed a wider variability as their positions are more spread in the PCA plot. No separation was evident between genotypes collected from SA1 and SA3, which revealed a more similar phenotypic traits distribution that those from SA2.

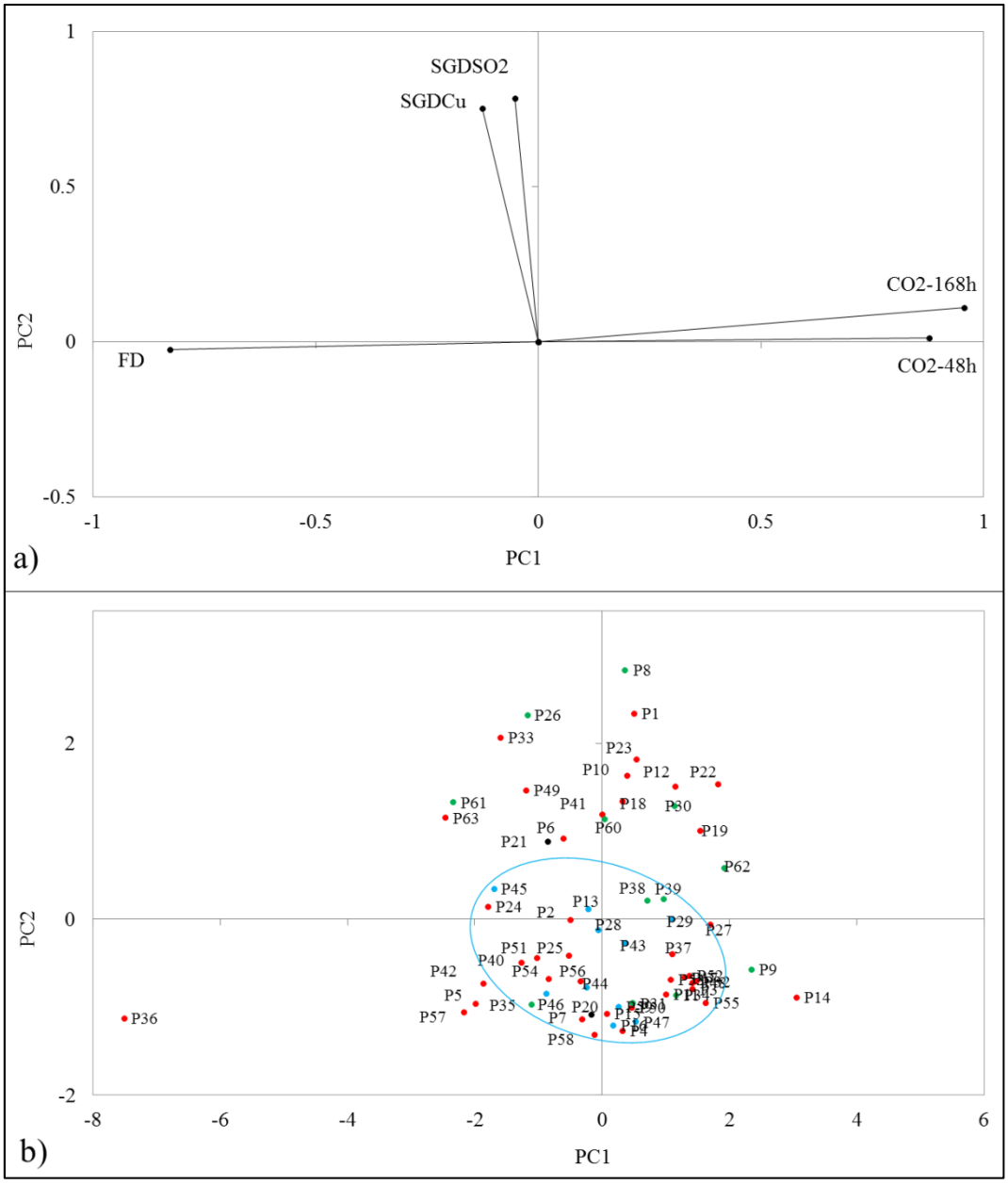


Figure 1.10

Principal component analysis of phenotypic traits: loadings plot (a) and scores plot (b). Different coloured dots indicates the yeast origin (sampling area): red for SA1, blue for SA2, green for SA3 and black for genotypes collected from both SA1 and SA3. The blue circle groups genotypes collected from SA2

2.4 Discussion

At the moment, *S. cerevisiae* strain variability in the vineyards of new winemaking countries is unexplored and its potential impact on new wine yeast selection is still unraveled. In this work, a sampling campaign of Merlot grape bunches collected from three vineyards of Brazilian winemaking area Vale dos Vinhedos have been carried out. Results suggested a strong presence of *S. cerevisiae* in the vineyards as 49% single bunch fermentations allowed the isolation of this species. Among the three vineyards, despite the low number of collected samples, SA2 showed the highest percentage of single bunch fermentation (5 out of 6) where this species was isolated. On the contrary, other sampling campaigns in traditional winemaking countries showed a very limited *S. cerevisiae* presence in single bunch fermentations (Viel *et*. al., 2017). These results seem to limit the influence of a long-standing viticulture, that is peculiar of traditional winemaking countries, on the abundance of wine yeast in vineyards. As mentioned above, strain isolation was performed from fermented grape bunches. This enrichment procedure was necessary due to the scarcity of yeast on sound grapes (Martini *et* al., 1996; Mortimer and Polsinelli, 1999), but it determined clonal replication. Therefore, isolates with identical mtDNA genotype were considered to be originated from the same cell.

By means of mtDNA RFLP analysis, 63 different genotypes have been found. Comparing the mtDNA electrophoretic profiles with those of the industrial strains most used in this area, no overlapping was found, evidencing no industrial strain dissemination in the sampled vineyards. The presence of industrial strains in vineyard has been wildly reported in traditional winemaking countries (Martiniuk *et* al., 2016; Valero *et* al., 2005, 2007; Viel *et* al., 2017). In this work, the lack of industrial strains in vineyard is probably due to the recent introduction of industrial winemaking process in this area and, therefore, of industrial wine starter.

Regarding genotypes frequencies among *S. cerevisiae* isolates, 58% of the genotypes identified a single isolate, indicating a high level of genetic variability in vineyards, a great genetic reservoir that can be exploited for technological purposes.

Concerning the distribution of the genotypes in the single bunch fermentations, most of the genotypes were found only in one sample, indicating they were vine-specific. This finding strengthened the modern French "*terroir*" notion that includes vineyard microbiota together with grape varieties and wine makers know how in this definition (Bokulich *et* al., 2013; Gayevskiy and Gottard, 2012; Van Leeuwen *et* al., 2004).

In the case of 10 genotypes, each one has been found in two samples (all but one, P21, collected in the same SA). Four were collected from neighboring vines. The most spread genotypes in the vineyards were P24, P20 and P1. P24 was found in 4 samples all collected from SA1; both P1 and P20 have been fund in 3 different samples, but P1 was isolated only in SA1, while P20 was found in SA1 and SA3. These results evidence a limited diffusion of the genotypes into and between the sampled areas.

MtDNA restriction profiles allowed performing a computer-assisted cluster analysis. On the base of similarity percentage, the genotypes were split in 3 main clusters, each including similar genotype numbers. The genotypes isolated from SA1 and SA3 were present in all the three clusters, while those from SA2 only in the first (I) and the second (II). This suggests that the genotypes of SA1 e SA3 were more similar than those of SA2. This observation is supported by the territorial morphology of Vale dos Vinhedos Appellation of Origin. In fact, SA2 is geographically separated from the others by a wide valley that it is crossed by several rivers and includes wooded areas. The effect of a physical barrier is confirmed by the fact that the only two genotypes present in more than one SA (P20, P21) were isolated in SA1 and SA3.

As above mentioned, the strain isolation was performed from fermented grape bunches. This enrichment procedure was necessary due to the scarcity of yeast on sound grapes (Martini *et* al., 1996; Mortimer and Polsinelli, 1999), but it determined clonal replication. Isolates with identical mtDNA genotype were considered to be originated from the same cell. Thus, one isolate for each of the 63 genotypes found has been selected to evaluate phenotypic variability. In this way the reduction in yeast number to be analysed was achieved, although strain genetic variability might be slightly under estimated.

Most of the strains shared an intermediate fermentation performance, in terms of fermentation vigour, CO_2 production during middle/late stationary phase and fermentation time. Among them, P33 and P35 left a limited residual sugars. Only P36 showed a poor fermentation performance, although was able to transform all the sugars.

Studies on industrial wine yeast populations have highlighted the process of adaptation of yeast lineages to the chemical treatments, mainly based on the use of copper and SO_2, imposed by viticulture and winemaking during the years (Hodgins-Davis *et* al., 2012; Townsend *et* al., 2003). In viticulture, the control of downy mildew (*Plasmopara viticola*) and grey mold *(Botrytis cinerea*) is commonly achieved by means of copper-containing fungal pesticides (copper oxychloride) (Aziz *et* al., 2006; Judet-Correia *et* al., 2011). Yeast copper tolerance is due to the amplification of *CUP1* genes and such genetic signature has been reported for wine yeast strains in previous studies (Legras *et* al., 2014, 2018; Strope *et* al., 2015). $CuSO_4$ tolerance of the 63 Brazilian strains tested was generally very high. This result is remarkable, considering that the highest tested concentration (1mM) was higher than the European MRL (Maximum Residue Level) for wine grape juice (EC – Annex I to Reg. 396/2005) and about 50 times higher than the maximum level legally allowed in Europe in wines (Reg. No 606/2009). Moreover in this Brazilian winemaking area, due to high humidity and frequent rain, copper sulphate

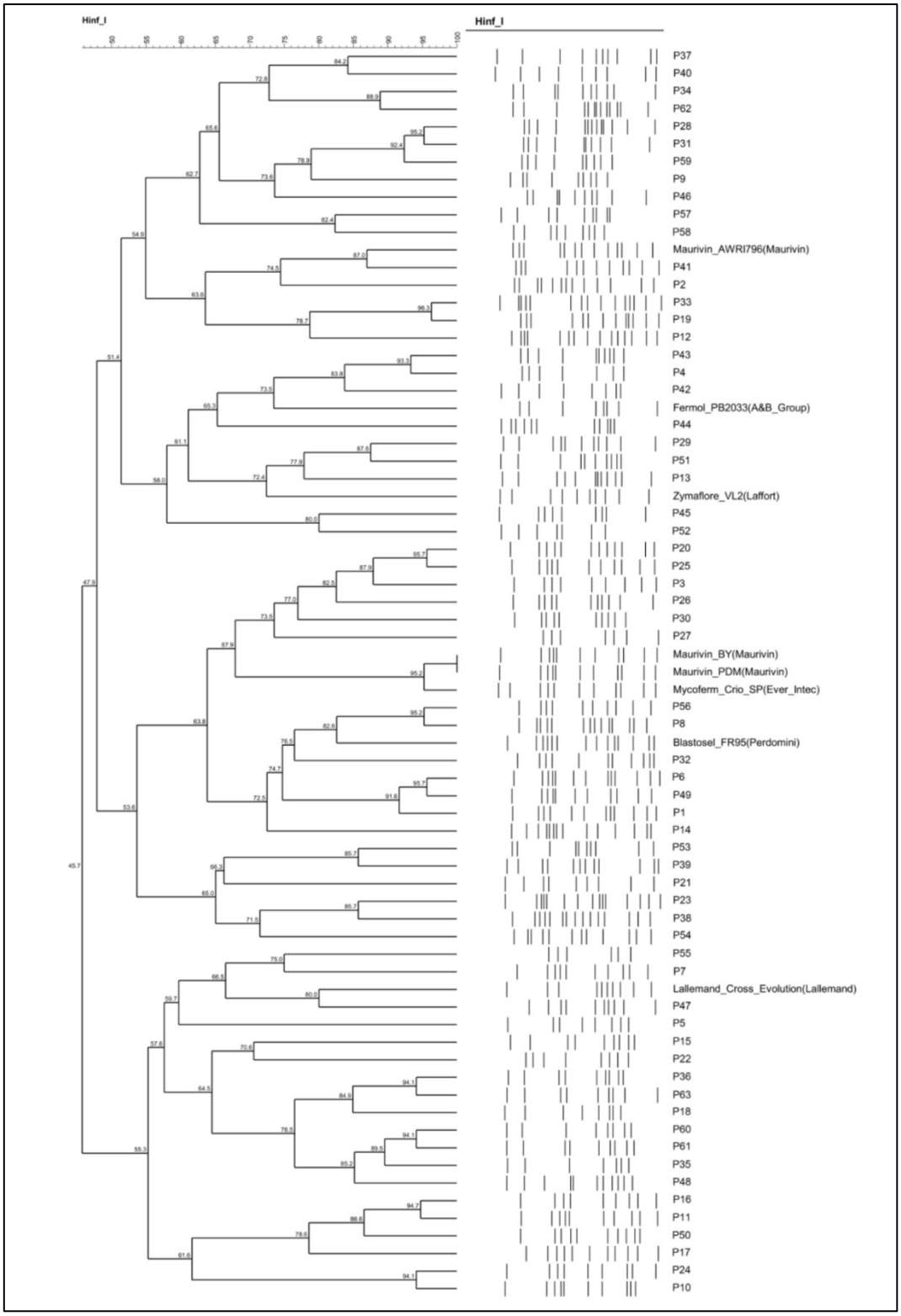
application can reach 80 kg ha^{-1} years, which surpasses the dosage of this fungicide in regions with temperate climate by several times (Brun *et* al., 1998; Mirlean *et* al., 2009). As consequence, a level of copper residues up to 3215.6 mg kg^{-1} can be found in these soils (Mirlean *et* al., 2007). A recent study reported that the maximum copper contamination in European vineyard soils ranges from 435 to 689 mg kg^{-1} (Ruyters *et* al., 2013). The highest level of copper in vineyard soils reported in literature is of about 1500 mg kg^{-1} in France (Mirlean *et* al., 2007), which is still far from Brazilian values. Therefore, it can be assumed that high level of strain copper tolerance found is due to the high copper residue in the vineyard.

Regarding SO_2 tolerance, 73% of the strains were resistant to 100 mg L^{-1} that is twice the concentration generally used in winemaking to avoid the growth of spoilage microorganisms (Divol *et* al., 2012; Henick-Kling *et* al., 1998; Hood, 1983).

Finally, PCA analysis including all the phenotypic traits reveals that the group of strains isolated from SA2, physically separated from the other SAs, showed a lower level of phenotypic variability that those isolated from SA1 and SA3 that evidenced a similar phenotypic trait distribution.

2.5 Conclusions

In conclusion, in this work high genotypic variability was found in the sampled vineyards of the Vale dos Vinhedos area and most of the genotypes revealed to be vine-specific. From the phenotypic traits analysis the high copper tolerance level suggested an environmental adaptation to the strong use of copper-based fungicides. Finally, as 33% of the tested strains showed good fermentation performance, high copper and sulphite tolerance, Vale dos Vinhedos vineyards seem to be an interesting yeast genotype reservoir for the selection of starters that match the need of local winemaking.



Supplementary.1: MtDNA RFLP cluster analysis using Dice's similarity coefficient and setting as optimization and tolerance the values recommended by the software (optimization 0.0%; tolerance 1.55%). Dendrogram was generated by the Unweighted Pair Group Method using Arithmetic Average (UPGMA) clustering algorithm, calculated by BioNumerics® software 6.6 (Applied Maths).

Supplementary 2: Strains fermentation performances and hydrogen sulphide production in standard synthetic must. Data are expressed as the average of three replicates ± standard deviations.

Genotype	CO_2 production at 48 h (g (100mL)$^{-1}$)	CO_2 production at 168 h (g (100mL)$^{-1}$)	Days of fermentation	H_2S*
P14	3.30 ± 0.04	8.79 ± 0.16	7	2
P55	2.66 ± 0.10	8.10 ± 0.22	8	2
P32	2.60 ± 0.09	7.85 ± 0.29	8	1
P48	2.56 ± 0.18	7.99 ± 0.12	8	3
P3	2.51 ± 0.14	7.98 ± 0.11	8	1
P62	3.08 ± 0.05	8.51 ± 0.11	9	3
P9	3.03 ± 0.12	8.67 ± 0.12	9	3
P22	2.76 ± 0.22	8.39 ± 0.26	9	1
P11	2.58 ± 0.18	8.48 ± 0.31	9	4
P37	2.57 ± 0.19	8.61 ± 0.07	9	1
P34	2.55 ± 0.04	7.69 ± 0.09	9	2
P16	2.47 ± 0.02	7.94 ± 0.07	9	2
P50	2.46 ± 0.10	8.11 ± 0.14	9	1
P60	2.34 ± 0.05	7.80 ± 0.03	9	1
P38	2.33 ± 0.10	8.01 ± 0.43	9	2
P31	2.29 ± 0.09	7.46 ± 0.15	9	1
P1	2.25 ± 0.08	7.83 ± 0.08	9	1
P23	2.24 ± 0.03	7.84 ± 0.14	9	0
P28	2.10 ± 0.27	7.50 ± 0.13	9	3
P27	2.69 ± 0.24	8.08 ± 0.64	10	3
P39	2.68 ± 0.13	8.23 ± 0.23	10	3
P19	2.64 ± 0.12	8.29 ± 0.45	10	1
P17	2.58 ± 0.13	8.64 ± 0.35	10	0
P4	2.54 ± 0.36	7.43 ± 0.56	10	2
P41	2.42 ± 0.15	8.10 ± 0.24	10	0
P8	2.35 ± 0.18	7.78 ± 0.18	10	1
P18	2.33 ± 0.11	7.91 ± 0.09	10	2
P43	2.10 ± 0.14	7.50 ± 0.23	10	3
P59	2.02 ± 0.22	7.41 ± 0.36	10	1
P49	1.92 ± 0.26	7.02 ± 0.54	10	1
P47	2.54 ± 0.12	7.23 ± 0.23	11	2
P53	2.52 ± 0.17	7.76 ± 0.65	11	2
P12	2.36 ± 0.25	7.92 ± 0.95	11	0
P6	2.25 ± 0.26	7.94 ± 0.74	11	0
P10	2.02 ± 0.40	7.64 ± 0.95	11	0

Genotype	CO_2 production at 48 h (g (100mL)$^{-1}$)	CO_2 production at 168 h (g (100mL)$^{-1}$)	Days of fermentation	H_2S*
P20	2.02 ± 0.30	7.47 ± 0.62	11	3
P44	2.00 ± 0.29	6.73 ± 0.72	11	3
P26	1.93 ± 0.19	6.63 ± 0.17	11	3
P5	1.84 ± 0.20	6.43 ± 0.59	11	0
P57	1.82 ± 0.45	6.58 ± 0.67	11	1
P21	1.79 ± 0.30	6.50 ± 0.52	11	0
P30	2.69 ± 0.14	8.29 ± 0.19	12	3
P52	2.63 ± 0.01	8.25 ± 0.02	12	3
P29	2.58 ± 0.03	8.00 ± 0.04	12	2
P63	2.08 ± 0.10	7.14 ± 0.21	12	1
P24	1.37 ± 0.23	6.72 ± 0.53	12	1
P46	2.32 ± 0.21	7.12 ± 0.52	13	4
P25	2.21 ± 0.09	7.23 ± 0.19	13	3
P35	2.18 ± 0.08	6.36 ± 0.08	13	2
P15	2.13 ± 0.14	7.05 ± 0.28	13	4
P13	2.06 ± 0.53	7.29 ± 0.48	13	2
P58	2.04 ± 0.51	7.09 ± 0.82	13	3
P61	1.90 ± 0.06	6.92 ± 0.04	13	2
P33	1.68 ± 0.03	5.90 ± 0.12	13	1
P7	1.58 ± 0.21	6.34 ± 0.75	13	0
P2	2.31 ± 0.14	7.12 ± 0.16	14	2
P56	2.23 ± 0.15	7.10 ± 0.35	14	3
P45	2.21 ± 0.24	6.82 ± 0.46	14	3
P42	1.89 ± 0.34	6.51 ± 0.46	14	2
P54	2.26 ± 0.07	7.39 ± 0.14	16	3
P40	2.17 ± 0.20	6.83 ± 0.22	16	1
P51	2.01 ± 0.27	6.23 ± 0.51	16	2
P36	0.39 ± 0.05	1.69 ± 0.14	19	1

*** The production of hydrogen sulphide was determined on BIGGY agar (DIFCO), scoring the browning degree of the yeast streak according to the following scale: 0 = white; 1 = light brown; 2 = brown; 3 = dark-brown; 4= black.**

3. Chapter II

Saccharomyces cerevisiae tolerance to copper and sulphites: an unexpected association

3.1. Introduction

The importance of *Saccharomyces cerevisiae* in winemaking has long been established: this species, within grape microbiota, is the main responsible for the transformation of sugars into ethanol and for most of the chemical and sensory properties of wines (Fleet, 2003; Romano *et* al., 2003; Camarasa *et* al., 2011). Extensive ecological surveys using molecular identification methods demonstrated the presence of *S. cerevisiae* strains in vineyards and the strain differentiation on the territory depending on grape variety, geographical distances and agronomical practices (Cordero-Bueso *et* al., 2011; Schuller *et* al., 2012; Garofalo *et* al., 2016). More recently, regional delineations were identified able to separate *S. cerevisiae* vineyard populations from different winemaking area (van der Westhuizen *et* al., 2017; Viel *et* al., 2017; Bokulich *et* al., 2014). In this framework Knight *et* al. (2015) demonstrated significant correlation between the region of isolation of *S. cerevisiae* and aroma profile in wines. These findings allowed including the vineyard microbiota component as part of wine terroir, which classically involves grape variety, climate and soil. As grapevine cultivation and winemaking are activities that humankind has developed for millennia, the possible role of these technological practices both in vineyard and cellar on yeast evolution was investigated. Recently, the impact of human activity on yeast diversity has been assessed at gene and genome level (Fay and Benavides, 2005; Legras *et* al., 2007; Liti *et* al., 2009; Schacherer *et* al., 2009) evidencing

several events of domestication (Fay and Benavides, 2005, Almeida *et* al., 2015). Some studies revealed signs of domestication in wine strains, such as an increased resistance to copper (present in grapevine pesticides) and sulphite (used as a preservative in wine) and correlated them with the presence of specific genetic variants (Pérez-Ortín *et* al., 2002; Warringer *et* al., 2011).

Among plant protection against fungal pathogens, the relevance of using copper-based fungicides in vineyard is a well-known awareness. Copper is a toxic element for many pathogenic organisms, including fungi that usually affect the vegetative portion of vines, the wood or the ripening grapes. Thanks to its broad mode of action, copper is an essential component of the plant protection, as it allows reducing the risk of pesticides resistance and, considering its efficacy on several pathogens, the number of interventions per year. In this background, copper in vineyard has a heavy impact also on the non-pathogenic fungal indigenous microflora, which includes *Saccharomyces cerevisiae*, the most important yeast specie in winemaking.

At low concentration, copper serves as a micronutrient. Yeasts have developed mechanisms to maintain copper homeostasis and detoxification to face surpluses, but when it reaches toxic levels it leads cells to death. Metallothioneins, low molecular weight molecules rich in cysteine-residues are cytoplasmic proteins designated to bind metal ions, resolving their toxic effects. In *Saccharomyces cerevisiae* has been observed that the *CUP1* gene encodes for the most effective copper chelating metallothionein. Several studies propose a positive correlation between high copper tolerance and an increased *CUP1*-gene copy-number in the yeast genome, due to an adaptation strategy of the yeasts to the increasing copper presence in their environment (Strope et al., 2015; Adamo *et* al., 2012).

Among chemicals routinely used in wine production, sulphur dioxide is the most common in cellars. Sulphites are widespread additives in wineries, where they are used with different purposes: as antioxidasic, in order to avoid the oxidation of flavours and phenolic compounds, or/and as antimicrobial, in order to inhibit bacteria and spoilage yeasts before the alcoholic fermentation of grape musts to support *S. cerevisiae* growth and after to reach the wine microbiological stability (Ribéreau-Gayon *et* al., 2006). The effect of SO_2 on the growth of *Saccharomyces cerevisiae* yeasts, usually added as fermentation starters, differs among strains and has a crucial influence on the ability of the inoculated yeasts to trigger the fermentation process (Nadai *et* al., 2016).

Sulphites are toxic compound for yeast cell, which overcome their presence in grape must by means of three major mechanisms: the production of acetaldehyde in order to bind sulphites (Stratford *et* al., 1987; Pilkington and Rose, 1988; Liu and Pilone, 2000), the incorporation of sulphur by means of sulphur aminoacids biosynthetic pathway (Casalone *et* al., 1992; Nardi *et* al., 2010), and the activation Ssu1p sulphite transporter, located in the plasmatic membrane of the yeast cell (Avram and Bakalinsky, 1997; Park and Bakalinsky, 2000). The expression of *SSU1* gene can be enhanced by a genomic translocation between chromosome XVI and VIII, which is widespread among wine *S. cerevisiae* strains. This translocation generated a dominant allele of the sulphite pump, SSU1-R1, expressed at much higher levels than SSU1 and, therefore, confers high level of sulfite resistance (Goto-Yamamoto, 1998; Pérez-Ortín *et* al., 2002; Yuasa *et* al., 2004). Another translocation, localized between chromosomes XV and XVI and involving the *SSU1* gene, has been recently identified by Treu *et* al. (2014).

Although copper- and sulphite-based formulations are the most used in vine cultivation and winemaking only few studies investigated the presence of the genetic variances involved in copper and sulphite resistance in wine yeasts. Moreover, these studies

considered a pool of *Saccharomyces* strains from very different geographical and technological origins (Yuasa *et* al., 2004; Warringer *et* al., 2011), while the distribution of these variants within *S. cerevisiae* populations present in vineyard is still unknown. Indeed no information is available on the impact of local agricultural practices on variant distributions.

In the present work *S. cerevisiae* vineyard populations isolated from three winemaking areas with different clime and agronomical history were studied: Conegliano Valdobbiadene Prosecco superior (CVPAO) and Piave (PAO) from the North-East of Italy, Vale dos Vinhedos (VVAO) from Southern Brazil. Italian and Brazilian vineyards mainly differ for the winemaking tradition. In the North-East of Italy winemaking has ancient origin: the first testimonies date back to the seventh century B.C., but only early in the sixties of the last century a real industrial production started. In Southern Brazil around 1970, thanks to several investments from foreign wine companies, industrial winemaking took place and only in the last 20 years Brazilian wine companies started to face first the local and, very recently, the international market (Fensterseifer, 2007; Filiputti, 2016). The two geographical areas have similar climatic conditions, according to Köppen classification (Köppen, 1936) CVPAO and PAO have humid subtropical climate (Cfa) (Salata *et* al., 2017) and VVAO has marine west coast climate (Cfb) (Flores *et* al., 2013). The two climatic categories differs only for summer temperatures: hot summer with maximum temperature higher than 22°C for Cfa and warm summer with at least 4 monthly temperatures higher than 10°C for Cfb (Kottek *et* al., 2006). The effect is higher humidity level in Brazilian vineyard that in North-East of Italy that can impact agronomical practices.

A total of 273 *S. cerevisiae* strains were analysed in order to determine the presence of the genomic translocations XVI_VIII and XV_XVI together with the *CUP1* gene copy-

number. Twenty commercial fermentation starters have been included to this study. For each strain copper and sulphite resistance level was determined. Afterward, the correlation between the resistance level and the presence of genetic variations was investigated considering both the strain origin and the territory history. Sulphur dioxide produced by burning sulphur fumes has been used for millennia to clean wine containers that were copper-made (Pérez-Ortín *et* al., 2002). Moreover copper is traditionally spread in vineyard as copper sulphate. Sulphate anion is an intermediate of sulphur metabolism in yeasts, leading to the intracellular production of sulphites, sulphide and sulphur-containing amino acids (Thomas and Surdin-Kerjan, 1997; Stratford *et* al., 1987).

Therefore, the presence of association between copper and sulphite tolerance levels was investigated to understand if any correlation within the genomic variants did exist among vineyard *S. cerevisiae* yeast strains.

3.2 Material and method

3.2.1 Areas of yeasts collection and *Saccharomyces cerevisiae* strains

In the present work 273 *Saccharomyces cerevisiae* strains have been used, from different areas: 20 of them have industrial origin and they are used in winemaking and alcoholic fermentation industrial processes (**Table 2.1**), the others are vineyard strains. Among the other strains, 190 of them come from Italy (34 from Conegliano Valdobbiadene Appellation of Origin – CVPAO – and 156 from Piave Appellation of Origin – PAO) and 63 of them come from Brazil (Vale dos Vinhedos Appellation of Origin – VVAO).

Table 2.1

Industrial strains used in this study:

Strain name	Description	Origin	Producer
Lalvin 71B	Wine strain	Narbonne, France	Lallemand Inc. (Montreal, Canada)
AWRI 1631	Wine strain	Derived from N96	AWRI collection (Adelaide, Australia)
Mycoferm Pro Crio SP	Wine strain	Champagne, France	EVER s.r.l. (Pramaggiore, Italy)
Mycoferm CRU 31	Wine strain	-	EVER s.r.l. (Pramaggiore, Italy)
Lalvin ICV D47™	Wine strain	Côtes du Rhône, France	Lallemand Inc. (Montreal, Canada)
Lalvin DV10®	Wine strain	Champagne, France	Lallemand Inc. (Montreal, Canada)
Lalvin EC-1118	Wine strain	Champagne, France	Lallemand Inc. (Montreal, Canada)
Zymaflore ® F15	Wine Strain	Bordeaux, France	LAFFORT® (Bordeaux, France)
Blastosel FR 95	Wine strain	Loire Valley, France	Perdomini-IOC S.p.A (Verona, Italy)
Blastosel Grand Cru	Wine strain	La Rioja, Spain	Perdomini-IOC S.p.A (Verona, Italy)

Strain name	Description	Origin	Producer
GY	Wine strain	Europe	-
LV10	Wine strain	Europe	EVER s.r.l. (Pramaggiore, Italy)
Mycoferm Cru 611	Wine strain	Europe	EVER s.r.l. (Pramaggiore, Italy)
Nouveaux Ferments	Wine strain	Europe	Enologica Vason (Verona, Italy)
NT116	Wine strain	Stellenbosch, South Africa	Oenobrands SAS (Montpellier, France)
Premium Prosecco 444	Wine strain	ISMA	Enologica Vason (Verona, Italy)
Lalvin QA23	Wine strain	Portugal	Lallemand Inc. (Montreal, Canada)
UC5	Sake strain	Japan	-
Premium® Blanc 12V	Wine strain	Alsace, France	Enologica Vason (Verona, Italy)
Uvaferm VRB	Wine strain	Logroño, Spain	Lallemand Inc. (Montreal, Canada)

3.2.2 Yeast isolation and identification

Yeast sampling has been carried out as reported by Viel *et* al., (2017). Briefly, after single-bunch fermentations ended, samples were diluted and spread on WL agar Petri dishes. Sixteen colonies were randomly chosen and tested for species belonging using High Resolution Melting (HRM) analysis (Nadai *et* al., 2018).

3.2.3 DNA extraction and mtDNA RFLP analysis

DNA extraction, *Hinf*I digestion and electrophoretic fragments separation, in order to identify the different mitochondrial DNA RFLP genotypes, were carried out as reported by Bovo *et* al. (2011). Isolates with identical mtDNA genotype were considered to be

originated from the same cell, so one isolate for each genotype has been chosen to evaluate phenotypical variability.

3.2.4 Copper and sulphites tolerance test

Copper tolerance and sulphites tolerance have been evaluated as described by Crosato *et* al. (2018).

Copper tolerance

Stationary-phase yeast precultures have been used to inoculate 96-wells sterile microplates (Greiner Bio-One, Germany) filled with 300 µl of growth liquid media. YNB broth (Yeast Nitrogen Base 6.7 g/L – Sigma®, glucose 20 g/L) has been used as control condition, while treatments were prepared using YNB broth with the addition of $CuSO_4$ at proper doses in order to obtain the final Cu^{2+} concentration of 0.1, 0.4, 0.8 or 1mM. After 18 and 48 hours from inoculum and incubation at 30°C cell turbidity at 600_{nm} has been monitored using a microtiter plate reader (Shimadzu UV-1601 – Japan). The assay was performed in triplicate. The effect of copper on yeast growth was evaluated using the ratio between the OD630nm values measured in present of $CuSO_4$ and in the control condition (0 mM Cu^{2+}).

Sulphites tolerance

For each isolate a loopful of 3 days YPD plated culture has been used to inoculate 10 mL of liquid YPD broth (yeast extract 10 g/L , peptone 10 g/L, dextrose 20 g/L) and grown for 24 h at 25 °C. 15 mL capacity tubes have been sterilized, filled with 11.2 mL of SM (Delfini and Formica, 2001) with 50 or 100 mg/L SO_2, and then inoculated with 800 µL

of pre-cultures in order to obtain a starting OD 600nm of 0.1 (approx. 10 6 cells/mL). Control yeast cultures were prepared with the same procedure without the SO_2 addition. Cell turbidity (OD 600nm) was monitored at 24 and 48 h using a spectrophotometer (UV–Vis Spectrophotometer UV-2600, Schimadzu—Japan). The effect of SO_2 on yeast growth was evaluated using the ratio between the OD 600nm values measured in present of SO_2 and in the control condition.

3.2.5 Real-Time PCR quantification of *CUP1* gene number of copies

Real time PCR was performed in a thermocycler CFX96 cycler – Real Time PCR Detection in 96-well PCR white plates. PCR reaction volume was set at 15 µl and each reaction mixture contained a 0.4 µM concentration of each primer, 1× SsoFast EvaGreen Supermix (Bio-Rad), and 10 ng of DNA. PCR primer couples CUP1_fw/rev (87 bp) and FBA1_fw/rev (125 bp) (**Table 2.2**) were designed using Primer Select software (DNAstar, Madison, WI) and synthesized by MWG-Biotech.

The amplification conditions were the following:

- Initial denaturation of DNA — 98° C for 2 minutes
- 40 cycles of — Denaturation — 98° C for 5 seconds
  Annealing of primers — 58° C for 40 seconds

The amount of fluorescence for each sample analysed, given by the incorporation of EvaGreen into dsDNA, was measured at the end of each cycle and analysed via CFX-Manager Software v2.0 (Bio-Rad Laboratories, Inc.). Melting curves of PCR products were obtained in the temperature range of 65-95°C, setting a fluorescence measurement at the end of each step of temperature increment (0.2°C for 5 seconds). Fluorescence

obtained from *FBA1* gene amplification has been set as reference for the quantification of the *CUP1* gene number of copies (Nadai *et* al., 2015). Each sample had a NTC control, which was run in the same quantitative PCR (No Template Control). Relative gene copy-number quantification was determined using the $2^{-\Delta\Delta Ct}$ formula, where Ct is defined as the cycle at which fluorescence is determined to be statistically significantly above background; ΔCt is the difference in Ct between the investigated gene (*CUP1*) and the reference gene (*FBA1*); ΔΔCt is the difference in ΔCt between the samples and the calibrator (genomic DNA extracted from the sequenced strain *Saccharomyces cerevisiae* P283 – ENA Assembly). All samples were analysed in triplicate and the quantification values were averaged by the CFX-Manager Software 2.0 (Bio-Rad Laboratories, Inc.).

3.2.6 Diffusion of the genomic translocations XVI-VIII and XV_XVI

End point PCR and electrophoresis on agarose gel has been used to check the presence of the two genomic translocations involving the sulphites efflux pump gene *SSU1* among all the selected strains: the first, between chromosomes XVI and VIII (Goto-Yamamoto *et* al., 1998; Pérez-Ortín *et* al., 2002), and the second, between chromosomes XV and XVI (Treu *et* al., 2014). Two couples of primers have been used for each target. The first couple (AB) amplifies the genomic DNA fragment in the same chromosome (XVI for the XVI-VIII translocation and XV for the XV-XVI translocation). In this way, the positive response of the PCR amplification meant the absence of the translocation. When no amplification was present, a second PCR has been performed using the second couple of primers (BC for the XVI-VIII translocation and AC for the XV-XVI translocation), which amplify the genomic DNA in presence of the specific translocation. Primers pairs used for this purpose are listed in **Table 2.2**.

Table 2.2

Primer pairs used in this work.

Primer Name	Sequence (5'-3')
CUP1_fw	TCATGTAGCTGCCCAACGG
CUP1_rew	AGAGCAGCATGACTTCTTGGTT
FBA1_fw	GGTTTGTACGCTGGTGACATCGC
FBA1_rew	CCGGAACCACCGTGGAAGACCA
A) chr16_fw	AGAACCGTGCTGCTCGTAAG
B) chr16_rev	GCAAGCGATAGCAAACATGA
C) chr8_rev	CATGGCAGCTAGAACCATCA
A) chr15_fw	GCCGTATACCGTTGCTCATT
B) chr15_rev	CAAGGTTTACCCTGCGCTAA
C) chr16_rev	ACCAGCGGAATGATATCCAG

3.2.7 Global Chi-square, Chi-square per cell and Relative Risk

The Chi-square for each experimental condition has been calculated in order to analyse the relationship between strains copper tolerance whit respect to their *CUP1*-gene copy number, the relationship between the strains sulphite tolerance with respect to the presence of the XVI-VIII translocation and the relationship between the copper and sulphites tolerance. Global Chi-square has been used for testing the independence between rows (respectively: *CUP1* copy number class, Presence or absence of the translocation, copper tolerant phenotype and sulphites tolerant phenotype) and columns (number of Tolerant and Sensitive strains) of the contingency tables. When global Chi-square was significant the source of its variation has been analysed by means of the command "Chi-square per cell" of XLSTAT software, vers.7.5.2 (Addinsoft, Paris, France). This tool produces a table showing, for each cell of the contingency table, if the

observed value of each cell is significantly higher, lower or equal to the theoretical values (Symoneaux *et* al., 2012).

Reported relative risks (*RR*) were used to quantify the association between the copper tolerance and the number of copies of the *CUP1* gene, between the sulphites tolerance and the presence of the XVI-VIII translocation, and between the copper and sulphites tolerance phenotypes.

3.3 Results

A pool of 253 *S. cerevisiae* strains collected from vineyards of three winemaking regions has been studied. Yeast collection involved two Italian regions where winemaking is a long-standing tradition: the Conegliano-Valdobbiadene Prosecco superior Appellation of Origin (CVPAO) and the Piave Appellation of Origin (PAO) (Viel *et* al., 2017). The third sampled area was Vale dos Vinhedos Appellation of Origin (VVAO), the most important emerging winemaking region in Rio Grande do Sul (Brazil) (Crosato *et* al., 2018). Despite the similar number of *S. cerevisiae* isolates collected during the sampling campaigns, the three areas showed high variability in terms of different genotypes (**Table 2.3**).

Table 2.3

Number of different *Saccharomyces cerevisiae* genotypes obtained by mtDNA RFLP analysis for each area.

	Number of colonies	mtDNA RFLP genotypes
CVPAO	295	34
PAO	254	156
VVAO	223	63

Among each area, one isolate per genotype has been chosen for genetic and phenotypical tests, and 20 commercial *S. cerevisiae* wine starter strains have been added to the sample.

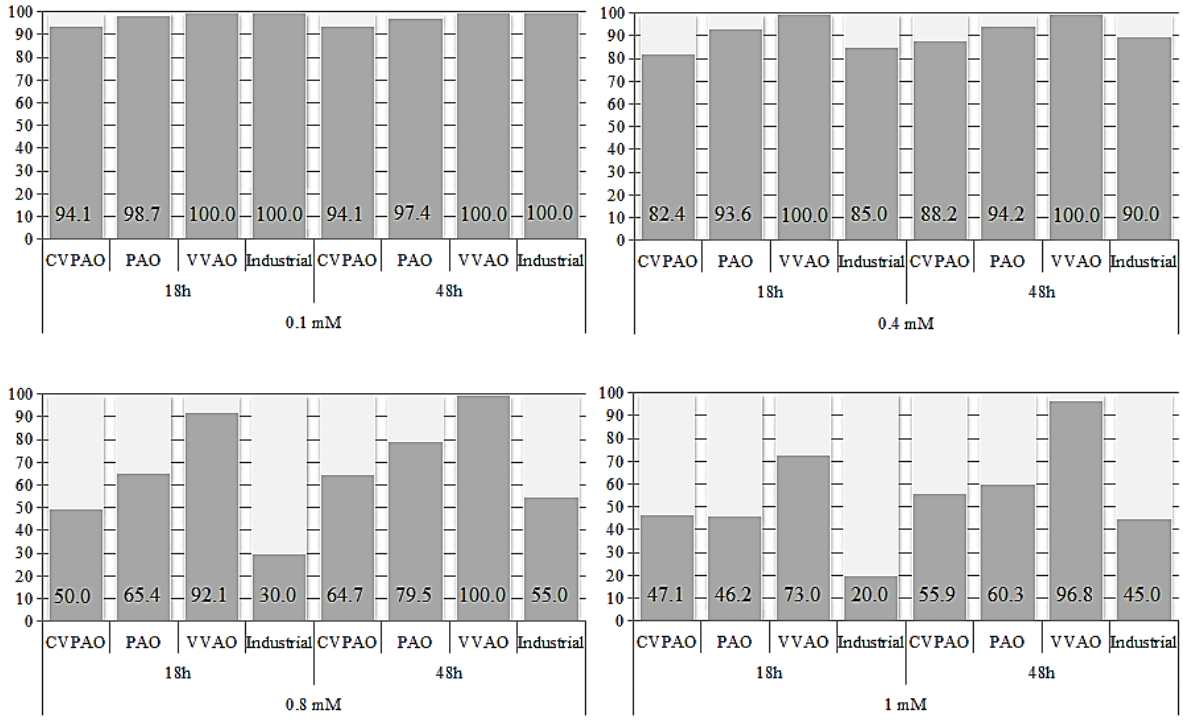
3.3.1 Copper tolerance among *Saccharomyces cerevisiae* strains

Strain copper tolerance has been tested in minimal liquid medium (YNB) and the optical density (600_{nm}) at two time points (18 and 48 hours from inoculum) was monitored. To discriminate copper tolerant from sensitive strains the values 0.5 of the ratio between the OD_{630nm} measured in presence of $CuSO_4$ and in the control condition was considered as threshold. This means that sensitive strains in presence of $CuSO_4$ at least halve the cell concentration measured in control condition (0 mM Cu^{2+}).

The Brazilian strains (VVAO) showed the highest percentages of tolerant yeasts in all conditions (**Figure 2.1**). The percentage of tolerant strains at 0.1 mM, the lowest copper concentration, was always higher than 90%. All VVAO and Industrial strains showed OD_{600} ratio values higher than 0.5, while CVPAO and PAO showed some sensitive strains (the percentage at 48 h was 5.9 and 2.6, respectively). At the copper concentration of 0.4 mM only VVAO showed 100% tolerant strains, while at 48 h the percentages of sensitive strains were 11.8, 5.8 and 10% in CVPAO, PAO and Industrial, respectively. At 0.8 mM $CuSO_4$ the differences became more noticeable both at 18 and 48 h. After 48 h from the yeast inoculum the sensitive strains rose to 35.3, 20.5 and 45% in CVPAO, PAO and Industrial, respectively. On the contrary, at the same time point VVAO showed 100% tolerant strains. At the highest copper concentration (1 mM) at 18 h, 27% of VVAO strains showed OD_{600} ratio values lower than 0.5. This percentage dropped to 3.2 at 48 h. The highest presence of sensitive strain (55%) was among the Industrial, followed by CVPAO (44.1%) and PAO (39.7%).

Figure 2.1.

Effect of different copper doses on yeast growth after 18 and 48 hours of incubation in minimal medium. Dark grey bars represent the percentage of tolerant strains while light grey bars represent the sensitive ones.



3.3.2 Influence of *CUP1*-gene copy-number on copper tolerance

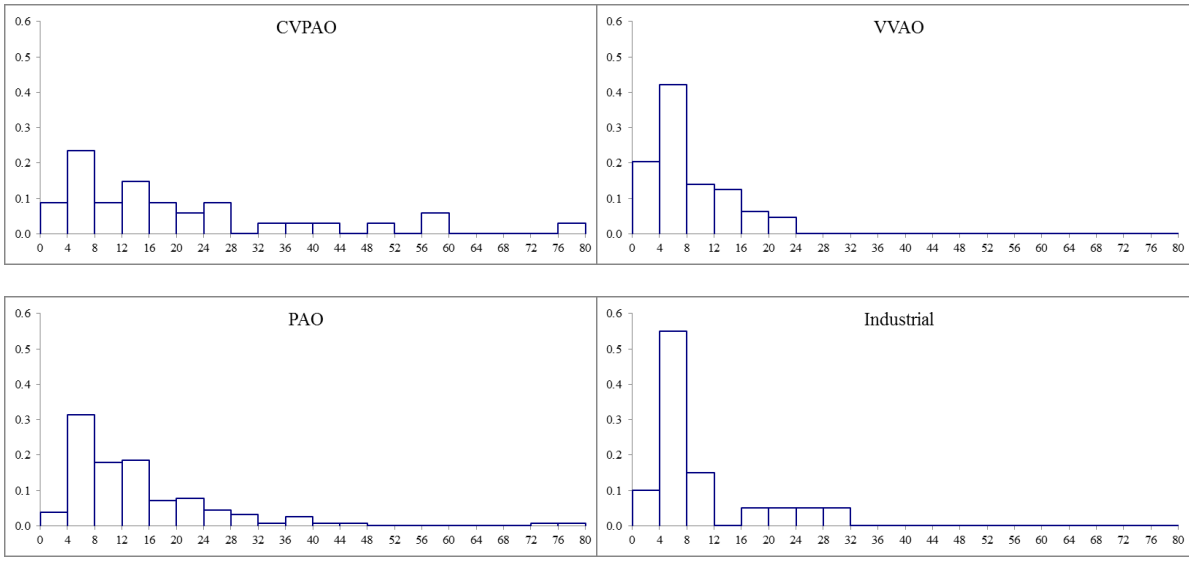
To better understand the influence of *CUP1* gene number of copies in the yeast genome on copper tolerance phenotype, qRT-PCR has been performed for each strain.

The relative frequency of the *CUP1* gene number of copies (**Figure 2.2**) showed a strongly right-skewed distribution: the 41.2%, 53.2%, 76.6% and 80.0% of the strains of, respectively, CVPAO, PAO, VVAO and Industrial presented up to twelve copies of the gene. The majority of the strains presented a copy-number value between 4 and 8.

CUP1 gene number of copies was highly variable among strains from different origin: it ranged from 0 to 77 in CVPAO, from 2 to 79 in PAO, from 2 to 22 in VVAO and from 1 to 30 in the Industrial strains. Strains with the highest *CUP1*-gene copy-number were found in CVPAO (B125.2, with 77 copies) and PAO (R103.5 and R146.4, with 79 and 72 copies, respectively). In CVPAO and PAO, the two Italian Appellations of Origin (AO), the copy-number appeared more variable than in VVAO and Industrial.

Figure 2.2

Relative frequency of CUP1 gene number of copies (x axe) for each strains origin.



Chi-square test has been performed to evaluate whether, among the tested strains, the copper tolerance was independent or not from the *CUP1* gene number of copies. In the analysis this trait has been considered as a categorical variable and the values corresponding to each strain were grouped in classes (**Tables 2.4 and 2.5**). The strains showing an OD ratio value higher than 0.5 have been considered tolerant. At *alpha* value

of 0.01, strain copper tolerance was significantly associated to *CUP1* gene number of copies when copper concentration was 0.8 and 1 mM $CuSO_4$ (**Table 2.4**).

Table 2.4

Global Chi-square values, for each experimental condition, calculated on the basis of contingency tables reporting the classes of *CUP1* gene copy-number and the frequency of tolerant or sensitive strains.

	18 hours		48 hours	
$CuSO_4$ concentration	Chi-square	*p*-value	Chi-square	*p*-value
0.1 mM	9.544	0.847	8.818	0.887
0.4 mM	22.413	0.097	19.586	0.188
0.8 mM	55.244	<0.0001 (***)	42.814	0.000 (***)
1 mM	58.026	<0.0001 (***)	39.764	0.000 (***)

Chi-square values were considered significant when higher than 30.578, corresponding to *alpha* level 0.01 (***).

At 0.8 and 1 mM $CuSO_4$, in the classes were the number of copies of *CUP1* gene was up to 8, the observed frequency of tolerant strains (T) was significantly lower than the expected theoretical frequency, while the observed frequency of sensitive strains (S) was significantly higher than the expected theoretical frequency (*alpha*=0.01). On the other hand, at these copper concentrations, strains copper tolerance was positively influenced by the number of copies of *CUP1* gene when its value was higher than 8. In fact, the observed frequencies of tolerant strains having more than 8 copies of the *CUP1*gene are higher than the correspondent theoretical frequencies (**Table 2.4**).

Table 2.5

Frequencies of tolerant and sensitive strains at 0.8 and 1 mM CuSO4.

		0.8 mM				1 mM			
		18 hours		48 hours		18 hours		48 hours	
N	*CUP1*-gene copy-number class	T	S	T	S	T	S	T	S
44	0-4	21(-)***	23(+)***	25(-)***	19(+)***	13(-)***	31(+)***	22(-)**	22(+)**
88	5-8	40(-)***	48(+)***	61(-)***	27(+)***	26(-)***	62(+)***	42(-)***	46(+)***
35	9-12	31(+)***	4(-)***	33(+)**	2(-)**	19(+)	16(-)	26(+)	9(-)
39	13-16	33(+)**	6(-)**	36(+)*	3(-)*	29(+)***	10(-)***	31(+)*	8(-)*
22	17-20	19(+)*	3(-)*	22(+)**	0(-)**	15(+)	7(-)	18(+)	4(-)
13	21-24	11(+)	2(-)	12(+)	1(-)	10(+)*	3(-)*	11(+)	2(-)
13	25-28	12(+)*	1(-)*	12(+)	1(-)	10(+)*	3(-)*	12(+)*	1(-)*
3	29-32	3(+)	0(-)	3(+)	0(-)	3(+)	0(-)	3(+)	0(-)
4	33-36	2(-)	2(+)	4(+)	0(-)	3(+)	1(-)	4(+)	0(-)
3	37-40	3(+)	0(-)	3(+)	0(-)	3(+)	0(-)	3(+)	0(-)
3	41-44	3(+)	0(-)	3(+)	0(-)	3(+)	0(-)	3(+)	0(-)
1	49-52	1(+)	0(-)	1(+)	0(-)	1(+)	0(-)	1(+)	0(-)
1	53-56	1(+)	0(-)	1(+)	0(-)	1(+)	0(-)	1(+)	0(-)
1	57-60	1(+)	0(-)	1(+)	0(-)	1(+)	0(-)	1(+)	0(-)
1	69-72	1(+)	0(-)	1(+)	0(-)	1(+)	0(-)	1(+)	0(-)
2	77-80	1(-)	1(+)	2(+)	0(-)	0(-)	2(+)	1(-)	1(+)

(+) or (-) indicate that the observed frequencies are higher or lower than the expected theoretical values.
* $p \leq 0.1$; effect of the Chi-square per cell
** $p \leq 0.05$; effect of the Chi-square per cell
*** $p \leq 0.01$; effect of the Chi-square per cell

To evaluate how the number of *CUP1* gene copies influences the copper tolerance in strains from different origins, they were grouped in two classes. The first class included strains with more than 8 copies and the second strains with a copy-number up to 8. The

RR (Relative Risk) has been calculated in order to assess if the positive effect of *CUP1*-gene copy-number is origin-related (**Table 2.6**).

Table 2.6

Effect of more than 8 copies of the CUP1 gene on yeasts copper tolerance. Risk Ratio (RR) > 1 indicates positive association.

Growth conditions and strains origin			*RR*	SE(ln*RR*)	*alpha*=0.05 (*) *alpha*=0.01 (**)
All strains (N=273)	18h	0.1 mM	1.00	0.015	ns
		0.4 mM	1.01	0.024	ns
		0.8 mM	1.87	0.100	**
		1 mM	2.37	0.145	**
	48h	0.1 mM	1.02	0.018	ns
		0.4 mM	1.06	0.030	ns
		0.8 mM	1.46	0.067	**
		1 mM	1.69	0.098	**
CVPAO (N=34)	18h	0.1 mM	1.20	0.129	ns
		0.4 mM	1.64	0.248	*
		0.8 mM	4.09	0.662	*
		1 mM	3.82	0.665	*
	48h	0.1 mM	1.20	0.129	ns
		0.4 mM	1.50	0.204	*
		0.8 mM	5.45	0.649	**
		1 mM	4.64	0.656	*
PAO (N=156)	18h	0.1 mM	0.98	0.016	ns
		0.4 mM	1.08	0.047	ns
		0.8 mM	2.60	0.172	**
		1 mM	4.07	0.285	**
	48h	0.1 mM	1.01	0.027	ns
		0.4 mM	1.03	0.042	ns
		0.8 mM	1.60	0.106	**
		1 mM	2.78	0.201	**
VVAO (N=63)	18h	0.1 mM	1.00	-	ns
		0.4 mM	1.00	-	ns
		0.8 mM	1.15	0.061	*
		1 mM	1.37	0.143	*
	48h	0.1 mM	1.00	-	ns
		0.4 mM	1.00	-	ns
		0.8 mM	1.00	-	ns
		1 mM	0.98	0.050	ns
Industrial (N=20)	18h	0.1 mM	1.00	-	ns
		0.4 mM	1.25	0.129	ns
		0.8 mM	15.00	0.966	**
		1 mM	9.00	1.033	*
	48h	0.1 mM	1.00	-	ns
		0.4 mM	1.15	0.101	ns
		0.8 mM	2.50	0.316	**
		1 mM	2.40	0.428	*

RR coefficients reported in **Table 2.6** evidence that the association between the number of copies of the *CUP1* gene and the copper tolerance varied among strains from different origin, in terms of significance and strength. The association was stronger at 18 than at 48 h in all cases, and in particular among Industrial strains at 0.8 and 1 mM. CVPAO, PAO and Industrial showed *RR* values significantly higher than one when copper concentration was 0.8 and 1 mM (0.4 mM only in CVPAO), confirming that a high number of copies of the *CUP1* gene is responsible for strain copper tolerance. On the other hand, VVAO showed a very weak association, with *RR* values significantly higher than one only at 18 h, when the copper concentration was 0.8 and 1 mM.

3.3.3 Sulphite tolerance and presence of the XVI-VIII and XV-XVI translocations

The sulphite tolerance has been tested on all strains in synthetic must (Delfini and Formica, 2001), mimicking oenological condition where this chemical is added before the fermentation. Sulphur dioxide has been added to sterile synthetic must to reach the final concentration of 50 or 100 mg/l, which are generally used in winemaking (Divol *et* al., 2012; Ribereau-Gayon *et* al., 2006). The optical density (600_{nm}) of each culture has been monitored during fermentation at two time points (24 and 48 hours from inoculum). When the ratio between the OD_{600nm} value measured in presence of SO_2 and in the control condition was higher than 0.5, strains have been considered tolerant.

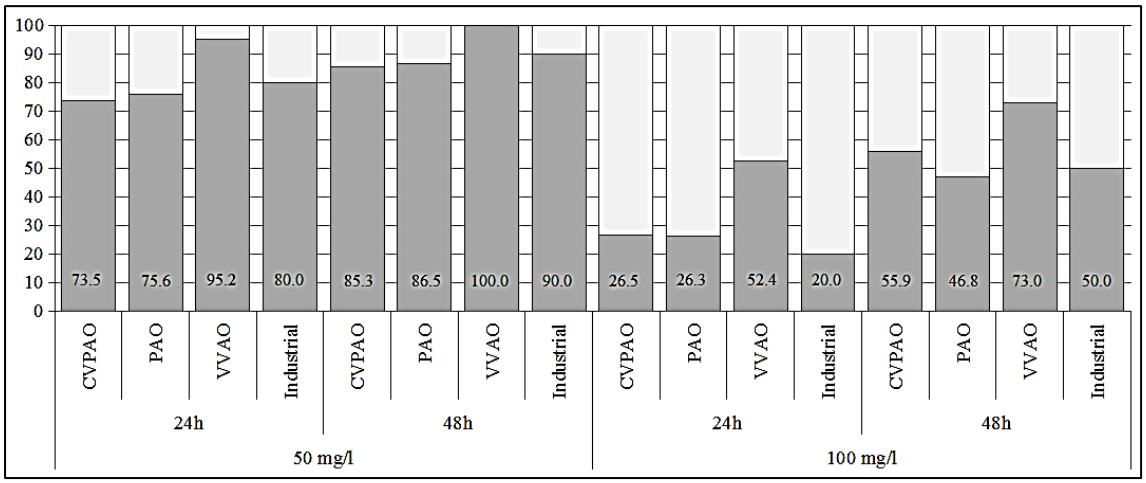
Sulphite tolerance test showed high percentages of tolerant strains at 50 mg/l of SO_2 (higher than 70% in all cases). On the contrary, the concentration of 100 mg/l of SO_2 revealed to be critical for the majority of the strains at 24 h, and for almost half of them at 48 h from inoculum: sensitive strains were, on the whole, the 68.1% at 24 h and the

45.8% at 48 h. VVAO strains revealed to be the most tolerant at both 50 mg/l (sensitive strains were 4.8% after 24 h) and 100 mg/l SO_2 (sensitive strains were 47.6% after 24 h and 27% after 48 h). At 24 hours and SO_2 concentration of 100 mg/l, the highest magnitude of the difference among VVAO strains and the others has been observed: CVPAO, PAO and Industrial sensitive strains were 73.5, 73.7 and 80.0%, respectively, while VVAO sensitive strains were 47.6%.

CVPAO, PAO and Industrial strains showed lower SO_2 tolerance than VVAO ones, especially at 100 mg/l SO_2. In fact, at this SO_2 concentration after 24 hours from inoculum VVAO sensitive strains were 47.6%, while CVPAO, PAO and Industrial sensitive strains were 73.5, 73.7 and 80.0%, respectively (**Figure 2.3**).

Figure 2.3

Effect of different sulphite doses on yeast growth after 24 and 48 hours of incubation in synthetic must. Dark grey bars represent the percentage of tolerant strains while light grey bars represent the sensitive ones.



The diffusion of the two genomic translocations involving *SSU1* gene, coding for the sulphites efflux pump correlated to sulphites tolerance, has been investigated among vineyard and Industrial strains and results are shown in **Table 2.7**.

Table 2.7

Diffusion of the genomic translocations XVI-VIII and XV-XVI.

	Chromosomes XVI-VIII translocation (%)	Chromosomes XV-XVI translocation (%)
CVPAO	29.4	2.9
PAO	57.7	0.6
VVAO	34.9	-
Industrial	60.0	-
Total	*49.1*	*0.7*

The XVI-VIII translocation has been found in 134 strains among 273, while the XV-XVI translocation only in two strains: P283.4 from CVPAO and R16.2 from PAO.

Frequency of the translocation among Industrial strains is similar to that among Italian vineyard strains (CVPAO and PAO: 52.6%), and in particular among PAO strains.

In order to examine the relationship between the sulphite tolerance of the strains and the presence of the XVI-VIII translocation, a Chi-square test of independence has been performed. The relation between these variables was significant, showing at 24 hours and 50 mg/l of SO_2 a χ^2 value (df=1, *N*=273) of 25.164, *p*=<0.0001, and at 100 mg/l SO_2 a χ^2 value (df=1, *N*=273) of 12.570, *p*=0.000. At 48 hours the χ^2 (df=1, *N*=273) at 50 mg/l SO_2 was 14.514, *p*=0.000, and at 100 mg/l SO_2 the χ^2 (df=1, *N*=273) was 22.120, *p*=<0.0001. *RRs* have been calculated in order to understand how the XVI-VIII translocation influences the SO_2 tolerance of strains from different origin (**Table 2.7**).

Table 2.8

Effect of the XVI-VIII translocation presence on strains tolerance to SO2 at 48 hours from inoculum. Relative Risk (RR) value > 1 indicates positive association.

Growth condition and strains group			Relative Risk			Chi-square	
			RR	ES(ln*RR*)	alpha=0.05(*) alpha=0.01(**) alpha=0.001(***)	$\chi 2$	*p*-value
All strains	50 mg/l	24 h	1.35	0.063	***	25.164	<0.0001
		48 h	1.18	0.040	***	14.514	0.000
	100 mg/l	24 h	1.85	0.181	***	12.570	0.000
		48 h	1.69	0.115	***	22.120	<0.0001
CVPAO	50 mg/l	24 h	1.35	0.179	ns	0.958	0.328
		48 h	1.08	0.139	ns	0.001	0.975
	100 mg/l	24 h	1.20	0.599	ns	0.016	0.900
		48 h	2.16	0.264	***	4.872	0.027
PAO	50 mg/l	24 h	1.74	0.120	***	29.651	<0.0001
		48 h	1.32	0.072	***	16.625	<0.0001
	100 mg/l	24 h	3.56	0.382	***	13.141	0.000
		48 h	1.91	0.194	***	11.752	0.001
VVAO	50 mg/l	24 h	1.07	0.044	ns	0.462	0.497
		48 h	1.00	-	-	-	-
	100 mg/l	24 h	1.68	0.189	**	5.222	0.022
		48 h	1.68	0.131	***	10.477	0.001
Industrial	50 mg/l	24 h	1.11	0.242	ns	0.013	0.909
		48 h	1.33	0.204	ns	1.134	0.287
	100 mg/l	24 h	6.23	1.426	ns	1.576	0.209
		48 h	1.56	0.518	ns	0.208	0.648
Vineyard	50 mg/l	24 h	1.38	0.065	***	25.911	<0.0001
		48 h	1.17	0.041	***	11.963	0.001
	100 mg/l	24 h	1.79	0.181	**	11.018	0.001
		48 h	1.71	0.118	***	21.469	<0.0001
Italian	50 mg/l	24 h	1.62	0.097	***	29.894	<0.0001
		48 h	1.27	0.060	***	15.179	<0.0001
	100 mg/l	24 h	2.56	0.288	**	11.292	0.001
		48 h	1.86	0.162	***	15.298	<0.0001

RR coefficients reported in **Table 2.7** evidenced that the association between the presence of the XVI-VIII translocation and the sulphites tolerance varied among strains from different origin. In all cases (except VVAO at 50 mg/l, where all strains were tolerant) a positive association has been found. The strongest association was at 100 mg/l of SO_2, and in particular among Italian strains CVPAO and PAO (*RR* value of 2.16 and 1.91,

respectively). In Industrial strains the presence of the XVI-VIII translocation does not significantly increase the probability to show sulphite tolerance.

3.3.4 Copper and sulphites tolerance association

In order to understand if there is any relationship between copper and sulphites tolerance among the tested oenological yeast strains, an association test has been performed. In this analysis the strains have been classified as "tolerant" to copper and/or to sulphites if they showed a tolerant phenotype in all the experimental conditions (time points and chemical concentration). They have been classified as "sensitive" to copper and/or to sulphites if they showed a sensitive phenotype in at least one of the experimental conditions.

Chi-square test of independence has been performed. The relation between the copper tolerance and the sulphites tolerance was significant, showing a χ^2 value (df=1, N=273) of 4.461, p=0.035.

The Chi-square per cell observation allowed identifying which kind of association exists between the tolerance phenotypes among the investigated strains. Results indicated that the strains showing a tolerant or a sensitive phenotype for both the chemicals are many more than expected, while those strains showing a mixed phenotype are less than expected ($p \leq 0.05$).

RR coefficients have been calculated in order to understand how the tolerance phenotype for one of the two chemicals influences the tolerance phenotype related to the other chemical. Results evidenced that the copper tolerant phenotype has a positive influence on sulphites tolerance and the sulphites tolerant phenotype has a positive influence on copper tolerance (**Table 2.9**).

The relationship between the tolerance phenotypes has been deeper studied, in order to assess if it could be affect by the presence of the translocation between the chromosomes XVI and VIII and / or the different number of *CUP1* gene copies. The analysis showed that among strains with less up to 8 copies of *CUP1* gene the relationship between copper tolerance and sulphites tolerance was significant, showing a χ^2 value (df=1, *N*=132) of 5.198, *p*=0.023. *RR* coefficients have been calculated in order to know the magnitude of the reciprocal effect of the tolerance to copper and to sulphites among the strains having up to 8 copies of *CUP1* gene. Results evidenced that the relationship between the two phenotypes among these strains is strengthened with respect to the totality of the tested yeasts, as, among strains with up to 8 *CUP1* gene copies, the *RR* values were higher than the value of the totality of the strains (**Table 2.8**).

Table 2.9

Effect of the tolerance phenotype to one chemical on the tolerance phenotype to the other one. Risk Ratio (RR) value > 1 indicates positive association.

	RR	SE(Ln*RR*)	*alpha*=0.05 (*) *alpha*=0.01 (**)	*N*
cT phenotype on sT	1.46	0.182	*	273
sT phenotype on cT	1.31	0.123	*	273
cT phenotype on sT (up to 8 *CUP1* gene copies)	1.81	0.229	**	132
sT phenotype on cT (up to 8 *CUP1* gene copies)	1.97	0.274	*	132
cT phenotype on sT (up to 8 *CUP1* gene copies)	1.61	0.333	ns	141
sT phenotype on cT (up to 8 *CUP1* gene copies)	1.20	0.113	ns	141

cT) copper tolerance. sT) sulphites tolerance.

3.4 Discussion

Three groups of indigenous *Saccharomyces cerevisiae* yeasts isolated from different winemaking areas and a pool of twenty commercial *S. cerevisiae* oenological starter strains have been studied, focusing on the copper and sulphite tolerance, the *CUP1*-gene copy-number and the diffusion of the genomic translocations XVI_VIII and XV_XVI which involve the sulphites efflux pump *SSU1* gene.

CVPAO and PAO are areas of very ancient winemaking tradition, and they are recognized in Italian Appellation of Origin system as DOCG (Controlled and Guaranteed Designation of Origin – D.M. 17.07.2009; D.M. 22.12.2010). They are located in the North-East of Italy in Veneto region, where viticulture started around 181 b. C., when the Roman army advanced towards Aquileia. Some archaeological finds suggest an even older viticulture (Bianchin *et* al., 2016). On the contrary, Brazil has a recent winemaking history, starting in 1532 with the arrival of Portuguese colonizers. Around 1875, Italian immigrants carried their winemaking knowledge in the Serra Gaucha area, where VVAO (Vale dos Vinhedos) area lies. In the beginning of the twentieth century Rio Grande do Sul intensified the planting of vine varieties and from here onwards the quality of wines improved considerably until the 1990s, which can be considered the years of the first great evolution of Brazilian wine (Wurz *et* al., 2017; Fensterseifer, 2007). Vale dos Vinhedos raised to Denomination of Origin in 2012 (Registro de Indicação Geográfica N°IG201008 – INPI).

3.4.1 *CUP1* copies number and copper tolerance

In CVPAO and PAO, the two Italian (and with more ancient vine-growing tradition) Appellations of Origin (AO), the *CUP1* gene copy-number appeared more variable than in VVAO and Industrial, and results of copper tolerance test evidenced the higher tolerance of Brazilian *Saccharomyces cerevisiae* yeast strains with respect to Italian and Industrial ones. Industrial strains showed a lower tolerance to copper than the vineyard strains: this could be due to the irrelevance attributed, in the past, to the copper tolerance for yeast strains to be used in winemaking (Regódon *et* al., 1997) and, when recognized as an advantage for fermenting grape musts with high copper residues, it has been pointed out that such characteristic could encourage vine-growers to disrespect the recommended fungicide withholding periods (Rainieri and Pretorius, 2000; Pretorius, 2000).

Results from Chi-square association test between copper tolerance and the *CUP1* gene copies number evidenced that the effect of the *CUP1* gene copies number is significant when copper concentration is higher than 0.4 mM. Chi-square per cell in 0.8 and 1 mM copper concentrations evidenced that, in this pool of *Saccharomyces cerevisiae* strains, a *CUP1* gene copies number higher than 8 positively affects the copper tolerance. This result confirms the positive association between the copper tolerance and a high *CUP1* gene copies number. Moreover, the higher the copper concentration the higher is the strength of that association.

This is particularly true among industrial and Italian indigenous strains. Brazilian strains (VVAO) seems to have a copper excess management which do not involve the *CUP1* gene duplication: in fact, they showed the highest tolerance in the growth test in YNB medium, but the weakest association between the gene copies number and tolerant phenotype. The copper tolerance in these strains could be linked to a higher basal

expression of the *CUP1* gene (which is the mechanism for increasing sulphites tolerance *via* overexpression of the *SSU1* gene) or other copper tolerance mechanisms.

3.4.2 Diffusion of XVI-VIII translocation and sulphites tolerance

Brazilian *Saccharomyces cerevisiae* yeast strains showed higher tolerance to sulphites with respect to Italian and Industrial ones. Chi-square test evidenced that the sulphite tolerance is significantly associated to the presence of the XVI-VII genomic translocation, confirming the high impact of the Ssu1p on sulphites resistance in *S. cerevisiae* yeasts. Despite expectations, in Industrial strains the presence of the XVI-VIII translocation does not significantly increase the probability to show sulphite tolerance. On the contrary in the other strains, which are all isolated from vineyards, the association between the presence of the translocation and the sulphite tolerance is significant in almost all the experimental conditions, and it is stronger in Italian PAO strains.

Results in this work suggest that the XVI-VIII translocation is an evolutionary success not only for winery strains, as suggested in previous works (Pérez-Ortín *et* al., 2002; Zimmer *et* al., 2014), but also for vineyard strains, where the translocation has been found in the 48.2% of the strains isolated from vineyard. The frequency in vineyard is very high, considering that in the winery the yeasts are directly exposed to sulphites, and so it is plausible that sulphites had acted as selective agent. On the contrary, the vineyard environment is very different from that of winery, so the mechanism leading to the strong diffusion of this genomic variation could be different.

The translocation between chromosomes XVI and VIII is highly probably due to a fortuitous double-strand DNA break (DSB) in either one of the two promoter regions of *ECM34* and *SSU1* genes, which show micro-homology. This event, occurred in one of

these promoter regions, facilitated the illegitimate recombination with the other promoter region through a short homologous region (Pèrez-Ortín *et* al., 2002). In a similar way, Zimmer *et* al. in 2014 found that the DNA break-points which originated the translocation between chromosomes XV and XVI occurred in a low complexity AT rich region, without evident homology between chromosomes. In these two works, focused on genomic variation involving the *SSU1* gene (translocations XV-t-XVI and *t(VIII;XVI)*), authors speculated that the most probable origin of the translocations could be the adaptation of the yeasts to the large use of sulphites in cellars, which are a very common practice in the winemaking process, performed since the historical first evidences of wine production.

Nevertheless, results in the present study showed that sulphite tolerance of Industrial strains seems to be poorly influenced by the presence of the genomic translocation involving *SSU1* gene than the vineyard strains are. In the light of these considerations, along with the results presented in this study, the vineyard environment seems to have a higher impact on the wine yeasts genome variations than cellar and cellar practises have. In fact, Industrial strains showed the highest percentage of strains carrying the *t(VIII;XVI)*, and this agrees with the hypothesis claiming that the yeasts selection favoured strains showing good fermentation performances and sulphites tolerance; on the other hand, Industrial strains didn't show a significant association between the presence of the *t(VIII;XVI)* translocation and the sulphite tolerance, where both Italian and Brazilian vineyard strains did. This fact could be explained assuming that this genomic variation, is originated in the vineyard and only later, in the cellar, it has been unwittingly selected by oenologists' winemaking practises, such as the addition of SO_2 on crushed grapes or already on intact grapes to prevent oxidations (Fugelsang and Edwards, 2007).

3.4.3 Copper and sulphites tolerance association

To date, the wine world is approaching new tendencies: among all topics, vine-growers are on organic farming case (with the aim to reduce spreading pesticides), while oenologists are pressed by the wine market for reducing sulphites usage and for increasing the link between wine and *terroir*, which can be promoted by spontaneously fermenting grape musts or by using yeasts strains selected in the cultivation vineyard.

These goals represent a hard challenge for producers: they need to combine the organic farming to an agricultural practise allowing the microbial preservation, in order to reduce the risk of environmental disequilibrium and problems in spontaneous grape musts fermentation. The organic agricultural practices in viticulture need a heavy use of copper for controlling *Plasmopara viticola* (downy mildew) and other fungal diseases, like *Phomopsis viticola* (dead arm), *Guignardia bidwellii* (black rot), and others, but the toxic effect of copper is extended to oenological yeasts too. From this point of view, results in this study on yeast tolerance to copper, united to previously results of oenological yeasts biodiversity and fermentative aptitude for the same winemaking area (Crosato *et* al., 2018), are encouraging.

About sulphites reduction in winemaking, the effort is to satisfy the healthiness of the wine considering two main aspects: a low addition of sulphites, toxic for humans, and the management of spoilage microorganisms, for which the sulphites adding is requested and, to date, not totally replaceable. For this reasons the sulphites tolerance of oenological yeasts is still an important character in yeasts selection for winemaking. The best solution is finding new autochthonous yeast strains to be used by winemakers as added value to their products, increasing the linkage to the grapes area of origin without disregarding the technological requirements.

The analysis on the association between the tolerance to copper and the tolerance to sulphites allowed understanding that this is a positive and significant relationship, in particular among strains with up to 8 copies of the *CUP1* gene, while the presence of the XVI_VIII translocation is irrelevant with respect to the association between copper and sulphites tolerance. As previously described, the copper tolerance is associated to a number of *CUP1* gene copies of 9 or more, while among strains with up to 8 *CUP1* gene copies there is higher copper sensitivity and a positive and significant association between copper and sulphites tolerance.

Such result could be explained assuming that the strains with up to 8 copies of *CUP1* gene have, besides an efficient sulphites tolerance mechanism, an efficient mechanism to face copper toxicity too, alternative to *CUP1* gene duplication. On the other hand, our results suggest that the copper tolerance of the strains with 9 *CUP1* gene copies or more is based on the duplication of this gene only. This adaptation increased the copper tolerance without any effect on the sulphites tolerance: in fact, in those strains presenting 9 copies of the *CUP1* gene or more the association between the copper tolerance and the sulphites tolerance is positive but non-significant (**Table 7**).

Our result agrees with the assumption that the *CUP1* gene duplication is a specific and effectiveness response to face the copper toxicity in wine yeasts.

Moreover, as Adamo *et* al. (2012) found, the duplication of the *CUP1* gene is associated to an evolutionary adaptation to copper, due to the exposure to increasing doses of this metal. The period of exposure of Italian yeast population is very different from the Brazilian one: the spread of copper-based treatments in vineyard started in the end of the 1800-s, rapidly adopted in Europe, and so in Italy, after Alexis Millardet discovered its effectiveness on downy mildew control (Ayres, 2004). In Brazil, and in particular in the

Vale dos Vinhedos, the start of the copper heavy usage in viticulture started few decades ago only, when vine-growers converted their vineyards and their winemaking processes in order to obtain the admission as "Appellation of Origin". In this way, the Italian strains underwent a longer adaptation process than Brazilian ones, and assuming that the duplication of the *CUP1* gene is due to copper exposure, this could be the reason why we found a generally lower *CUP1* gene number of copies among Brazilian strains than among Italian ones. Besides a low *CUP1* gene copies number, a high copper tolerance had been found in VVAO strains: the possible explanation is that the selective pressure of copper on yeasts had rapidly selected all tolerant phenotypes, and the duplication of the *CUP1* gene is not yet enough effectiveness, in this population, to actually be identified as a successful copper tolerance strategy. Among Italian yeasts population, a high number of the *CUP1* gene copies is associated to the copper tolerance: on the basis of our results, this finding could be blamed to the longer time that the less tolerant yeast strains had for rising the sufficient amount of *CUP1* gene copies to deal with copper toxicity.

Another possible explanation for this association could be linked to the copper sulphate as fungicide. The sulphur metabolism in yeast lead to the production of sulphites inside the cell itself (Thomas and Surdin-Kerjan, 1997; Park and Bakalinski, 2000), and yeasts which naturally produce high quantity of intracellular sulphites are plausibly tolerant to high quantity of sulphites in the growth media too. Our results suggest that the exposure to copper sulphate could influence the tolerance of vineyard yeasts to both copper and sulphites: in this case, copper stimulates copper tolerance *via* the *CUP1* gene duplication and sulphate stimulates the sulphites tolerance *via* the occurrence of the XVI-VIII translocation (overexpression of Ssu1p).

3.5 Conclusions

In the present work a population of 273 *Saccharomyces cerevisiae* strains has been tested for copper and sulphites tolerance: 153 came from vineyard and 20 were commercial wine starters. Copper tolerance has been analysed with respect to the number of copies of the *CUP1* gene, encoding for a metallothionein involved in copper detoxification. Our results agree with previous literature findings, and indicate that the *CUP1* gene is associated to copper tolerance when the number of copies oversteps a certain threshold, that is our study was 8 copies. The higher influence of *CUP1* gene copies number on copper tolerance has been found among Italian vineyard strains: Brazilian strains, despite they were more tolerant to copper, presented a low *CUP1* gene number of copies, suggesting that they developed a different copper tolerance mechanism. Since the strongest association between copper tolerance and *CUP1* gene copies number has been found in strains from Italian vineyards, which have a longer winemaking tradition than Brazilian ones, the adaptation of autochthonous yeasts to copper could be linked to the exposure duration to this element.

Sulphites tolerance has been analysed with respect to the presence of the XVI-VIII genomic translocation, which causes the overexpression of the sulphites efflux pump Ssu1p. This genetic trait resulted generally associated to yeasts sulphites tolerance. Despite expectations, among Industrial strains the presence of the translocation is not associated to the tolerant phenotype, while all vineyard strains did.

Finally, an association between copper and sulphites tolerance has been found among the tested wine yeasts, suggesting that the development of tolerance mechanisms against these chemicals could be connected. A possible common point could be the high use of copper sulphate as antifungal pesticide in vine-growing, leading to a multiple resistance

strategy: if copper stimulates copper tolerance *via* the *CUP1* gene duplication, sulphate stimulates the sulphites tolerance *via* the occurrence of the XVI-VIII translocation (overexpression of Ssu1p).

4. Chapter III

The gene expression of the hexoses facilitators affects the strains fitness for fermentation: the fructose transporter *FSY1* improves the carbon sources utilization in *Saccharomyces cerevisiae*

4.1 Introduction

In wine alcoholic fermentation, glucose and fructose present in grape must are co-fermented by yeasts to ethanol and carbon dioxide. Grape must usually contains equal or very similar amounts of both sugars (Guillaume *et* al., 2007). Glucose is known to be the preferred carbon source for *Saccharomyces cerevisiae*. Although fructose is used concomitantly with glucose, the latter is the first sugar to be depleted from the medium during fermentation, and their consumption during fermentation show a discrepancy (Berthels *et* al., 2004; Galeote *et* al., 2010). Consequently, fructose becomes the main sugar present during the late stages of alcoholic fermentation, and wine yeasts have to ferment this non-preferred sugar after long periods of starvation and in the presence of large amounts of ethanol. The stress associated with these conditions, together with nutritional imbalances, may result in sluggish or stuck fermentations (Guillaume *et* al., 2007; Blatayron and Sablayrolles, 2001). It has been reported that stuck fermentations are frequently characterized by an unusually high fructose-to-glucose ratio (Berthels *et* al., 2004). The low fructose utilization capacity of *S. cerevisiae* is thought to contribute to the

low fermentation rate. The ability of wine yeasts to ferment fructose is therefore critically important for the maintenance of a high rate of fermentation at the end of the process and for fermentation of the must to dryness. The reasons why the difference between the glucose fermentation rate and the fructose fermentation rate are unclear, but one of the first steps in hexose metabolism is generally thought to be involved (Guillaume *et* al., 2007).

The first essential movement towards the utilization of hexose sugars is their uptake by yeast cells. In yeast, hexose uptake may proceed through facilitated diffusion carriers and energy-dependent active proton-sugar symporters (Anjos *et* al., 2013; Galeote *et* al., 2010; de Sousa *et* al., 2004). Hexose transport in *S. cerevisiae* occurs via facilitated diffusion carriers and these are encoded by several genes, including the *HXT* genes, the *GAL2* gene encoding a galactose transporter and *SNF3* and *RGT2* encoding two glucose sensors (Anjos *et* al., 2013; de Sousa *et* al., 2004). Among the 17 *HXT* genes in *S. cerevisiae*, only seven of them, Hxt1p– Hxt7p, are required for growth on glucose or fructose (Galeote *et* al., 2010; Luyten *et* al., 2002). Although all of the hexose transporters in *S. cerevisiae* can also transport fructose, glucose is the preferential sugar for Hxt carriers (Galeote *et* al., 2010). The catabolic hexose transporters exhibit different affinities for their substrates; furthermore, the expression of their corresponding genes is controlled by the glucose sensors, according to the availability of carbon sources (Boles and Hollenberg, 1997).

Recently, Galeote *et* al. (2010) demonstrated that the new *FSY1* gene found in *S. cerevisiae* encodes a high-affinity fructose/H+ symporter. Moreover, *FSY1* expression is repressed by high concentrations of glucose or fructose and is induced by ethanol as the sole carbon source.

Glucose-signaling pathways and transcriptional regulation

Glucose present in the medium rapidly represses the expression of genes that are required for the metabolism of alternative sugars, and induces the transcription of genes that facilitate its uptake and metabolism (Pires and Brányik, 2015; Mosley *et* al., 2003). Glucose regulation of *HXT* gene expression is mediated via signals emanating from the glucose receptors Snf3 and Rgt2: they are *HXT* homologous able to mediate the active glucose carriers by low and high glucose concentrations, respectively (Flick *et* al., 2003; Özcan *et* al., 1996, 1998). The expression of individual *HXT* genes depends on environmental factors, such as the hexose concentration sensed by the yeast cell (Guillaume *et* al., 2007).

Glucose ensures its own efficient metabolism by serving as an environmental stimulus that regulates the quantity, types, and activity of glucose transporters, both at the transcriptional and post-translational levels. Expression of the *HXTs*, all of which exhibit different levels of glucose affinity, is differentially regulated depending on extracellular glucose concentrations.

High glucose concentration

In high glucose concentrations there are two key proteins: one is the protein-kinase Snf1, and the second is the Mig1 (and the Mig1 complex: Tup1/Ssn6/Mig1). In high glucose concentration the Glc7-Reg1/2 phosphatases cleave the P-group from Snf1p, which results inactivated. Inactive Snf1 is unable to phosphorylate the cytosolic Mig1p, which can enter into the nucleus and find and bind the general repressors Tup1 and Ssn6. The so-formed Mig1-complex binds the promotors of genes including those involved in gluconeogenesis, respiration and alternative carbon sources uptake and utilization (e.g.

SUC, *GAL* and *MAL* genes). The starting point of such regulatory chain is the Snf1p dephosphorylation, and this event is induced by the glucose phosphorylation: in high glucose concentration, indeed, the Hxt-mediated glucose uptake and the immediate glucose phosphorylation by the Hxk1 or Hxk2 hexokinases (but also AMP depletion) induces the Snf1p inactivation (Pires and Brányik, 2015).

Moreover, the high glucose concentration activates the plasma membrane sensor Grp1, which can bind the Gpa2p to form an activated receptor system. The Grp1-Gpa2 receptor activates the Cyr1p allowing the ATP conversion to cAMP. The increased cAMP concentration leads to the PKA activation and, consequently: STRE-controlled genes repression and activation of growth genes and cell cycle progression genes (**Figure 3.1**) (Geladé *et* al., 2003).

The glucose-mediate gene repression, as reported, is a strong and redundant regulation system, mostly concerning the inhibition of the others carbon sources uptake and utilization (*GAL* and *MAL* genes).

Besides a gene repression, the high glucose concentration can also leads to a positive gene regulation: is this the case of the Rgt2 sensor pathway. In high glucose concentration the Rgt2 sensor activation leads to a partial Rgt1p inactivation: in this case the Mth1p and Std1p are inactivated and degraded by SCF-Grr1p and the Rgt1p is turned into a transcriptional activator of *HXT1*.

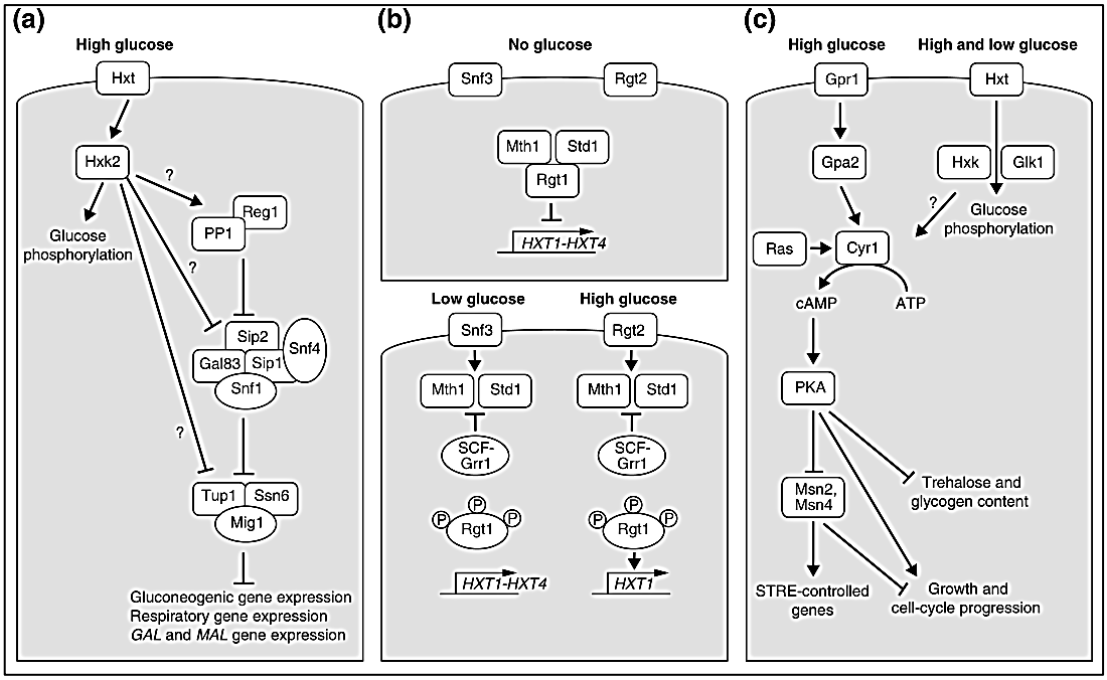


Figure 3.1

A simplified schematic representation of the three well-characterized glucose-response pathways in *S. cerevisiae*. (a) The main glucose repression pathway. (b) The Snf3/Rgt2 glucose-sensing pathway. (c) The Gpr1/Gpa2 glucose-sensing pathway (Figure 1 in Geladé et al., 2003).

Glucose absence

In glucose absence the Snf3/Rgt2 glucose-sensing pathway is involved. In the case of glucose absence the Snf3p and Rgt2p are inactive. In these conditions the Rgt1 complex (Mth1/Std1/Rgt1) is active and represses the transcriptions of the *HXT1-HXT4* genes (**Table 3.1** and **Figure 3.1**).

ORF	Gene name	Protein function or characteristic	Ratio of spot intensity of the mutant to that of the wild type[b]				No. of Rgt1 sites/ no. of conserved sites[c]
			*rgt1*Δ Gal	*RGT2-1* Gal	*SNF3-1* Gal	*snf3*Δ *rgt2*Δ Glu	
YHR092C	***HXT4***	Glucose transporter	↑ 23.1 (↑ 10.5)	↑ 2.4	↑ 10.6	↓ 0.2	5/2
YDR345C	***HXT3***	Glucose transporter	↑ 13.3 (↑ 16.9)	↑ 2.8	↑ 5.3	↓ 0.2	11/7
YMR011W	***HXT2***	Glucose transporter	↑ 13.0 (NC)	↑ 1.9	↑ 5.2	↓ 0.3	3/2
YKR075C		Similarity to N terminus of Reg1	↑ 12.6 (↑ 2.1)	↑ 2.0	↑ 8.1	↓ 0.2	7/5
YGL157W		Similarity to dihydroflavonol 4-reductase	↑ 7.9 (↑ 6.6)	↑ 2.5	↑ 4.6	↓ 0.1	2/1
YHR094C	***HXT1***	Glucose transporter	↑ 7.2 (↑ 51.4)	↑ 2.4	↑ 3.8	↓ 0.1	11/4
YOR047C	***STD1***	Regulator of Rgt1	↑ 4.5 (↑ 3.5)	NC	↑ 2.7	↓ 0.4	2/2
YHR096C	***HXT5***	Glucose transporter	↑ 4.0 (NC)	NC	↑ 2.1	↓ 0.3	4/0
YGL209W	***MIG2***	Glucose-dependent repressor	↑ 3.4 (↑ 12.5)	↑ 2.0	↑ 6.8	NC	9/4
YNL234W		Heme-binding globin-like protein	↑ 3.1 (NC)	NC	↑ 1.8	↓ 0.5	2/1
YOR062C		Similarity to N terminus of Reg1	↑ 3.0 (↑ 5)	NC	↑ 1.7	↓ 0.2	4/1
YNL065W	***AQR1***	MFS[e] transporter; resistance to monocarboxylic acids	↑ 2.6 (NC)	NC	↑ 1.7	NC	5/4
YLR109W	***AHP1***	Alkyl hydroperoxide reductase; redox homeostasis	↑ 2.5 (NC)	NC	↑ 1.7	↓ 0.1	2/2
YER037W	***PHM8***	Involved in phosphate metabolism?	↑ 2.4 (NC)	↑ 2.2	↑ 2.3	↓ 0.2	1/1
YOR338W		Uncharacterized ORF (SGD)	↑ 2.2 (NC)	NC	NC	NC	0
YKL036C		Dubious ORF (SGD)	↑ 2.1 (A)	NC	NC	NC	2
YCR005C	***CIT2***	Peroxisomal citrate synthase	↑ 2.1 (↑ 4)	↑ 1.7	NC	↓ 0.5	3/2[d]
YER028C	***MIG3***	Possible glucose-dependent repressor	↑ 2.0 (↑ 4.6)	NC	NC	NC	4/2
YGL039W		Similarity to dihydroflavonol 4-reductase	↑ 2.0 (NC)	NC	NC	↓ 0.5	2/0
YKL035W	*UGP1*	UDP-glucose pyrophosphorylase	↑ 2.0 (NC)	NC	NC	NC	1/0
YOL016C	***CMK2***	Calmodulin-dependent protein kinase	↑ 2.0 (↑ 3.4)	↑ 1.7	NC	NC	1/0
YHR087W		Uncharacterized ORF (SGD)	↑ 2.0 (NC)	NC	NC	↓ 0.2	1/0
YMR316W	***DIA1***	Regulation of invasive growth?	↑ 1.9 (NC)	↑ 1.7	NC	NC	3/0
YER062C	***HOR2***	Glycerol-1-phosphatase	↑ 1.9 (NC)	NC	NC	↓ 0.3	2/1
YJL214W	***HXT8***	Glucose transporter	↑ 1.9 (↑ 1.8)	NC	↑ 2.9	NC	5/0
YBR105C	***VID24***	Vacuolar protein targeting	↑ 1.9 (↑ 2.6)	NC	NC	NC	4/3
YDR423C	*CAD1*	Jun family of transcription factors	↑ 1.9 (NC)	NC	NC	NC	0
YHR097C		Uncharacterized ORF (SGD)	↑ 1.9 (NC)	NC	NC	↓ 0.5	0
YOL046C		Dubious ORF (SGD)	↑ 1.9 (A)	NC	NC	NC	2
YBR067C	*TIP1*	Cell wall mannoprotein	↑ 1.9 (NC)	NC	NC	↑ 3.8	5/1
YDR277C	***MTH1***	Regulator of Rgt1	↑ 1.8 (↑ 2.1)	NC	↑ 2.0	NC	4/2
YOL136C	***PFK2***	6-Phosphofructo-2-kinase; regulation of glycolysis	↑ 1.8 (NC)	NC	↑ 1.8	NC	2/2
YFL054C		Glycerol transporter	↑ 1.8 (↑ 3.5)	NC	↑ 2.0	NC	3/2
YDR001C	*NTH1*	Neutral trehalase; stress response	↑ 1.8 (NC)	NC	NC	NC	2/2
YPL026C	*SKS1*	Protein kinase; multicopy suppressor of *snf3*	↑ 1.8 (NC)	NC	NC	NC	6/3[e]
YLR413W		Uncharacterized ORF (SGD)	↑ 1.8 (NC)	NC	NC	NC	1/0
YDL062W		Dubious ORF (SGD)	↑ 1.8 (A)	NC	NC	NC	1
YMR136W	*GAT2*	GATA zinc finger toxin factor	↑ 1.8 (NC)	NC	NC	NC	2/1
YFR016C		Uncharacterized ORF (SGD)	↑ 1.8 (NC)	NC	NC	NC	0
YKR098C	*UBP11*	Ubiquitin-specific protease	↑ 1.8 (NC)	NC	NC	NC	4/3
YKR076W	*ECM4*	Cell wall organization	↑ 1.8 (NC)	NC	NC	NC	7/5[f]
YLR194C		Uncharacterized ORF (SGD)	↑ 1.8 (↑ 1.8)	NC	NC	↓ 0.3	1/0
YAL061W		Putative polyol dehydrogenase	↑ 1.8 (↑ 1.7)	NC	NC	NC	1/0

Table 3.1

Known and potential targets of Rgt1 (Figure 3 in Kaniak et al., 2004).

Low glucose concentration

When low amounts of glucose are presents the Snf3 sensor is turned active. The main Snf3 role is the Rgt1 inactivation, which is reached in two cooperating ways: the hyper-phosphorylation of Rgt1p and the inactivation and degradation of Mth1p and Std1p via SCF-Grr1. By the Snf3p action, the transcription of *HXT1-HXT4* is turned active.

With respect to the sugars transporters genes, the resulting regulatory system turnover during the fermentation should be similar to the proposed following one:

- High glucose concentration: the glucose income into the cell (with consequent immediate phosphorylation, etc.) leads to the alternative carbon sources transcriptional inactivation via the Snf1p/Mig1p regulation and to the *HXT1* transcriptional activation via the conversion of Rgt1p into a transcriptional activator (mediated by Rgt2p).
- Medium/low glucose concentration: the Snf3 sensor activation leads to the transcriptional activation of *HXT1-HXT4* genes.
- Glucose depletion: *HXT1-HXT4* transcriptional repression mediated by Snf3/Rgt2.

The fermentation rate and the ability to ferment grape must sugars to dryness depend on the level of expression of the transporters. Good fermenting strains express transporter genes at high level, thus their gene expression study it's an important feature to select the best strains for fermentation.

Generally, gene expression studies are focused just on a single strain during the fermentation process (Alexandre *et* al., 2001; Boer *et* al., 2003; Gibson *et* al., 2008; Wu *et* al., 2004).

This work is focused on one of the most important trait of the fermentative aptitude of *Saccharomyces cerevisiae* species: the fermentable carbon sources uptake from the growth media. This essential feature involves several plasma membrane hexoses transporters and a web of highly complex regulation pathways, which results in a fine expression calibration of the transporter genes. In this work we evaluated the correlation between stationary phase and the gene expression of the different transporters in four different *S. cerevisiae* strains, to evaluate how the different expression influences the ability to sustain and to end fermentation.

The normalized relative expression of the hexoses transporters genes has been analysed by the quantitative $2^{-\Delta\Delta CT}$ (Livak) Method for Real Time PCR data. The gene expression patterns have been studied at two time points of the stationary phase during a fermentation trial performed for four strains (P301.9, R31.3, R008, and EC1118). The study involved the following genes: *HXT1*, *2*, *3*, *4*, *5*, *6/7*, *8*, *MAL31*, *GAL2* and *FSY1*.

The *FSY1* gene encodes for a novel high affinity transporter protein. The diffusion of *FSY1* among the Italian vineyard strains and among a pool of commercial strains has been evaluated. The results showed a quite high diffusion in vineyard and a strong presence in the industrial group. Gene expression analysis showed deep differences in the sugar transporters utilization, in particular for the fructose transporter genes: strains carrying the fructose transporter gene showed different expression of *FSY1*, which can turns into a enhanced carbon sources utilization ability at the end of the fermentation.

4.2 Material and Method

4.2.1 Yeast strains

Wine *Saccharomyces cerevisiae* strains EC1118, P301.9, R008 (Treu *et* al., 2014) and R31.3 have been used in the present work. The strains P301.9, R31.3 and R008 come from Conegliano Valdobbiadene Prosecco Superiore DOCG (CVPAO) and Raboso Piave DOC (PAO) regions in North East Italy, and they have been isolated during a yeast selection program that isolated about 600 yeasts, which lead to around 200 mitochondrial DNA RFLP genotypes. In the genome of the industrial strain EC1118 (Lallemand Inc. – Montreal, Canada) Galeote *et* al. in 2010 found the presence of the high affinity fructose/H^+ symporter *FSY1* and in this study EC1118 served as positive control for this gene.

Thanks to the genome sequencing of four vineyard strains Treu *et* al. in 2014 identified the *FSY1* fructose symporter in the strain P301, and in the same work they assessed the absence of this gene in R008. The identified ORF in the cited work has been named by authors "*P301_O30021*". In the present work the name *FSY1* has been used as synonymous of *P301_O30021*.

Industrial yeast strains used in this work are listed in **Table 3.2**.

Table 3.2

Industrial wine yeast strains used in this work.

Strain name	Description	Origin
Lalvin 71B	Wine strain	Narbonne, France
AWRI 1631	Wine strain	Derived from N96
Mycoferm Pro Crio SP	Wine strain	Champagne, France
Mycoferm CRU 31	Wine strain	Europe
Lalvin ICV D47™	Wine strain	Côtes du Rhône, France

Strain name	Description	Origin
Lalvin DV10®	Wine strain	Champagne, France
Lalvin EC-1118	Wine strain	Champagne, France
Zymaflore ® F15	Wine Strain	Bordeaux, France
Blastosel FR 95	Wine strain	Loire Valley, France
Blastosel Grand Cru	Wine strain	La Rioja, Spain
GY	Wine strain	Europe
LV10	Wine strain	Europe
Mycoferm Cru 611	Wine strain	Europe
Nouveaux Ferments	Wine strain	Europe
NT116	Wine strain	Stellenbosch, South Africa
Premium Prosecco 444	Wine strain	ISMA
Lalvin QA23	Wine strain	Portugal
UC5	Sake strain	Japan
Premium® Blanc 12V	Wine strain	Alsace, France
Uvaferm VRB	Wine strain	Logroño, Spain

4.2.2 Fermentation trial

Fermentations were performed at 25°C in synthetic grape must MS300 (Bely *et* al., 1990) modified for the carbon source: 100 g/L of glucose and 100 g/L of fructose were added instead of 200 g/L of glucose. Fermentations took place in 1L bioreactors (Multifors, Infors HT, Basel, CH). These instruments are sensors equipped in order to allow setting and monitoring the temperature and provided with a flow meter in order to determine CO_2 outflow (red-y mod. GSM-A95A-BN00, Infors HT, Basel, CH, Switzerland) (range 1-20 ml/min). For each strain, approximately 3×10^6 cells/ml have been inoculated in 1L of MS300 must. For each strain cells were collected for Real Time-PCR assay at two sampling points during stationary phase, corresponding to when 45 and 60 g/L of CO_2 have been produced. After sampling, cells were centrifuged to remove the growth media

and immediately frozen at -80 °C. At the same sampling points, 50 ml of fermented media have been collected and frozen for HPLC analysis.

4.2.3 HPLC analysis

The residual sugars, the produced ethanol and glycerol have been determined by HPLC analysis using a Waters 1525 Binary HPLC Pump (Waters, Milford, MA) equipped with an Aminex HPX-87H HPLC column (Bio-Rad, Hercules, CA). Waters 2414 Refractive Index Detector (Waters, Milford, MA) set to 600 nm has been used.

4.2.4 RNA extraction and reverse transcription

Total RNA has been extracted using the TRIzol® Plus RNA Purification Kit (Ambion). Concentration, purity and integrity of RNA samples were determined by spectrophotometric analysis using SPARK® multimode microplate reader (Tecan Trading AG, Switzerland), considering the absorbance ratio at 260/280 nm and at 230/260 nm. The quality and integrity of RNAs were confirmed by electrophoresis on 1.5% agarose gels under denaturing conditions (2% vol. formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7).

4.2.5 DNAse treatment

To obtain DNA-free RNA the total RNA previously extracted was treated as follows:

Total RNA	1 µg
10X Reaction buffer with $MgCl_2$ (Fermentas)	1 µl
DNAse I, RNAse-free (Fermentas)	1 µl (1U)
DMPC-treated water	to 10 µl

After 30 min incubation at 37° C, add 1 µl 50mM EDTA (Fermentas) and incubate at 65° C for 10 min to inactivate DNAse. The template can be used for reverse transcriptase.

4.2.6 Synthesis of cDNAs for PCR amplification

cDNA were synthesized using RevertAid M-MuLV Reverse Transcriptase (200 u/µl) (Fermentas) using poliT(16) primers (MWG-biotech, HPSF purified). Each reactions were assembled as follows:

Total RNA DNAse-free	11 µl
Random Primers (0.5µg/µl, Promega)	0.4 µl
Oligo(dT) Primer (0.5µg/µl, MWG)	1 µl
Nuclease-Free Water	0.6 µl
(incubation at 65°C for 10 minutes)	
RevertAid 5X Reaction Buffer (Fermentas)	4 µl
dNTP mix for RNA (Promega)	2 µl
RevertAid M-MuLV Reverse Transcriptase (200 u/µl) (Fermentas)	1 µl
final volume	*20 µl*

The reactions were incubated at room temperature (22–25°C) for 10 minutes and at 42°C for 2 hours. Afterwards for enzyme inactivation tubes were incubated at 70°C for 15

minutes. Each step was performed with PTC200thermal cycler (MJ Research Inc.). Samples have been stored at -20°C until Real Time-PCR has been run.

4.2.7 Polymerase Chain Reaction and gel electrophoresis

To check quality control of cDNAs a PCR reaction was performed in a PTC200 thermal cycler (MJ Research Inc.). Reagents for the amplification reactions were added as follows:

Primers 50 µM	0.2 µl (each)
dNTPs 1,25 mM	4 µl
GoTaq® DNA Polymerase (5u/µl) (Promega)	0.1 µl
GoTaq reaction buffer (Promega)	5 µl
Nuclease free water	13.5 µl
cDNA (dil. 1:10)	2 µl
final volume	*25 µl*

Amplification of the gene *APE2* was performed on cDNAs both for checking the reverse-transcription efficiency and for excluding genomic DNA contamination.

Primer Sequence (5'-3')

APE2 F TGCGCATCAATGTAATGTGGAAGCAGAGTA

APE2 R TGAAATCAGGTTCCACGGTTAAATCGTAGTGT

Thermal protocol was set as follows:

Cycle1	(1x)	95°C – 3’
Cycle2	(35x)	95°C – 15"
		60°C – 30"
		72°C – 1'
Cycle3	(1x)	72°C – 5’
4°C	for ever	

Amplified samples were run on 1.5% agarose gel containing 1X GelRedTM Nucleic Acid Gel Stain (Biotium). Run was performed on horizontal electrophoresis apparatus with TBE 0.5x as running buffer (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) and the bands were visualized by UV trans-illumination. Digital images were acquired with EDAS290 capturing system (Kodak).

4.2.8 Primer design

PCR primers of the investigated genes for real-time assays are listed in **Table 3.3**. They have been designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). This software uses Primer3 to generate the candidate primer pairs for a given template sequence, and then it submits them to BLAST search against a user-selected database. Yeast database has been used to check primer specificity on sequences of other yeast species. Special attention has been paid to primer length (15–25 bp), annealing temperature (58°C–62°C), nucleotides composition, 3′-end stability and amplicon size (80–200 bp). All primers have been synthesized and OPC purified by Metabion International AG (Germany). Primer pair for *FSY1* has been designed on the *P301_O30021* ORF sequence and then tested for the amplification on both *FSY1* (EC1118) and *P301_O30021* (vineyard strains) sequences. After synthesis, the primer specificity has been also tested by end point PCR and gel electrophoresis.

Table 3.3

List of the investigated genes and details of primers and amplicons for each gene.

Gene	Molecular function (SGD curated)	Reference	Primer Sequence [5′ → 3′]	Amplicon Length	Efficiency %	R^2
HXT1	HeXose Transporter (low-affinity glucose transporter)	Present work	F: 5'-GAA GCT GGC AGA ATC GAC GA-3' R: 5'-TAT GGA TGG TCA GGT GGG CA-3'	71 bp	102.9	0.996
HXT2	HeXose Transporter (high-affinity glucose transporter)	Present work	F: 5'-TGA ACT CCC AGC AAA GCC AA-3' R: 5'-TCC CAA CCA AAG ACA AAC CCA-3'	90 bp	104.9	0.992
HXT3	HeXose Transporter (low affinity glucose transporter)	Present work	F: 5'-CAC GTT ATT TGG TTG AAG CTG GT-3' R: 5'-GTT GAA TGA ATG GAT GGT CTG GG-3'	93 bp	101.3	0.998
HXT4	HeXose Transporter (high-affinity glucose transporter)	Present work	F: 5'-TGG TGG TAT GAC ATT CGT TCC-3' R: 5'-CGC TGA CCT TAT TTG AAA GAG CA-3'	101 bp	104.7	0.989
HXT5	HeXose Transporter (moderate affinity for glucose)	Present work	F: 5'-TCC AAA TCG CCT CCA TTG ACA-3' R: 5'-AAT ACC ACC AAC GCC CAG TC-3'	77 bp	102.5	0.983
HXT6/7	HeXose Transporter (high-affinity glucose transporter)	Present work	F: 5'-GAC TTT GGA AGA AGT CAA CAC CA-3' R: 5'-TTC TTC AGC GTC GTA GTT GGC-3'	106 bp	100.0	0.998
HXT8	Protein of unknown function with similarity to hexose transporters	Present work	F: 5'-AAT TCT GTC CAG TGG CGT GT-3' R: 5'-CGG AAC AAA CGT CAT ACC ACC-3'	81 bp	95.2	0.991
FSY1	Fructose/H^+ symporter (Galeote *et* al., 2010)	Present work	F: 5'-CGA TGT TAA AGG CGG GTG GA-3' R: 5'-AAC GTG GTG ACT CGG GTA AG-3'	98 bp	95.1	0.989
MAL31	MALtose fermentation; high-affinity maltose transporter (alpha-glucoside transporter)	Present work	F: 5'-AGC TTT CTA TGC CCT GCC TG-3' R: 5'-CCG ACA ATC TCA CCT GCC AT-3'	129 bp	102.2	0.990
GAL2	GALactose metabolism (also able to transport glucose)	Present work	F: 5'-GGG TCT GAA GGC TCC CAA AG-3' R: 5'-ACA AAC AAA GCA AGG AAA CGG T-3'	85 bp	104.7	0.981

For each different primers pair, efficiency of RT-PCR (E), slope values and correlation coefficients (R^2) were determined, using serial 1:5 dilutions of template cDNA, on CFX96 cycler – RealTime PCR Detection System (Bio-Rad Laboratories, Inc).

Efficiency was considered adequate when ranging from 95% to 105%, R^2 was considered acceptable when greater than 0.98-0.99.

4.2.9 Diffusion of the *FSY1* gene in vineyard

The primer pair for *FSY1* gene (**Table 3.3**) has been used in Real Time-PCR experiment to assess the diffusion of the fructose transporter gene in the Conegliano-Valdobbiadene Prosecco Superiore DOCG and the Raboso Piave DOC *Saccharomyces cerevisiae* populations, and in a pool of 20 industrial wine strains (**Table 3.2**). One isolate per mitochondrial DNA RFLP genotype has been tested. PCR reaction has been carried out using CFX96 cycler – RealTime PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), in white-walled PCR plates (96 wells). Reactions were prepared in a total volume of 15 µl containing 400 nM each primer, 1× SsoFast EvaGreen Supermix 2× (Bio-Rad), 5 µl of cDNA and RNase/DNase-free water to adjust reaction volume. Thermal cycle conditions have been set as follows: initial template denaturation at 98°C for 2 minutes, followed by 40 cycles of denaturation at 98°C for 5 seconds, and combined primer annealing/elongation at 60°C for 40 seconds.

4.2.10 Gene Expression Analysis

Real-Time PCR was carried out on a CFX96 Cycler-Real Time-PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA), in white-walled PCR plates (96 wells). A ready to use master-mix containing a fast proof-reading Polymerase, dNTPs, stabilizers, $MgCl_2$ and EvaGreen dye was used according to the manufacturer's instructions (Bio-Rad).

Reactions were prepared in a total volume of 15 µl containing:

400 nM each primer

1× SsoFast EvaGreen Supermix 2× (Bio-Rad)

5 µl of cDNA

RNase/DNase-free water to adjust reaction volume

Thermal cycle conditions were set as follows:

initial template denaturation:		98°C – 30 seconds
40 cycles of	denaturation:	98°C – 2 seconds
	primer annealing/elongation	60°C – 10 seconds

The amount of fluorescence, given by the incorporation of EvaGreen into dsDNA, has been measured at the end of each cycle and analysed by CFX-Manager Software v2.0 (Bio-Rad Laboratories, Inc.). To calculate efficiency (E) of Real Time-PCR and correlation coefficients (R^2) of each primer pair a PCR amplification has been run using serial 1:5 dilutions of cDNA template, obtained by pooling all the samples, on CFX96 cycler-Real Time-PCR Detection System. Efficiency has been calculated from the slope of the standard curve using the formulas:

$$E = 10^{-1/slope} \quad \text{and} \quad \% E = (E^{-1}) \times 100$$

Gene expression analysis was performed using the CFX-Manager Software v2.0 and adopting the $2^{-\Delta\Delta CT}$ method.

Four housekeeping genes have been used in Real Time-PCR gene expression analysis: the ALG9 and UBC6 primers have been designed by Teste *et* al. (2009); the FBA1 and PFK1 primers have been designed by Cankorur-Cetinkaya *et* al. (2012) and Nadai *et* al. (2015), respectively (**Table 3.4**). In this study the lettering "total expression" stands to indicate the sum of the normalized expression values of the genes, for each strain, relative to one of the two sampling points (45 or 60 g/L of produced CO_2).

Table 3.4

List of the reference genes and details of primers and amplicons for each gene.

Gene	Molecular function (SGD curated)	Reference	Primer Sequence [5′ → 3′]	Amplicon Length	Efficiency %	R^2
ALG9	Mannosyltransferase activity	Teste *et* al. (2009)	F: 5′-CAC GGA TAG TGG CTT TGG TGA ACA ATT AC-3′ R: 5′-TAT GAT TAT CTG GCA GCA GGA AAG AAC TTG GG-3′	156 bp	95.7	0.996
UBC6	Ubiquitin-protein ligase activity	Teste *et* al. (2009)	F: 5′-GAT ACT TGG AAT CCT GGC TGG TCT GTC TC-3′ R: 5′-AAA GGG TCT TCT GTT TCA TCA CCT GTA TTT GC-3′	272 bp	99.0	0.985
FBA1	Fructose-bisphosphate aldolase activity	Cankorur-Cetinkaya *et* al. (2012)	F: 5′-GGT TTG TAC GCT GGT GAC ATC GC-3′ R: 5′-CCG GAA CCA CCG TGG AAG ACC A-3′	125 bp	102.4	0.998
PFK1	6-Phosphofructokinase activity	Nadai *et* al. (2015)	F: 5′-GAG GTT GAT GCT TCT GGG TTC CGT-3′ R: 5′-TGT GGC GGT TTC GTT GGT GTC G-3′	138 bp	97.7	0.998

4.2.11 Statistical analysis

Statistical analysis has been carried out on chemical and gene expression data. The student's *t* test or the one-way analysis of variance (ANOVA) followed by the Tukey *post-hoc* test has been performed using the XLSTAT 7.5.2 (Addinsoft SARL, Paris, France) software. The analysis has been carried out comparing the averages of three independent replicates, and differences were considered statistically significant for *p* value lower than 0.05.

4.3 Results

4.3.1 *FSY1* diffusion in vineyard population

The *FSY1* gene has been found in 6 out of 34 strains (18%) in the Conegliano-Valdobbiadene Prosecco Superiore DOCG area, in 42 out of 156 strains (27%) in the Raboso Piave DOC area, for a total of 48 out of 190 vineyard strains (25%); it has been also found in 9 out of the 20 industrial wine strains (45%). On the hole, 57 strains out of 210 strains carried one of the two fructose transporter genes, corresponding to the 27% of the tested strains (**Figure 3.2**).

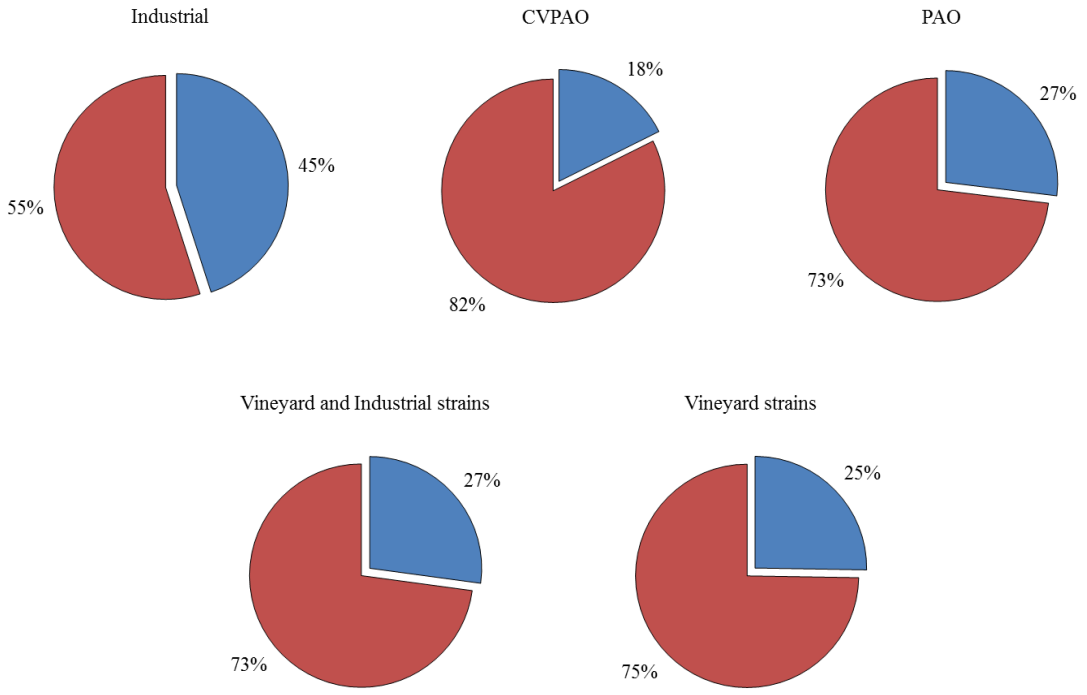


Figure 3.2

***FSY1* diffusion in vineyard and industrial strains. (■) presence of *FSY1* (■) no fructose transporter gene**

4.3.2 Fermentation process and HPLC analysis

The four tested strains showed different fermentation behaviour. The first strain which ended the fermentation was EC1118, followed by P301.9, R31.3 and R008. The highest peak of CO_2 production rate has been obtained for P301.9 (1.93 gCO_2/L/h), reached in 15.58 hours from inoculum, while the lowest has been obtained for EC1118 (1.53 gCO_2/L/h) and reached in 16.75 hours from inoculum (**Table 3.5**).

Table 3.5

Parameters of the fermentation kinetic.

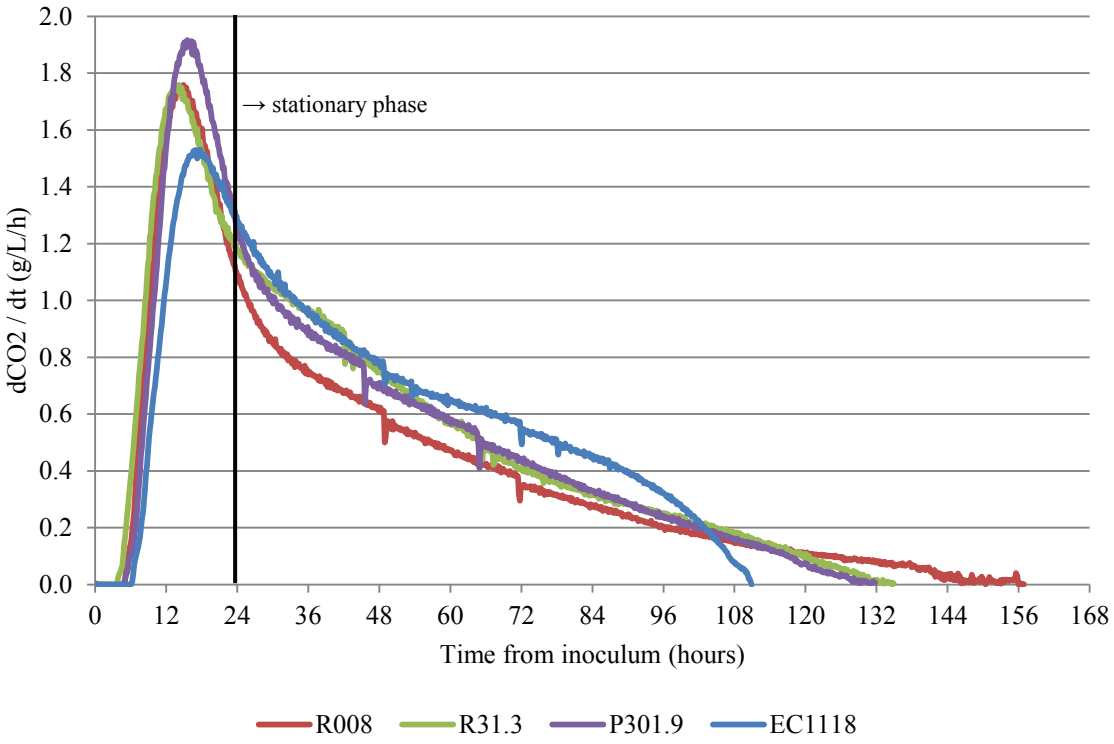
Strain	CO_2 production peak (g/L/h±sd)	Peak Time (h±sd)	Total Fermentation Time (h±sd)
EC1118	1.53 ± 0.05^{A}	16.75 ± 0.85^{B}	110.2 ± 1.7^{A}
R008	1.76 ± 0.00^{B}	14.91 ± 0.33^{A}	156.9 ± 1.0^{C}
R31.3	1.76 ± 0.05^{B}	14.08 ± 1.08^{A}	134.9 ± 4.2^{B}
P301.9	1.93 ± 0.05^{C}	15.58 ± 0.84^{AB}	131.7 ± 4.2^{B}

Letters indicate significant differences obtained from Tuckey post-hoc test after ANOVA analysis of variance between the strains (*alpha*=0.05).

The industrial strain EC1118 showed a very different CO_2 production kinetic with respect to the other strains: despite its lower CO_2 production peak it was the fastest strain to conclude the fermentation process, showing a high CO_2 production rate all long the stationary phase and a clear closure of the fermentation process. The other three strains showed a higher CO_2 production peak, especially P301.9, and the stationary phase was characterized by gradual decrease in CO_2 production rate which lead to elongation of the fermentation process and to a blurred end of the fermentation process, especially for R008 (**Figure 3.3**).

Figure 3.3

CO_2 production kinetics of the studied yeast strains. Data of dCO_2/dt are the average of three biological replicates.



The mean total sugar residue of the four strains was 76.69±0.93 g/L at 45 g/L of produced CO_2 and 40.80±4.09 g/L at 60 g/L of produced CO_2. ANOVA analysis of variance found no significant differences among strains sugar residue in both sampling points (*alpha*=0.05). This result indicates that the sampling points have been accurately chosen and performed in order to harvest cells in the same physiological state, relating to the residual sugar content. At 45 g/L of produced CO_2 the strain EC1118 showed the lowest ratio between fructose and the total sugar residue (60.30%), corresponding to the most balanced intake of fructose and glucose with respect to the other strains which have shown significantly higher ratios. The same pattern has been observed at 60 g/L of produced CO_2. For all strains this ratio increased from 45 to 60 g/L of produced CO_2,

indicating that the increasing preference for glucose instead of fructose in the late stationary phase is a character shared by all these strains (**Table 3.6**). The strain R31.3 sowed the highest increment of the ratio between fructose and the total sugar residue from 45 to 60 g/L of produced CO_2 (12.27 %), followed by P301.9 (10.11 %), R008 (8.92 %) and EC1118 (6.57 %), which confirms its best balance between glucose and fructose intake. Ethanol yield was similar for all strains at both 45 and 60 g/L of produced CO_2, while R31.3 showed the lowest glycerol amount at both time points.

Table 3.6

Results of HPLC analysis at 45 and 60 g/l of produced CO_2 expressed as the average of three biological replicates ± standard deviation.

	Fructose/Total sugars (%)			Ethanol (% vol.)		Glycerol (g/L)	
	45 g/L	**60 g/L**	***p***	**45 g/L**	**60 g/L**	**45 g/L**	**60 g/L**
EC1118	60.30±*0.43*^{A}	66.87±*0.70*^{A}	*<0.0001****	7.12±*0.20*^{A}	8.67±*0.42*^{A}	4.41±*0.03*^{B}	4.30±*0.11*^{B}
P301.9	66.56±*0.74*^{B}	76.67±*1.72*^{B}	*0.002***	6.92±*0.22*^{A}	8.31±*0.55*^{A}	4.28±*0.05*^{B}	4.22±*0.11*^{AB}
R31.3	67.01±*1.08*^{B}	79.27±*1.84*^{B}	*0.016**	6.97±*0.31*^{A}	8.70±*0.28*^{A}	3.94±*0.07*^{A}	3.98±*0.12*^{A}
R008	66.05±*0.29*^{B}	74.97±*1.65*^{B}	*0.012**	6.48±*0.65*^{A}	8.28±*0.28*^{A}	4.18±*0.17*^{AB}	4.41±*0.10*^{B}

(***) $p<0.001$, (**) $p<0.01$, (*) $p<0.05$ between the percentage of fructose on total sugar residue at 45 and 60 g/L of produced CO_2 by Student's *t* test. Letters indicate significant differences obtained from Tuckey *post-hoc* test after ANOVA analysis of variance between the strains (*alpha*=0.05).

At the end of the fermentation process three out of the four strains consumed all the sugars, while R008, the slowest strain in concluding fermentation process, left 7.62 g/L of fructose and produced the lowest amount of ethanol with respect to the other strains (**Table 3.7**).

Table 3.7

Results of HPLC analysis at the end of the fermentation expressed as the average of three biological replicates ± standard deviation.

	Ethanol (% vol.)	Glucose (g/L)	Fructose (g/L)	Glycerol (g/L)
EC1118	11.49±*0.04*^{B}	nd	nd	4.74±*0.03*^{B}
P301.9	11.45±*0.04*^{B}	nd	nd	4.35±*0.24*^{AB}
R31.3	11.38±*0.26*^{B}	nd	nd	3.93±*0.26*^{A}
R008	10.64±*0.07*^{A}	nd	7.62±*0.04*	4.37±*0.21*^{AB}

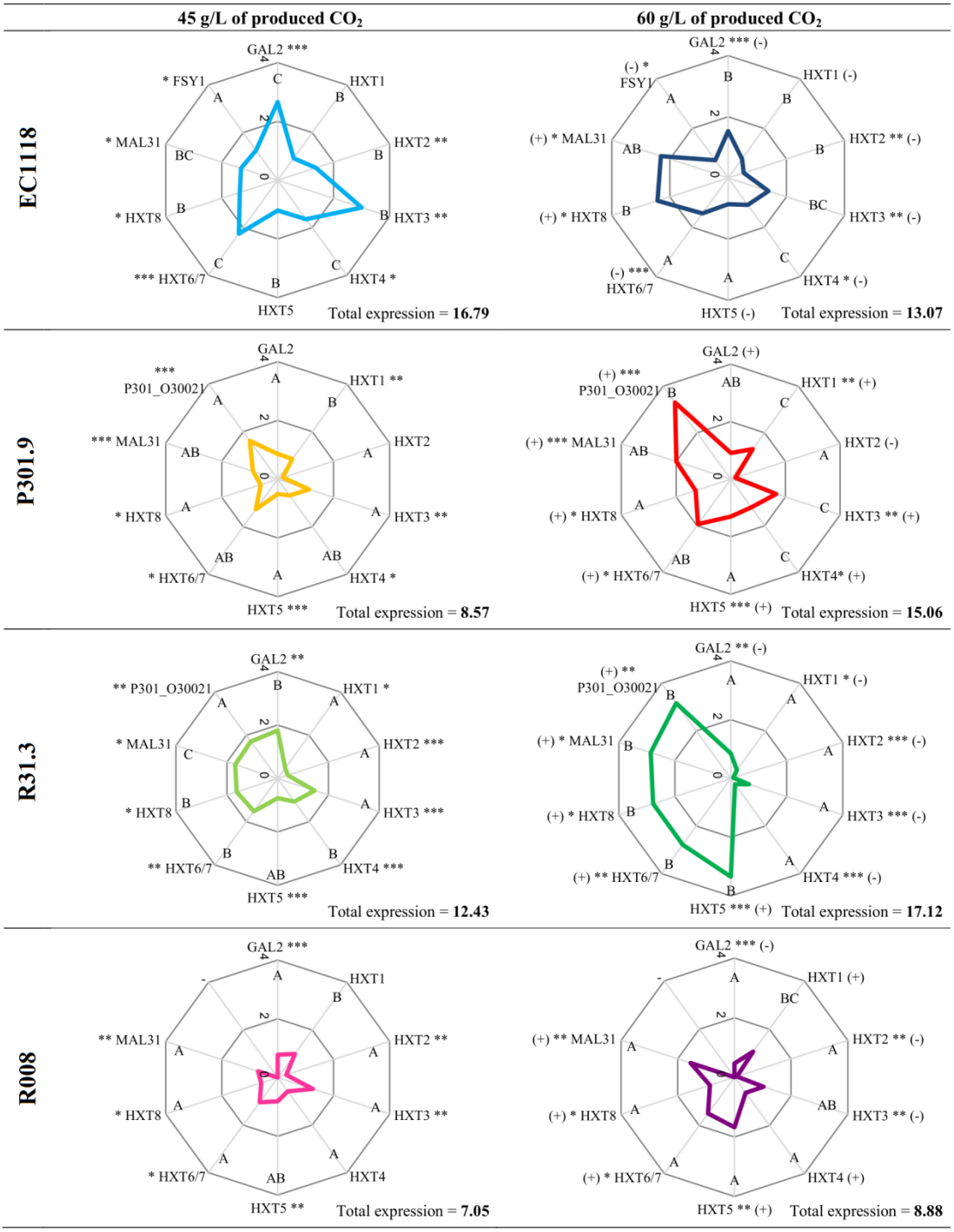
Letters indicate significant differences obtained from Tuckey *post-hoc* test after ANOVA analysis of variance between the strains (*alpha*=0.05).

4.3.3 Gene expression analysis

During stationary phase two samplings have been performed to collect cells for RNA extraction: they have been set when 45 and 60 g/L of CO_2 would have been produced. The aim of this experiment was to investigate the regulation of the most important hexose transporter gene during the stationary phase, in oenological conditions. After harvest, the cells have been washed and frozen at -80°C. Then, their RNA has been extracted and retro-transcribed to cDNA to be used in real-time PCR study in order to investigate how the expression of the hexoses transporters genes would have been changed passing from 45 to 60 g/L of produced CO_2. Results of gene expression analysis are shown in **Figure 3.4**.

Figure 3.4

Normalized expression of hexose transporter genes. Letters indicate significant differences in the gene expression values obtained from ANOVA analysis of variance followed by Tuckey *post-hoc* test between the strains, at 45 or 60 g/L of CO_2 (*alpha* = 0.05). (***) $p<0.001$, (**) $p<0.01$, (*) $p<0.05$ between gene expression values at 45 and 60 g/L of produced CO_2 by Student's *t* test for each yeast strain.



A strain-specific expression pattern has been observed (**Figure 3.4**). The strains EC1118 and R31.3 reduced the expression of *HXT1*, but on the contrary in the strains P301.9 and R008 its expression significantly increased. *HXT2* decreased its expression in all strains. *HXT3* increased its expression in the strain P301.9, while decreased in the other strains. *HXT4* decreased its expression in the strains EC1118 and R31.3 while in the strains P301.9 and R008 its expression increased. *HXT5* decreased its expression only in EC1118, and the same expression pattern has been observed for the *HXT6/7* genes. The gene *HXT8* increased its expression in all strains, and the same result has been obtained for the *MAL31* gene. The expression of *FSY1* has been increased in the strains P301.9 and R31.3, while it has been decreased for the strains EC1118; in the strain R008, which does not carry this gene, no expression has been detected. Finally, the gene *GAL2* showed an increased expression only in the strain P301.9, while in other strains its expression decreased.

Each strain, as clearly appearing in heat-map in **Figure 3.5**, differently uses the hexose transporter genes. The strain EC1118 reduced its total expression from 45 to 60 g/L of produced CO_2 (-22%), while P301.9 showed the highest increase in total expression (+76%), followed by R31.3 and R008. At 45 g/L of produced CO_2 the strain EC1118 showed the highest total expression followed by R31.3, P301.9 and R008, while at 60 g/L of produced CO_2 the strain R31.3 showed the highest total expression followed by P301.9, EC1118 and R008 (**Figure 3.4**).

Figure 3.5

Different utilization of the tested hexose transporter genes among yeast strains. Data per each gene are expressed as percentage calculated on the total expression, for each strain and time point (45-60 g/L of produced CO_2). The difference between the total expression values from 45 to 60 g/L of produced CO_2 is reported as percentage of variation.

	EC1118		P301.9		R31.3		R008	
	45 g/L	*60 g/L*	*45 g/L*	*60 g/L*	*45 g/L*	*60 g/L*	*45 g/L*	*60 g/L*
GAL2	15.8	11.7	10.1	6.1	14.5	5.0	11.0	5.2
HXT1	5.5	5.9	9.9	8.6	3.8	2.1	14.1	12.1
HXT2	8.1	4.1	2.1	1.1	3.2	0.5	4.3	1.0
HXT3	17.9	10.6	13.0	11.0	11.7	3.8	17.8	11.6
HXT4	9.8	8.3	8.0	8.0	8.6	1.4	8.2	7.2
HXT5	6.1	6.6	5.9	8.6	5.9	19.5	11.4	18.8
HXT6/7	13.4	11.0	14.8	12.8	12.2	16.3	14.8	16.9
HXT8	8.1	18.6	7.1	8.6	13.0	16.3	8.4	9.7
MAL31	7.8	17.7	10.3	13.2	13.5	16.8	9.9	17.4
FSY1 / P301_O30021	7.5	5.4	18.9	21.9	13.7	18.5		
Total (%)	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*
Variation (%)	***-22.2***		***+75.6***		***+37.7***		***+25.9***	

At 45 g/L of produced CO_2 the most expressed genes in EC1118 were *HXT3*, *GAL2* and *HXT6/7*; in P301.9 were *FSY1*, *HXT6/7* and *HXT3*; in R31.3 were *GAL2*, *FSY1*, *MAL31* and *HXT8*; in R008 were *HXT3*, *HXT6/7* and *HXT1*.

The most expressed genes at 60 g/L of produced CO_2 in EC1118 were *HXT8* and *MAL31*; in P301.9 were *FSY1* and *MAL31*; in R31.3 were *HXT5*, *FSY1*, *MAL31*, *HXT8* and *HXT6/7*; in R008 were *HXT5*, *MAL31* and *HXT6/7* (**Figure 3.5**; for details see **Supplementary 3.1**).

4.4 Discussion

Saccharomyces cerevisiae is the organism provided with the highest number of glucose (hexose) transporter genes. In its genome twenty genes encode for proteins similar to hexose transporters (*HXT1* to *HXT17*, *GAL2*, *SNF3*, and *RGT2*) (Bisson *et* al., 1993; Boles and Hollenberg, 1997; Kruckeber, 1996; Ciriacy and Reifenberger, 1997), plus genes encoding transporters for other sugars also able to transport glucose (such as *MAL31*) (Özcan and Johnston, 1999). The Hxt proteins belong to the major facilitator superfamily (MFS) of transporters and transport their substrates by passive, energy-independent facilitated diffusion, with glucose moving down a concentration gradient (Bisson *et* al., 1993). The molecular function of these hexose transporters is redundant, as none of these transporters are essential for growth on glucose. In *S. cerevisiae* two uptake systems have been described: the first, a constitutive low-affinity system, and the second, and a glucose-repressed high-affinity system. The effectiveness of these transporter systems depends on the cooperation of several transporters. Indeed, the yeast *Saccharomyces cerevisiae* is evolved in and adapted to different environments, but all of them are characterized by the turnover of high and low hexose concentrations: such environments are, for example, grape surface (low sugars) and grape must (high sugars, i.e. when biotic or abiotic factors damage the raisin skin and the juice outflows, or when grapes are crushed for wine production); after the first phases of the grape must fermentation the sugar concentration rapidly decreases, again. Similar conditions are faced by *S. cerevisiae* during beer, apple juice, rice or other substrates fermentations.

Only seven out of the 20 members of the *HXT* gene family encode functional glucose transporters. A strain lacking these seven *HXT* genes (*HXT1* through *HXT7*, called the *hxt* null mutant) is unable to grow on glucose, fructose, or mannose and has no glycolytic flux (Boles and Hollenberg, 1997; Liang and Gaber, 1996; Reifenberger *et* al., 1997).

HXT2, *HXT6*, and *HXT7* encode high-affinity transporters. *HXT1*, *HXT3*, or *HXT4* encode low-affinity glucose transporters (Reifenberger *et* al., 1997). *GAL2* encodes a galactose transporter similar to the Hxt proteins (Nehlin *et* al., 1989), is also able to transport glucose.

The *HXT8–17* genes encode proteins that either cannot transport glucose or are not expressed under the so far conditions tested (Özcan and Johnston, 1999), although Treu *et* al (2014) found that the *HXT8* gene was expressed during the first stage of synthetic grape must fermentation, even if at low levels comparing to the expression of the *HXT1–7* genes.

Recently, the *FSY1* gene, encoding for a fructose/H+ symporter, has been discovered and characterized in EC1118 *Saccharomyces cerevisiae* strain (Galeote *et* al., 2010; Anjos *et* al., 2013). In 2014 Treu *et* al. found out the fructose transporter a vineyard strain (P301), and in the subsequent transcriptomic analysis work the authors observed its expression under fermentation conditions, even if at lower levels with respect to other hexoses transporter genes.

4.4.1 *FSY1* diffusion in vineyard *Saccharomyces cerevisiae* populations

The diffusion of the fructose transporter gene *FSY1* regarded the 25 percent of the tested vineyard strains. This result suggests that the fructose transporter activity in *Saccharomyces cerevisiae* could have a positive effect in cell survival in vineyard. Moreover, the high diffusion of this trait in the industrial strains pool suggests that it has been non-consciously selected by humans, so it represents a genetic trait correlated or associated to good fermentation performances. Results from fructose transport genes diffusion are remarkable considering that the high affinity fructose uptake in *S. cerevisiae*

requires energy to the cell: indeed it is an H^+ symport transport mechanism, which can give ecological advantage to yeast cells carrying it in low sugar concentrations, i.e. on intact grape skins or at the end of the fermentation processes.

4.4.2 Fermentation performance

Kinetics of carbon dioxide production differs among the tested *Saccharomyces cerevisiae* yeast strains. EC1118 showed the best kinetic, with a good CO_2 production peak (1.53±0.05 g/L/h) and in particular during the stationary phase: indeed, differently from other strains it maintained high CO_2 flux rates until the complete exploiting of carbon sources (glucose and fructose), clearly identified in **Figure 1** thanks to the drop of the CO_2 flux. On the contrary, the strain P301.9 showed the highest CO_2 production peak, reaching 1.93±0.05 g/L/h of CO_2, but then it constantly decreased its CO_2 production flux until the end of the fermentation, without a clear closure point. Similar behaviour has been observed for R31.3 and R008. The moment when the CO_2 production peak has been registered differed among strains: R31.3 and R008 have been the fastest, followed by P301.9 (probably because of it reached a higher CO_2 production peak value that the other strains) and EC1118 (**Table 4**).

Interestingly, the three strains EC1118, P301.9 and R31.3, carrying the *FSY1* fructose transporter gene, have been able to completely ferment the sugars in the synthetic must; on the contrary, the strain R008, lacking for the fructose specific transport, left 7.62±0.04 grams of unfermented fructose. Moreover, it produced around 1% vol. less ethanol with respect to the other strains. These results suggest that the presence of a fructose specific transport system could give direct or indirect fermentative advantages to yeast cells, in oenological conditions.

The ratio between the residual fructose and the total residual sugars has been investigated during stationary phase, in order to evaluate the impact of the carbon source (glucose or fructose) preference of the four tested strains. At 45 g/L of produced CO_2 ranged from 60.30 to 67.01% (**Table 5**). The strain EC1118 showed the lowest ratio, followed by R008, P301.9 and R31.3. At 60 g/L of produced CO_2 the ratio increased in all strains, ranging from 66.87 to 79.27%. Again, the strain EC1118 showed the lowest ratio, followed by R008, P301.9 and R31.3. These findings confirmed the *S. cerevisiae* preference for glucose during stationary phase, but at the same time they underlined the difference that the strains can exhibit in this parameter.

ANOVA analysis of variance found no significant differences among the strains in total residual sugars, at both sampling points (*alpha*=0.05) (**Supplementary 5**). This result indicates that the sampling points have been appropriately chosen and performed in order to harvest cells in the same physiological state, relating to the residual sugar content. This assumption allowed comparing gene expression results between strains at the same sampling point and between the two sampling points for each strain.

4.4.3 Gene expression analysis

Real Time PCR has been used for analysis of relative gene expression. The results evidenced on the one hand high strain specificity in expression patterns, and on the other hand evidenced that all the hexose transporter genes were activated during the stationary phase in all the strains (except for the specific fructose transporters in R008, lacking in them). The latter observation indicates that at the sampled stages of stationary phase all the strains undergo intermediate sugars uptake regulation, as the high and low glucose regulation pathways seem simultaneously working.

In particular, EC1118 appeared to highly express *HXT3*, *GAL2* and *HXT6/7* at 45 g/L of produced CO_2, while at 60 g/L of produced CO_2 the highest expression has been detected for *HXT8* and *MAL31* genes. In this context the activity of the fructose transporter *FSY1* appeared limited to marginal role.

In P301.9 the total expression at 45 g/L of produced CO_2 was about the half of the value for EC1118. In this strain the role of the fructose transporter gene *FSY1* appeared more important than in EC1118, together with the *HXT6/7* genes. The expression in P301.9 increased for all the transporter genes (except for *HXT2*, that seems to be independent from Rgt1 regulation – Kaniak *et* al., 2004) at 60 g/L of produced CO_2 sampling point, but the expression increment for *FSY1* gene showed to be higher than the other genes, confirming the key role of this gene in P301.9.

In the strain R31.3 the transporters genes showed to be separated in two groups at both 45 and 60 g/L of produced CO_2: in the first time point the high expressed group included *GAL2*, *FSY1*, *MAL31* and *HXT8*, while in the second time point the high expressed group included *HXT5*, *FSY1*, *MAL31* and *HXT8*. The high expressed group remained almost identical from 45 to 60 g/L of produced CO_2, varying only for the *GAL2* and *HXT5* expression level.

Finally, R008 showed the lowest total gene expression at both 45 and 60 g/L of produced CO_2. The most expressed gene at 45 g/L of produced CO_2 was *HXT3*, followed by *HXT6/7*, while at 60 g/L of produced CO_2 the *MAL31*, *HXT5* and *HXT6/7* genes showed the highest expression levels.

Among the four strains R31.3 and P301.9 showed to have a slight variation in their gene expression pattern during the studied part of the stationary phase: instead of an expression pattern changing, they increased the expression level of the same group of genes (**Figure**

3.4 and **3.5**). On the contrary, the EC1118 and R008 changed both the expression pattern and the total expression level.

Despite the differences among the strains, these results indicated that the sugar decrease triggered an increase in expression levels in all the four yeasts, and thus is plausible to suppose that sensing of such sugar decrease caused a changing in the regulatory pathways of the yeasts cell. How each strain sensing system reacted to such signal entailed the detected changing in the genes expression. As a result, the main active glucose- mediated regulation pathway could be supposed in each strain.

At 45 g/L of produced CO_2 in EC1118 the most expressed genes were *GAL2* and *HXT3*. At this time point the residual glucose was 31.03±0.22 g/L and the residual fructose was 47.10±2.36 g/L (**Supplementary 5**). The ratio "fructose/total residual sugars" (from here onwards called F/T ratio) was 60.30%. In these conditions the high expression of *GAL2* suggests that the cell now perceives a "low glucose" signal: this gene is indeed inhibited by the Snf1/Mig1 pathway in high glucose concentration, so in low glucose conditions the transcription of *GAL2* is activated. A concomitant high expression of *HXT3* has been observed. The *HXT3* transporter gene is regulated by the Rgt1 transcriptional repressor (Flick *et* al., 2003; Kaniak *et* al., 2004) (**Figure 3.1**). Rgt1p is part of a complex together with Mth1p and Sdt1p, and such complex represses the transcription of the *HXT1-HXT4* genes in absence of glucose. Glucose promotes the hyper-phosphorylation of Rgt1p (through the Snf3p signalling which leads to the degradation of Mth1p and Sdt1p by SCF-Grr1), and therefore its dissociation from the *HXT* gene promoters. Moreover, the high expression level of the *HXT6/7* genes, which are known to be expressed during the latest phases of the fermentation (Luyten *et* al., 2002), suggests that the yeast cell is undergoing or it is going to undergo a "low glucose" signalling. At 60 g/L of produced CO_2 in EC1118 the most expressed genes were *HXT8* and *MAL31*. At this time point the residual

glucose was 11.77±1.37 g/L and the residual fructose was 23.71±1.90 g/L (**Supplementary 5**). The F/T ratio was 66.87%. In these conditions EC1118 seemed to definitely activate a glucose-starvation response. *HXT8* is indeed known to be induced by low levels of glucose (Özcan and Johnston, 1999) and seems to be target of the Rgt1 negative regulation, together with *HXT4* (Kaniak *et* al., 2004) which also was up-regulated in EC1118 (with respect to the percent weight on the total expression) from 45 to 60 g/L of produced CO_2. The *MAL31* high gene expression in the second sampling point indicates that at such sugars concentration the alternative carbon sources genes are turned activated, meaning that the Snf1/Mig1 high glucose repression in blurring in favour of the Snf3/Rgt1 low glucose regulation.

These results suggest that the "low-glucose" signal detected by Snf3p starts to turning up early in the stationary phase of the EC1118 cells, since when the glucose concentration is of about 30 g/L. In literature has been reported that the Snf3 sensor signalling pathway is active at "low glucose concentration", but which is the concentration range in which such pathway is working is unclear (cited literature of this chapter). The 30 g/L glucose concentration can be considered, in winemaking context, "high" rather than "low" concentration: besides a glucose residue of 30 g/L, as above mentioned, there was a fructose residue of almost 50 g/L.

In the experiment of the present work the *GAL2* and *HXT3* genes were highly regulated at the first sampling point while the highest regulated genes at the second sampling point were the *HXT8* and *MAL31*. Such result suggests that the EC1118 Snf3 sensor started switching up before that the whole fermentation process had overstepped the midpoint (45 g/L of produced CO_2) and that nearer to the fermentation end (60 g/L of produced CO_2) the sugars depletion could be carried out by the alternative carbon sources genes.

Regarding P301.9, at 45 g/L of produced CO_2 the most expressed genes were *FSY1* and *HXT6/7*, while the third most expressed gene is *HXT3*. At this time point the residual glucose was 26.76±2.08 g/L and the residual fructose was 53.20±2.05 g/L (**Supplementary 5**). The F/T ratio was 66.56%. As previously discussed on the EC1118 case, the *HXT3* and *HXT6/7* higher expression suggests that the yeast cells are effectively under intermediate sugars uptake regulation. However, the *FSY1* expression at this so high level was unexpected, especially in comparison with the relative expression level in EC1118: the *FSY1* gene seems playing an important role in the sugars uptake of this yeast strain. Such assumption is further supported by the relative gene expression results at 60 g/L of produced CO_2: despite a wide cross total expression increase from 45 to 60 g/L of produced CO_2 the *FSY1* contribution on total expression became greater, passing from 18.9 to 21.9% (**Figure 3.5**). Among the other most expressed genes, at the second time point, *MAL31*, *HXT6/7* and *HXT3* gave similar contributions, indicating that the glucose-dependent response of P301.9 highly differs from the one of EC1118. In EC1118, indeed, there has been clear turnover in the gene expression prevalence (*GAL2* and *HXT3* vs *HXT8* and *MAL31*). In P301.9, on the contrary, the expression pattern was substantially unchanged, only changing its intensity. This has been probably due to poorly effective gene expression regulation pathways.

Regarding R31.3, at 45 g/L of produced CO_2 the most expressed genes were *GAL2* and *FSY1*, while the third most expressed gene is *MAL31*. At this time point the residual glucose was 26.36±3.32 g/L and the residual fructose was 53.90±3.51 g/L (**Supplementary 5**). The F/T ratio was 67.01%.

The expression pattern of R31.3 transporters genes was completely different from the other strains ones, with a higher involvement of the genes of the late stationary phase and the alternative carbon sources (*HXT6/7* and *MAL31*) (**Figure 3.5**). Such difference could

reflect an early exit from the Snf1/Mig1 repression pathway with respect to EC1118. At 60 g/L of produced CO_2 the most expressed genes were *HXT5* and *FSY1*, but the genes *HXT6/7*, *HXT8* and *MAL31* were still expressed at high levels (**Figure 3.5**). At this time point the residual glucose was 8.36±2.03 g/L and the residual fructose was 31.56±3.46 g/L. Except for the interchange between *GAL2* and *HXT5* the strain R31.3 showed to adopt a transcriptional response similar to that of P301.9, as both preferred increasing the same gene expression pattern instead of changing the pattern itself.

Finally, regarding R008, at 45 g/L of produced CO_2 the most expressed genes were *HXT3* and *HXT6/7*, while the third most expressed gene was *HXT1*. At this time point the residual glucose was 26.36±3.32 g/L and the residual fructose was 53.90±3.51 g/L (**Supplementary 5**). The F/T ratio was 67.01%. At 60 g/L of produced CO_2 the most expressed genes were *HXT5* and *MAL31*, while the third most expressed gene was *HXT6/7*. At this time point the residual glucose was 11.83±2.34 g/L and the residual fructose was 35.13±3.66 g/L (**Supplementary 5**). The F/T ratio was 74.97%. The total gene expression of R008 was very low, with respect to the other strains, at both 45 and 60 g/L of produced CO_2 (**Figure 3.4**). These clues suggest that in this strain the glucose-based regulation systems are not optimized. About the first sampling point, the presence of *HXT1* among the most expressed genes, together with the very low expression of the alternative carbon sources genes, suggests a late exit from the Snf1/Mig1 repression pathway with respect to the other strains. The delay in the carbon sources uptake regulation could affect the proper utilization of the alternative carbon sources (i.e. fructose, in this case) at the latest fermentation stages. Such a speculation seems to fit also to the R008 expression pattern at the second sampling point. At 60 g/L of produced CO_2, indeed, strong expression decrease has been coherently observed for *HXT3*, along with the increase of *MAL31*, which represents the alternative carbon sources genes activation:

such a change indicates that the Snf1/Mig1 repression is now blurring. Among EC1118, P301.9 or R31.3 *HXT1* was not found among the most expressed genes at 45 (and neither at 60) g/L of produced CO_2. In particular, *HXT1* accounted for the 14% of the total R008 expression at 45 g/L of produced CO_2 and for the 12% at 60g/L of produced CO_2, meaning that in this strain its expression remained almost constant during stationary phase, relating to the other genes.

Thus, is fair concluding that the in EC1118, P301.9 and R31.3 the exit from the Snf1/Mig1 high glucose repression happened before the first sampling point. Contrary to the three other strains, therefore, R008 probably exit later from the high glucose repression pathway. The sub-optimal efficiency of the carbon sources genes exploitation by R008, especially for those concerning the alternative carbon sources uptake, could be responsible of the unfermented fructose residue that R008 left at the end of the CO_2 production kinetic (**Table 3.7**).

The Hxt5 transporter has moderate affinity for glucose, is induced by the presence of non-fermentable carbon sources and by a decrease in growth rate (Gibson *et* al., 2008; Diderich *et* al., 2001; Verwaal *et* al., 2002). Moreover, the *HXT5* expression is under the control of STRE elements in the *HXT5* promoter (Verwaal *et* al., 2004). Such data suggests that the high expression of this gene can represent the transcriptional marker of a suboptimal yeast strain adaptation to the fermentation conditions, leading to global yeast suffering which can turns in sluggish or stuck fermentation. In this work the *HXT5* gene was strongly expressed in R31.3 and R008 at 60 g/L of produced CO_2, while its expression decreased only in EC1118. These results seem confirming the excellent EC1118 fitness in fermentation conditions from the point of view of the expression

regulation of genes related to the carbon sources utilization. The three vineyard strains, in fact (especially R008; **Figure 2**), seem suffering the conditions of the latest phase of the fermentation, which determined the expression increase of *HXT5*.

Furthermore, it is interesting to note that in the vineyard strains P301.9, R31.3 and R008 cases the F/T ratios at 45 g/L of produced CO_2 were significantly higher than that of EC1118. The results of this work clearly showed that the vineyard strains provided with *FSY1* have used it much more than EC1118 did. Concluding on the *FSY1* expression: first, EC1118 is supposed to be a very well-fitted strain to the tested fermentation conditions (see previous paragraph); second, it showed the lower fructose/total sugars residue ratios; third, the vineyard strains used *FSY1* much more than the industrial strain EC1118; thus, the resulting interpretation leads speculating that the higher utilization of *FSY1* by P301.9 and R31.3 is needed to the counterbalancing of their higher glucose/fructose ratios. In this way, they could fix their sub-optimal hexoses transporters genes regulation pathways.

4.5 Conclusions

This work represents an effort to deep investigate the utilization of the hexoses transporter genes of four *Saccharomyces cerevisiae* strains, under fermentation conditions. Differently from the most of the literature on this theme, the investigated diploid strains were not artificially depleted for any gene. This peculiarity may make harder the understanding of the expression patterns, but on the other hand allowed to observe an actual data background.

About fermentation performances, no strong differences were detected among the strains at the two sampling points (45 and 60 g/L of produced CO_2) while at the end of the fermentation R008 left a sugar residue, consistent with its lower ethanol yield.

The gene expression patterns were very different among strains. Such result was expected, but at a lower extent: from this work emerged instead a deeply different utilization among all the investigated genes by the four strains, suggesting that the strain-specificity is not related to a single gene, but it depends on the main regulation pathways.

All the hexose transporter genes were activated during the stationary phase in all the strains (except for the specific fructose transporters in R008, lacking in them). The latter observation indicates that at the sampled stages of stationary phase all the strains undergo intermediate sugars uptake regulation, as the high and low glucose regulation pathways seem simultaneously working.

Despite the differences among the strains in the total expression levels, results indicated that the sugar decrease triggered an increase in expression levels in all the four yeasts. How each strain sensing system reacted to such signal entailed the detected changing in the genes expression.

The gene expression result in EC1118 at 45 g/L of produced CO_2 suggests that the yeast is undergoing a "low glucose" signalling, while at 60 g/L of produced CO_2 seemed to definitely activate a glucose-starvation response (high *HXT8* and *MAL31* transcription). Moreover, at such sampling point the alternative carbon sources genes were activated, meaning that the Snf1/Mig1 high glucose repression in blurring in favour of the Snf3/Rgt1 low glucose regulation

These results suggest that the "low-glucose" signal detected by Snf3p starts to turning up early in the stationary phase of the EC1118 cells, since when the glucose concentration is of about 30 g/L. In literature has been reported that the Snf3 sensor signalling pathway is active at "low glucose concentration", but which is the concentration range in which such pathway is working is unclear. The 30 g/L glucose concentration can be considered, in winemaking context, "high" because such glucose residue is coupled with a fructose residue of almost 50 g/L (in EC1118).

The result in this work suggests that the EC1118 Snf3 sensor switches up before that the whole fermentation process had overstepped the midpoint (45 g/L of produced CO_2) and that nearer to the fermentation end (60 g/L of produced CO_2) the sugars depletion is carried out mostly by the alternative carbon sources carriers.

In P301.9 the *FSY1* expression was unexpected high, especially in comparison with the relative expression level in EC1118: the *FSY1* gene seems playing an important role in the sugars uptake of this strain. Despite a wide cross total expression increase from 45 to 60 g/L of produced CO_2 the *FSY1* contribution on total expression became greater, passing from 18.9 to 21.9%. In P301.9 the expression pattern was substantially unchanged in the two sampling points, and only changed its intensity. This has been probably due to poorly effective gene expression regulation pathways. In P301.9, on the

contrary, the expression pattern was substantially unchanged, only changing its intensity. This has been probably due to poorly effective gene expression regulation pathways.

The expression pattern of R31.3 transporters genes at 45 g/L of produced CO_2 is completely different from the other strains, with a higher involvement of the genes of the late stationary phase and the alternative carbon sources. Such difference could reflect an early exit from the Snf1/Mig1 repression pathway. At 60 g/L of produced CO_2 the most expressed genes were *HXT5* and *FSY1*, but the genes *HXT6/7*, *HXT8* and *MAL31* were still expressed at high levels. The strain R31.3 showed to adopt a transcriptional response similar to that of P301.9, as both preferred increasing the same gene expression pattern instead of changing the pattern itself.

The total gene expression of R008 was very low, with respect to the other strains, at both 45 and 60 g/L of produced CO_2. These clues suggest that in this strain the glucose-based regulation systems are not optimized. About the first sampling point, the presence of *HXT1* among the most expressed genes, together with the very low expression of the alternative carbon sources genes, suggests a late exit from the Snf1/Mig1 repression pathway with respect to the other strains. The delay in the carbon sources uptake regulation could affect the proper utilization of the alternative carbon sources (i.e. fructose, in this case) at the latest fermentation stages. Such a speculation seems to fit also to the R008 expression pattern at the second sampling point. At 60 g/L of produced CO_2, indeed, strong expression decrease has been coherently observed for *HXT3*, along with the increase of *MAL31*, which represents the alternative carbon sources genes activation: such a change indicates that the Snf1/Mig1 repression is now blurring. Among EC1118, P301.9 or R31.3 *HXT1* was not found among the most expressed genes at 45 (and neither at 60) g/L of produced CO_2. In particular, *HXT1* accounted for the 14% of the total R008 expression at 45 g/L of produced CO_2 and for the 12% at 60g/L of produced CO_2,

meaning that in this strain its expression remained almost constant during stationary phase, relating to the other genes.

Thus, it is fair to conclude that the in EC1118, P301.9 and R31.3 the exit from the Snf1/Mig1 high-glucose repression happened before the first sampling point. Contrary to the three other strains, therefore, R008 probably exit later from the high glucose repression pathway. The sub-optimal efficiency of the carbon sources genes exploitation by R008, especially for those concerning the alternative carbon sources uptake, could be responsible of the unfermented fructose residue that R008 left at the end of the CO_2 production kinetic.

The Hxt5 transporter has moderate affinity for glucose, is induced by the presence of non-fermentable carbon sources and by a decrease in growth rate. Moreover, the *HXT5* expression is under the control of STRE elements in the *HXT5* promoter. The high expression of this gene can represent the transcriptional marker of a suboptimal yeast strain adaptation to the fermentation conditions, leading to global yeast suffering which can turns in sluggish or stuck fermentation. In this work the *HXT5* gene was strongly expressed in R31.3 and R008 at 60 g/L of produced CO_2, while its expression decreased only in EC1118, confirming the excellent EC1118 aptitude for winemaking environments.

Furthermore, in the vineyard strains P301.9, R31.3 and R008 cases the fructose/total residual sugars ratios were significantly higher than that of EC1118. Such observation needs to be considered together with the evidence that the vineyard strains provided with *FSY1* have used it much more than EC1118 did.

EC1118 is a very well-fitted strain to fermentation conditions, and in this work it showed the lower fructose/total sugars residue ratios; moreover, the vineyard strains used *FSY1*

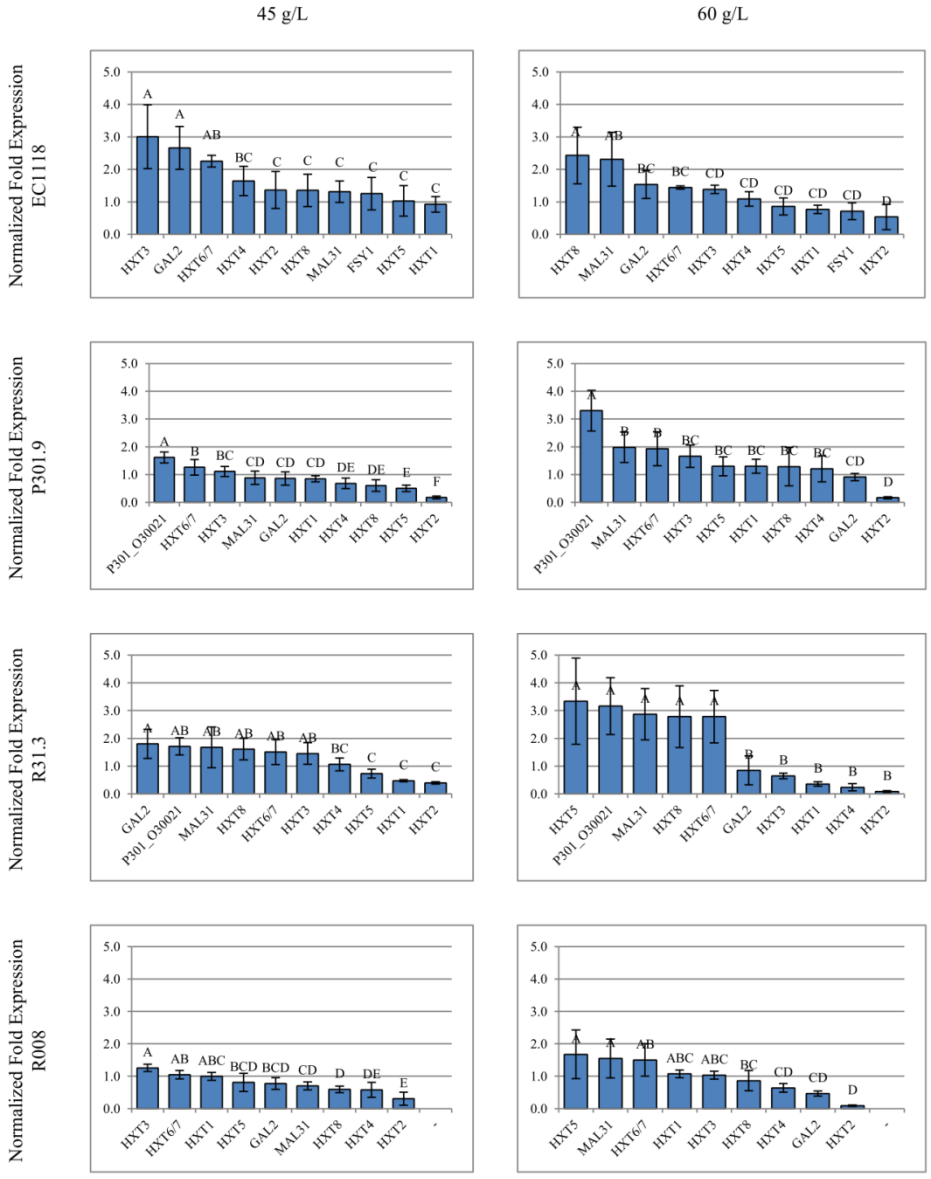
much more than the industrial strain EC1118. The results obtained in the present work leads to the following interpretation: the higher utilization of *FSY1* by P301.9 and R31.3 is needed to the counterbalancing of their higher glucose/fructose ratios. In this way, they could fix their sub-optimal hexoses transporters genes regulation pathways.

The *FSY1* moderate diffusion among vineyard strains suggests that its mere presence in the yeast genome doesn't provide an improved fitness in that environment. Differently, during the *Saccharomyces cerevisiae* yeasts selection for novel strains (that almost always starts from vineyards), the *FSY1* gene has been unconsciously selected together with the other genetic traits and genomic variations linked to the best winemaking performances.

However, if the moderate diffusion of *FSY1* seems indicate that this genetic trait is no needed for the *Saccharomyces cerevisiae* yeast cells survival in the vineyard, it is also reasonable that this gene has no negative implication: otherwise, such unfavourable genetic modification is deleted from the population.

The most remarkable results of this work are two. The first is the detection of the putative role of *FSY1* in the carbon sources utilization by *Saccharomyces cerevisiae*, where it seems acting as helper of more effective carbon sources utilization at the latest fermentation stages. The second is the possibility of improving the wine yeasts selection and characterization by distinguish the strains for their fitness in the grape must fermentation environment, at transcriptional level, by detecting the strain-specific expression pattern of the here studied hexoses carriers under fermentation conditions.

Supplementary 3: Gene expression analysis results. Letters indicate significant differences in the gene expression values obtained from ANOVA analysis of variance followed by Tuckey *post-hoc* test between the genes for each strain, at 45 or 60 g/L of produced CO_2 (*alpha* = 0.05).



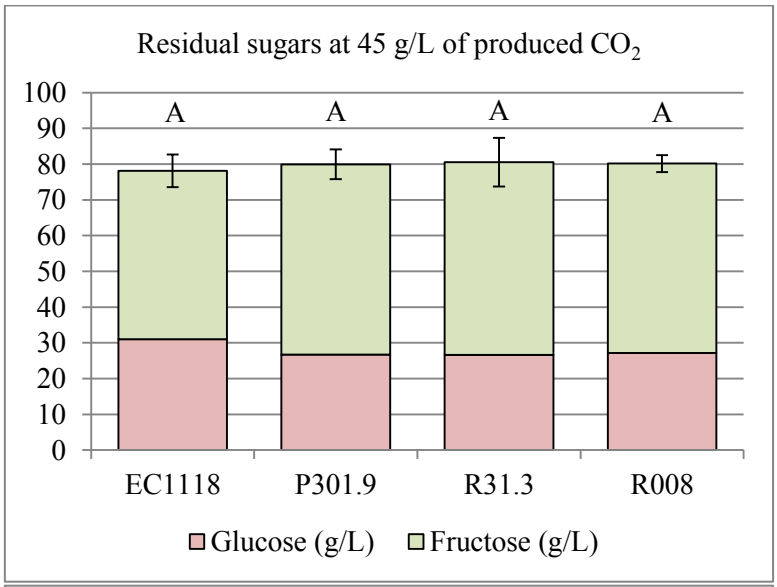
Supplementary 4 Normalized expression values of investigated hexose transporter genes.

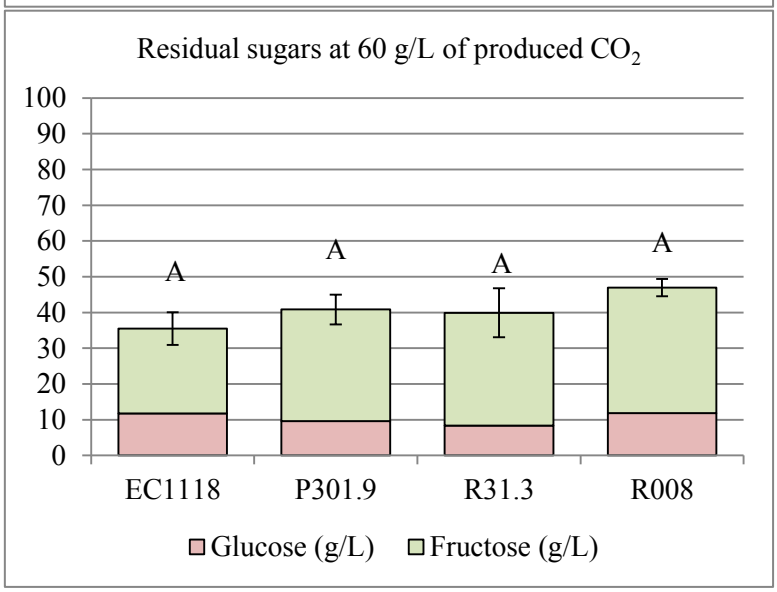
Gene	Strain	Expression at 45 g/L ±sd	Expression at 60 g/L ± sd	*p* value
GAL2	EC1118	2.660 ± *0.66*	1.533 ± *0.43*	0.0054 (**)
	P301.9	0.862 ± *0.24*	0.914 ± *0.13*	0.6883
	R31.3	1.801 ± *0.52*	0.851 ± *0.52*	0.0030 (**)
	R008	0.775 ± *0.18*	0.465 ± *0.08*	0.0005 (***)
HXT1	EC1118	0.925 ± *0.24*	0.771 ± *0.13*	0.1667
	P301.9	0.849 ± *0.11*	1.300 ± *0.25*	0.0045 (**)
	R31.3	0.475 ± *0.04*	0.356 ± *0.08*	0.0120 (*)
	R008	0.996 ± *0.12*	1.072 ± *0.12*	0.3426
HXT2	EC1118	1.364 ± *0.57*	0.563 ± *0.39*	0.0051 (**)
	P301.9	0.181 ± *0.05*	0.167 ± *0.04*	0.5508
	R31.3	0.399 ± *0.04*	0.084 ± *0.04*	0.0000 (***)
	R008	0.304 ± *0.20*	0.089 ± *0.02*	0.0076 (**)
HXT3	EC1118	3.004 ± *0.98*	1.385 ± *0.13*	0.0043 (**)
	P301.9	1.117 ± *0.18*	1.662 ± *0.40*	0.0029 (**)
	R31.3	1.454 ± *0.39*	0.648 ± *0.10*	0.0007 (***)
	R008	1.257 ± *0.11*	1.031 ± *0.12*	0.0037 (**)
HXT4	EC1118	1.642 ± *0.45*	1.090 ± *0.22*	0.0143 (*)
	P301.9	0.686 ± *0.19*	1.209 ± *0.47*	0.0326 (*)
	R31.3	1.065 ± *0.23*	0.240 ± *0.13*	0.0000 (***)
	R008	0.578 ± *0.23*	0.640 ± *0.13*	0.5209
HXT5	EC1118	1.028 ± *0.47*	0.859 ± *0.26*	0.4721
	P301.9	0.505 ± *0.12*	1.301 ± *0.34*	0.0000 (***)
	R31.3	0.731 ± *0.16*	3.341 ± *1.55*	0.0004 (***)
	R008	0.806 ± *0.28*	1.673 ± *0.75*	0.0076 (**)
HXT6/7	EC1118	2.252 ± *0.18*	1.439 ± *0.05*	0.0000 (***)
	P301.9	1.267 ± *0.28*	1.931 ± *0.61*	0.0167 (*)
	R31.3	1.511 ± *0.45*	2.782 ± *0.94*	0.0081 (**)
	R008	1.045 ± *0.13*	1.502 ± *0.50*	0.0446 (*)
HXT8	EC1118	1.354 ± *0.50*	2.430 ± *0.87*	0.0186 (*)
	P301.9	0.606 ± *0.21*	1.289 ± *0.69*	0.0167 (*)
	R31.3	1.613 ± *0.39*	2.782 ± *1.11*	0.0218 (*)
	R008	0.593 ± *0.10*	0.862 ± *0.31*	0.0367 (*)
MAL31	EC1118	1.310 ± 0.33	2.312 ± 0.83	0.0100 (*)
	P301.9	0.884 ± 0.24	1.985 ± 0.55	0.0001 (***)
	R31.3	1.675 ± 0.73	2.869 ± 0.92	0.0135 (*)
	R008	0.700 ± 0.12	1.548 ± 0.60	0.0012 (**)
FSY1	EC1118	1.254 ± 0.50	0.712 ± 0.26	0.0197 (*)
	P301.9	1.617 ± 0.20	3.301 ± 0.73	0.0000 (***)
	R31.3	1.709 ± 0.31	3.164 ± 1.02	0.0052 (**)

(***) $p<0.001$, (**) $p<0.01$, (*) $p<0.05$ between gene expression values at 45 and 60 g/L of produced CO_2 by Student's t test.

Supplementary 5. Residual sugars at 45 and 60 g/L of produced CO_2. Letters indicate significant differences in the gene expression values obtained from ANOVA analysis of variance followed by Tuckey *post-hoc* test between the strains, at 45 or 60 g/L of CO_2.

		Glucose (g/L)	Fructose (g/L)	Total sugar residue (g/L)
45 g/L	EC1118	31.03±2.22	47.10±2.36	78.13±4.58^{A}
	P301.9	26.76±2.08	53.20±2.05	79.96±4.14^{A}
	R31.3	26.63±3.32	53.90±3.51	80.53±6.83^{A}
	R008	27.21±0.64	52.95±1.79	80.16±2.40^{A}
60 g/L	EC1118	11.77±1.37	23.71±1.90	35.48±3.26^{A}
	P301.9	9.58±1.86	31.25±2.47	40.83±4.31^{A}
	R31.3	8.36±2.03	31.56±3.46	39.92±5.49^{A}
	R008	11.83±2.34	35.13±3.66	49.96±5.95^{A}





5. Chapter IV

The second fermentation for sparkling wine production following the Martinotti's (Charmat) method: insights in the *Saccharomyces cerevisiae* ethanol adaptation procedure (*pied-de-cuve*), pressure evolution in autoclave and yeast transcriptional response during the wine chilling at the end of the process.

5.1 Introduction

5.1.1 The grape must fermentation and wine second fermentation

The vinification is the biotechnological process through which the grape juice is transformed into wine. This biological process is allowed by the yeast fermentative metabolism. Oenologists can chose if let the grape must to spontaneously ferment, so that the indigenous microflora triggers the sugars consumption, or they can decide to use a commercialized specific yeast strain; they can decide how to prepare the inoculum, the modulation of the nitrogenous supply, and they can decide which technology has to be used. The wine, as just simply reported, is the resulting sum of adding all these elements and the oenologist can deal with them on the basis of the oenological goals.

The alcoholic fermentation usually happens at the expense of the grape must, where sugars will be consumed and ethanol will accumulate. The yeasts, the microbial agents leading this process, can usually find in must the nutrients necessary to ensure their vital functions: carbon sources i.e. glucose and fructose, free nitrogen sources, i.e. free aminoacids and ammonium, growth and survival factors, i.e. vitamins, sterols and lipids. However, often happens that such a nutrient requirements is not attended, so that oenologists supply the lack with external addition.

After the fermentation, when residual sugars are completely depleted or are at the proper level with respect to the oenologist's purposes, the wine can be sent to the fining procedure after that it can be bottled and sold to be consumed as steel wine, or it can be processed to undergo a second fermentation in order to obtain a fizzy or a sparkling wine (depending on the final pressure in the bottle). Such a process plans that the residual sugars in the base wine (the wine which will undergo the second fermentation) have to be adjusted to a certain concentration; then, the base wine has to be put in a pressure-tight recipient and added with a yeast inoculum previously adapted to ethanol (*pied de cuve*). In traditional method (Champenoise method) the second fermentation happens in bottles. Federico Martinotti, coordinator of the oenological school of Asti (Italy), at the end of the XIX century invented an alternative method for sparkling wine production which uses pressure-tight vessels, called autoclaves. Unfortunately, he couldn't be able to enjoy the benefits of his invention, as Eugène Charmat in 1907 patented this system before Martinotti. As consequence, this method is also called Martinotti-Charmat method.

5.1.2 Best yeast properties for the second fermentation

The base wine, i.e. the product resulting from the first fermentation dependent on the grape must, in the sparkling phase and then in the second fermentation, has the following characteristics:

- the sugar content originally present in grape must has been depleted during the first fermentation, consequently to support a second fermentation using the base wine as a medium it is necessary an addition of fermentable sugars in quantities depending on the objective of the oenologist : usually 24 g / L of sucrose is added to the classic method to guarantee a final pressure of 6 atmospheres;

- as far as sulfur dioxide is concerned, at this moment in the technological process it is almost entirely combined, ie it is not present in quantity and in such form as to exert an inhibiting action ;
- the base wine has a high alcohol content, often higher than 10% vol: this aspect is the most restrictive for the present microflora, but it can however allow the development of *Saccharomyces cerevisiae*;
- the second fermentation takes place under increasing pressure of CO2, up to 6 atmospheres. The carbon dioxide already at low pressures blocks the respiratory activity but not the fermentative one which is instead stimulated, and the productions of fermentation by-products such as isoamyl alcohols, isobutanol, etc. are also favoured:
- the fermentation temperature is under the oenologist's control: it is kept between 12 and 18 ° C and the lowering of the temperature involves a proportional slowing down of the fermentation, necessary in order to obtain a product with better quality characteristics;
- yeasts, regardless of the development method that can vary from stock to stock, tend to settle on the bottom and do not resuspend spontaneously, compromising the success of the froth; for this reason the autoclaves are equipped with stirrers that are frequently operated;
- containers in which second fermentation occurs, bottles or autoclaves, are hermetically sealed to prevent carbon dioxide from dispersing into the atmosphere (Zambonelli, 2003).

By virtue of the aforesaid considerations it is opportune to define some characteristics that the yeasts that will have to be used in the process of taking foam should possess, whether it is carried out according to the Martinotti Method rather than according to the

Classic Method. For the second fermentation in autoclave, the characteristics that must have the yeasts are:

- Species. *Saccharomyces cerevisiae* is the only species capable of providing sparkling wines given its resistance to ethanol and the organoleptic characteristics it gives to the finished product.
- Good fermentative vigour. Character essential because it favours the initiation and a rapid conclusion of the fermentation, even if in the case of the second fermentation it must not compete with other species thanks to the filtrations carried out on the sparkling base and the initial concentration in alcohol that prevents the development of other microbial species, decreasing the competition.
- Resistance to sulphur dioxide. A medium resistance is sufficient, since for the concentration in SO2 in the sparkling base it is not a limiting character of the development.
- High fermentation power. A resistance capacity of up to 14 ° alcohol should be sufficient, but an even higher resistance can guarantee a better completion of the fermentation.
- Fermentative purity. The production of acetic acid must be as low as possible.
- Type of development. Dusting development is preferred in the second fermentation.
- Foaming power. It is characterized by the presence of cells in the foam. According to Castellari *et* al. (2000) strains with high foaming power are the ones that give better results because their cells after stirring remain in suspension longer and assure better than others the speed of fermentation and its completion.
- Low sedimentation speed. Negative character for second fermentation.

- Killer factor. It is not necessary, as the species *Saccharomyces cerevisiae* is the only one to be present and to carry out the fermentative activity.
- Action on malic acid. A slight activity on malic acid does not compromise the quality of the product.
- Low production of sulphites. The sulphites form spontaneously with the fermentative activity, adding to those already contained in the base wine.
- Low production of hydrogen sulphide. The strains that produce high amounts of hydrogen sulphide determine a wine with a decidedly more bitter taste and reduce the sensory finesse, compromising the quality of the product
  (Zambonelli, 2003).

For the Classic Method the characteristics to be found are almost the same as in the Martinotti Method, except for some aspects:

• Type of development. In this case a flocculent development is preferable, as the cells, aggregating with each other, facilitate the operations of removing the fake deposit at the disgorgement.

• Foaming power. The foaming strains produce a light superficial veil that does not easily fragment and which hinders the operations of cell collection and clarification of the wine.

An important feature in favour of a quality second fermentation and a rapid sedimentation of yeasts is the use of so-called "immobilized yeasts". These are cells of *Saccharomyces cerevisiae*, endowed with vigorous and powdery growth, contained within calcium spheres alginate-sodium alginate-agar gel.

Fumi *et* al. in 1987 conducted experiments concerning the use of immobilized yeasts. They observed that there are no chemical-physical differences in the oenological product

obtained by second fermentation of base wine between free and immobilized cells. They noted that the conditions of alginate gel after fermentation change considerably in relation to alginate and cell concentrations within, and this may also be linked to the type of cells being used. Furthermore, the number of cells found in the wine at the end of the second fermentation highlighted the degree of variable integrity of the immobilizing matrix (Fumi *et* al., 1987). They concluded by expressing the concept that the ability of alginate beads containing the immobilized yeasts to maintain their shape is directly proportional to the concentration in alginate and inversely proportional to the quantity of immobilized yeast and their fermentative power (the greater the fermentation power, the greater tendency the cells to free themselves from the structure that keeps them immobilized). If the cells come to free, the wine turbidings occur logically.

This technique was born with the aim of simplifying the *remuage* without altering either the fermentation or the disgorging process, and to obtain perfectly limpid wines, without cells and with excellent organoleptic characteristics. However, the fact remains that this method, however ingenious it may be, has not yet found use in current practice.

Also important is the yeast strain used in sparkling wine production:

• the vast majority of *Saccharomyces cerevisiae* strains produce sparkling wines of acceptable quality, albeit with different characteristics.

• the strain can influence the quality of a sparkling wine equal to that of the vine: therefore, the choice of the strain must be put in relation with the type of product to be obtained. With some particularly flavouring strains, quality sparkling wines can be obtained, even starting from completely anonymous wines.

• Certain wines with a very marked primary flavour (such as Moscato and Malvasia), if second fermented with certain strains, give rise to extremely unpleasant products (Zambonelli, 2003; Tini *et* al., 1995).

The choice of the yeast strain must therefore be made on the basis of comparative tests and through tastings of the experimentations themselves.

5.1.3 The Prosecco sparkling wine production

The Martinotti's method is also called "the Prosecco method", because of its adoption in Prosecco Sparkling wine production. Such a wine proved to be the most suitable for this methodology and in the Prosecco wine area it has constantly been improved since '70s. Not only Prosecco wines can be processed under the Martinotti's method, but this technology is particularly suitable for aromatic grapes and wines too: the very short yeast lees contact, in comparison to that of the traditional method, allows to highlight the fruity and fresh aromas coming from the grapes, limiting the yeasty or bready aromas typical of the traditional method wines.

The Martinotti's method starts with the obtaining of a proper base wine. The base wine production steps are:

- If desired, a pre-fermentative grape juice contact with marc;
- Alcoholic fermentation conducted at 18°C, with a final ethanol concentration of 8-10% vol.;
- Avoiding of the malolactic fermentation, in order to preserve the grape fruity and flowered aromas and the freshness of the wine provided by the malic acid;
- Protein and tartaric acid stabilizations (very limited, to avoid wine impoverishment);

- Filling the autoclave with the sweetened base wine and preparation of the adapted yeast inoculum (also called *pied de cuve*);
- Second fermentation (or *prise de mousse*), usually lasting for 10-20 days at 16-18°C;
- Fermentation stop when the desired sugar residue is reached, basing on the internal autoclave pressure, thanks to the rapid chilling of the wine.
- First isobaric filtration to eliminate the rough lees;
- Tartaric acid stabilization, which can be spontaneous if enough stability level is given by the wine chilling, or by adding of stabilization agents;
- Sterile isobaric pre-bottling filtration (0.45 μm) and bottling.

After the second fermentation begins, oenologists usually depressurise the autoclaves when the first 0.5-1 bars (depending of oenologist's experience) of internal pressure is reached: this operation serves to eliminate the gases coming from the first phases of the fermentation, which is usually rich in unpleasant off-flavours. After the cited operation, the fermentation process is let go on until the proper internal pressure and sugar residue will be reached. The optimal pressure increase for a fine bubbles obtaining is 0.2-0.3 bars in 24 hours, so the temperature is controlled to increase or reduce the pressure increase. Paying attention to not go below the 0.2-0.3 rates is necessary to avoid sluggish or stuck fermentations. Yeast suspension using the autoclave equipment with regular intervals is recommended to ensure the homogeneity and to favour the noble lees contact with all the fermenting mass. At the end of the second fermentation the obtained wine has to be cooled: there are different oenological opinions on the better choice between the rapid or slow wine chilling, because the fearing risk is to let the yeasts to produce sulphur metabolites which, at this production stage, are very hard to overcome. However, the

most of the oenologists prefer a rapid temperature decrease. This goal depends, as is quite easy to realize, on the autoclave and chilling installation sizing, so the oenologist has to deal with this operative limit. The alternative could be an immediate yeast lees isobaric removal. On the other hand, this choice compromises the possibility to perform the recommended yeast contact: this stop on the noble yeast lees should not be as long as the traditional method requires, but it usually takes place in the Martinotti's method for 3-9 months long to improve wine stability. Depending on the duration of the stop on the lees the method is called "Short Martinotti" or "Long Martinotti", respectively. In the first one, the freshness, fruity and flowery aromas are sought-after, while in the latter the oenological goal is an increased structure, foaming stability and sophisticated aromas.

After the sparkling wine isobaric filtration and sulphur dioxide and/or other required adjustments the tartaric acid stabilization has to take place. This physical treatment needed in order to eliminate all the tartrates which can react with the potassium ions present in the wine, producing a sensory irrelevant but unesthetic precipitate. When all the stability requirements are reached, the sparkling wine produced by the Martinotti's method is ready for sterile isobaric filtration, bottling and selling.

5.1.4 Ethanol toxicity

Ethanol, the main compound deriving from alcoholic fermentation, is toxic to living organisms even at low concentrations (Fancellu *et* al., 2008). During its fermentation activity, yeast undergoes various stressful conditions, including the accumulation of ethanol (Chandler *et* al., 2004). In the case of spontaneous fermentation of must the microorganisms present are numerous and belonging to different species, such as *Brettanomyces*, *Candida*, *Hanseniaspora*, *Pichia*, *Schizosaccharomyces*, etc. (Pretorius *et* al., 1999; Khan *et* al., 2000; Valero *et* al., 2007). The less resistant species are destined to

disappear with the continuous progress of fermentation. At 4-6 ° alcohol usually only the species *Saccharomyces cerevisiae* is found (Fancellu *et* al., 2008), but also in this species ethanol exerts inhibition of cellular activity, acting on the function of the cell membrane.

The cell membrane is made up of phospholipids, characterized by a hydrophobic part and a hydrophilic part. The latter interacts with the water, which constitutes a layer of solvation around the hydrophilic heads. Ethanol, being a dipole like water, when it is found at high concentrations replaces the solvation water. Moreover, due to the structural characteristics of the ethanol molecule, it penetrates inside the membrane, settling among the phospholipids, compacting the membrane itself due to the reduction of the repulsion force between the polar heads and the restriction of the lateral movements of the chains of fatty acids that characterize the hydrophobic part: this results in a stiffening and a loss of fluidity of the membrane. The effect of ethanol on the cell membrane is similar to that caused by low temperatures: in this case, in fact, there is a stiffening of the membrane with loss of fluidity. The ethanol, however, due to the crushing of the phospholipids, also involves a compaction of the proteins present on the membrane, causing conformational variations and hindering the activity of transport of nutrients inside the cell and release of amino acids and other substances: in fact the presence of the ethanol molecule in the double phospholipidic layer affects the ability of the cell to control the influx of protons from the outside, and this affects the functionality of the transport proteins that use the simulation system, including many transporters dedicated to amino acids.

Among the functions of the cell membrane we find that to maintain the motor protonic force (FPM), generated and maintained by the cell with a considerable energy investment in terms of ATP consumption: the internal balance (cellular homeostasis) must be maintained to guarantee the survival of the cell itself. The membrane activity generates a positive charge on the outside and negative inside: in this way it allows the cell to manage

the flows of different substances and nutrients exploiting the concentration gradient of the substances themselves and / or the potential difference between cytoplasm and external environment. In particular, there are proteins (membrane ATPases) that maintain the FPM by consuming ATP and translocating H^+ ions to the outside: ethanol is produced internally in the cell during alcoholic fermentation and since it is a toxic compound it has to come out of it.

When it is at low concentrations it is able to cross the membrane without the need for transporters and therefore according to concentration gradient, but when its concentration outside the yeast increases, its outflow is slowed down, or even the flow can be reversed. In this case, ethanol is placed inside the membrane at the level of the phospholipids and its presence alters the integrity of the membrane itself: in this way it is possible to create easier paths, similar to channels, through which the protons enter uncontrolled within the cell. The ATPase will be in trouble because it is forced to perform its activity more quickly to expel the H^+, with a significant consumption of ATP, and cell growth is thus compromised.

Furthermore, ethanol involves the denaturing of proteins: the protein chains remain permanently bent and maintain the native configuration thanks to interactions or chemical bonds between different parts of a single chain but also thanks to the interaction with the surrounding environment, especially with the molecules of water in which proteins are normally immersed; the presence of ethyl alcohol interferes with these interactions since it tends to substitute for water molecules, having dimensions and electrical charge similar to it: the protein molecules can then undergo changes in shape and structure because the native conformation is no longer sustainable under the new conditions environmental.

Yeast cells try to respond to ethanol-induced stress through the synthesis of molecules with protective function and the activation of alternative ways of responding to the

stressful condition, which include the promotion of transcription of genes that code for transcription factors that they in turn regulate the expression of other genes involved in the response mechanism (Hohmann and Mager, 1997; Estruch, 2000). It is a complex molecular mechanism that allows the cell to survive and resume its cellular activity (Chandler *et* al., 2004). Beyond a certain concentration of ethanol the genes coding for heat shock proteins (Hsp) are activated, while those that were previously synthesized are blocked. Hsp are part of a much conserved protein family and have been characterized for the first time by studying the reaction to a strong thermal shock (Piper *et* al., 1995), however they are also synthesized in response to other types of stress. The Hsp involved in the ethanol response are respectively: Hsp70, induced at ethanol concentrations between 4-6% vol. with fermentation temperature of 25°C; Hsp104, Hsp70, Hsp26, Hsp12 are induced one after another as the alcohol concentration gradually increases from 4 to 10% vol.; Hsp82 and Hsp30 are induced at concentrations of 6% vol. They are therefore induced differently and in relation to different concentrations of ethanol. Their function may be different, for example Hsp104 acts as a refolding agent in the disaggregation of denatured proteins (Glover *et* al., 1998), while Hsp12 is associated with membrane protection against ethanol dehydration (Sales *et* al., 2000; Stanley *et* al., 2010). In order to cope with the loss of fluidity of the membrane, the cell transforms the saturated fatty acids of the hydrophobic chains of phospholipids into unsaturated fatty acids: unsaturated fatty acid does not have a linear and rigid structure like the saturated fat, but has at least one correspondence of each double link contained in it. This structure makes unsaturated fatty acid an element of the membrane which, thanks to its steric encumbrance, in turn forces the molecules close to it to move, thus partially restoring the fluidic character.

5.1.5 The *pied de cuve* adaptation protocol

The second fermentation in autoclave takes place in objectively difficult conditions due to the considerable volume of wine and its static nature (Zambonelli, 2003). In order to make the froth and the organoleptic characteristics of the final product successful, the correct preparation of the pied de cuve is very important. The purchased yeasts are generally dehydrated (Active Dry Yeasts or LSA) and need to be rehydrated and activated before they can be added to the base wine. This phase is as delicate as it is crucial, since the conditions of the oenological product to be sparked are very difficult, starting from the ethanol concentration of the sparkling base which is high and which can immediately jeopardize the growth and fermentation activity of the yeast, if not properly prepared through a good fermentation foot (***pied de cuve***). Here is reported a common-used procedure for the preparation of the *pied de cuve*:

- Pour the amount of LSA indicated by the manufacturer into a container containing about one third of its volume of 3% aqueous solution of sucrose and at a temperature of 35 ° C.
- Allow to stand for 30-60 minutes to promote rehydration.
- Shake the suspension and double the volume with the same sparkling wine already sweetened. It is advisable that the latter has a temperature of about 20 ° C so that the final one of the pied de cuve falls to about 25 ° C.
- The fermentation is allowed to start, and when the yeast population reaches the end of the exponential phase, the volume is doubled again with the same sparkling wine.

- Continue to repeat the same procedure until obtaining a volume of parent culture, that is, of starter, sufficient to inoculate the total mass (usually the inoculum / mass ratio is 1:10).

It is advisable, if the quantity of starter is low for the mass to be sparkling, continue to multiply the concentration of yeasts as just indicated, to reduce the risk of a sluggish fermentation or even that the fermentation cannot start.

5.1.6 Sulphur disulphide, sulphur compounds and the role of copper

The two most important oenological problems are the wine oxidation or reduction. The first is mostly due to the oxygen intervention, but also the light excess can cause wine oxidation. For example, the "*goût de lumière*", to avoid which the dark green glass is requested for sparkling wines bottling. On the contrary, the wine reduction is due to the presence of the hydrogen disulphide and other sulphur compounds, which are produced by the yeast cells during the fermentation and/or during the stop on the less. If the oxidation risk can be quite easily avoided preserving the wine from oxygen and light, the wine reduction is very difficult to handle, in particular for sparkling wines.

5.1.7 Sulpur metabolism and sulphur dioxide toxicity

The "reduction" wines off-flavour produced during or after fermentation is due to the yeasts production of volatile sulphur compounds: hydrogen disulphide (H_2S), thiols, mercaptans and sulphur dioxide.

Sulphur is a very important element for the yeast growth: it's involved in the synthesis of sulphur aminoacids (cysteine and methionine) which are essential for the proteins shaping. Yeast cells uptake inorganic sulphur compounds, like sulphate ions, and then incorporate them into sulphur aminoacids. The origin of H_2S or SO_2 is strongly linked to the sulphite reductase enzyme activity: the more it is active, the more sulphur ion and, consequently, the more hydrogen sulphide will be produced. On the contrary, a slight activity of the sulphite reductase can reduce the amount of produced hydrogen sulphide, but it also leads to the overproduction of sulphur sulphite and a consequently overproduction of sulphur dioxide.

Sulphites are widely used in winemaking for their antimicrobial, antioxidasic and antioxidant effects. Yeasts usually produce low or medium SO_2 amounts, depending on their genetic features and fermentation conditions. High-SO_2 productive yeasts are usually adapted to tolerate high amounts of this compound, as they produce high amounts themselves. Low-SO_2 productive yeasts can handle SO_2 using four major ways: the acetaldehyde production (that irreversibly binds SO_2, inactivating it); the glutathione production (sulphur aminoacid incorporation) (Surdin-Kerjan, 1987, 1997; Stratford *et* al., 1987); sulphite uptake and reduction (Yoshimoto and Sato, 1968; Kobayashi and Yoshimoto, 1982) or SO_2 efflux thanks to a specific plasma membrane transporter (Ssu1 pump) (Park and Bakalinsky, 2000) (**Figure 4.1**).

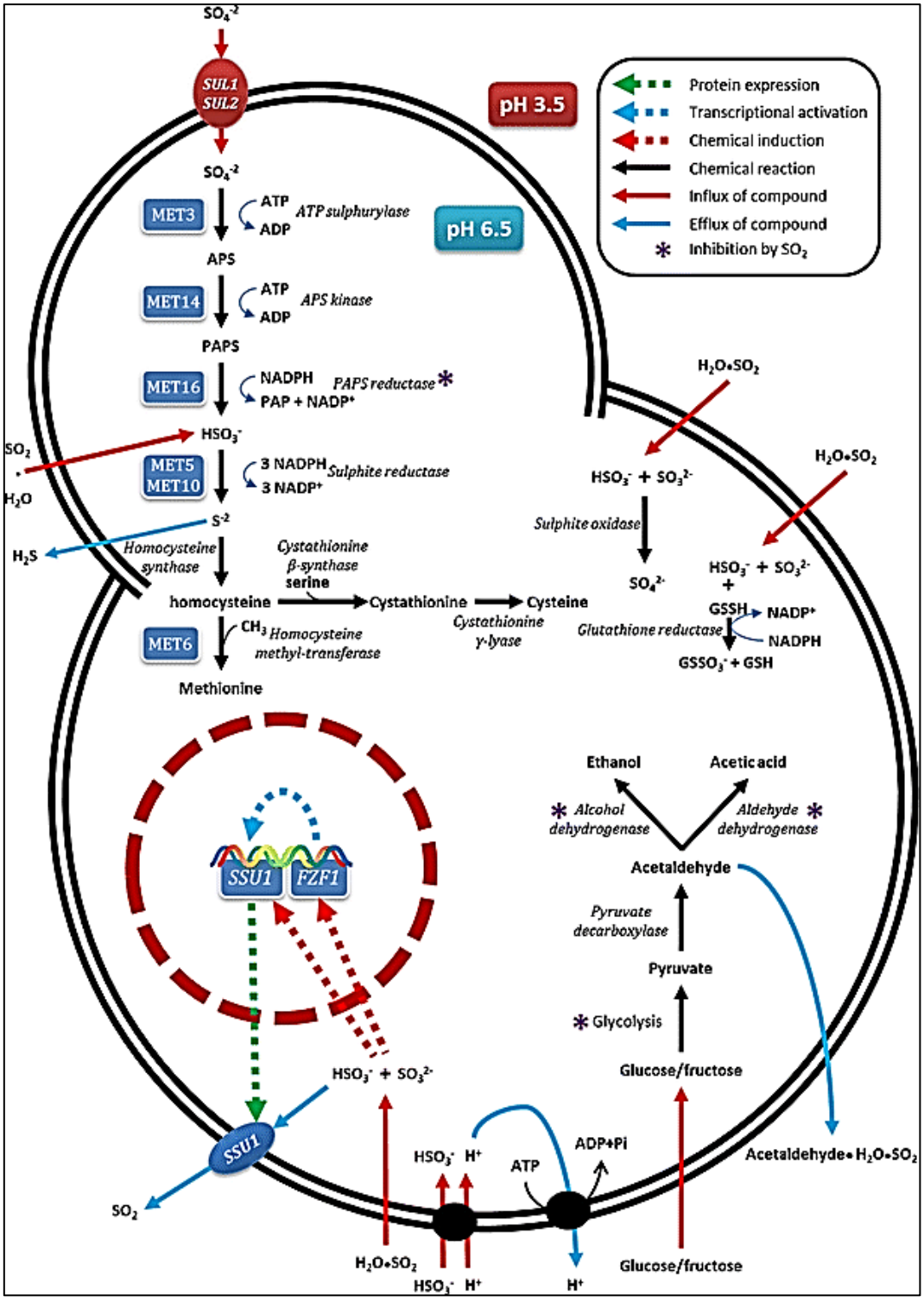


Figure 4.1

A summary of the sulphate assimilation pathway and the cellular and molecular responses of S. cerevisiae to the presence of SO2. SAAB sulphur amino acid biosynthesis, SR sulphur reduction. (Figure 2 in: Park and Bakalinsky, 2000)

5.1.8 Hydrogen sulphide production

Hydrogen sulphide production in wine has been also associated to unfavourable abiotic conditions, and nutrient starvation: presence of free SO_2 (Acree *et* al., 1972; Stratford and Rose, 1985), which is usually added to grape musts before the alcoholic fermentation; presence of other sulphur compounds (Acree *et* al., 1972); vitamins starvation (Bohlscheid *et* al., 2007) and nitrogen starvation (Schutz and Kunkee, 1977; Thomas *et* al., 1993), which represents a poor presence of carbon skeletons from serine and aspartate aminoacids. In these conditions the sulphur ion is thrown outside of the plasma membrane as H_2S. The H_2S production can be translated as the yeast cells reaction to the nitrogen starvation and such a mechanism acts because of sulphite reductase is a nitrogen-dependent enzyme: when nitrogen sources are limiting the sulphite reductase synthesis increases, followed by an increasing in sulphur ion concentration, that, without carbon skeleton to react with, effluxes outside the cell as hydrogen disulphide, in the growth media.

5.1.9 The negative role of hydrogen sulphide in wines

The hydrogen disulphide represents a relevant problem in oenology: compound confers "reduction" off flavours, reminding to rotten eggs (Mestres *et* al., 2000). Moreover, it is an extremely volatile compound, showing 1.1–1.6 μg/L as sensory thresholds in red and white wines, respectively (Siebert *et* al., 2010).

If the hydrogen disulphide produced during the grape must fermentation is stripped away from the liquid thanks to the carbon dioxide flux, the hydrogen disulphide produced during the second fermentation, in sealed pressure-tight vessels, has no ways to flow out and after the head-space saturation hydrogen disulphide will dissolve into the wine together with the carbon dioxide.

In the same way, the others undesirable sulphur compounds, cannot flow out to the vessel too, increasing the risk of wine "reduction".

Besides its production, the H_2S tends to stay in the wine: in this way other heavy sulphur compounds (mercaptans) will be formed, such as ethyl-mercaptan which is easily formed by reaction of H_2S with ethanol. With respect to the H_2S, mercaptans are more difficult to overcome and can also worst the problem if oxidized by oxygen: in this case disulphide will be produced, another class of undesirable sulphur compounds in wine.

5.1.10 Principal strategies for hydrogen sulphide overcome

Oxygen

Thanks to its volatility, H_2S can be easily removed by dissolving oxygen. Such a solution is not suitable for mercaptans, which are heavier and more persistent than H_2S.

Copper

Copper can resolve problems of H_2S and its derivative thiols (mercaptans) (Kreitman *et* al., 2016). It's a very useful tool in the Martinotti's method, because in this case a pouring for oxygenation is not possible. This heavy metal is present in almost all grape juices as plant protection treatments residue on grapes skin. The most of copper precipitates during grape must fermentation and eliminated with rough lees removal as copper sulphide (CuS). As a result, the obtained wine will contain a copper residue lower than the legal limit of 1 mg/L (Reg. CE N. 606/2009). Copper in Italian oenology can be added as pure metal or as copper sulphate solution ($CuSO_4$). Reacting with sulphur group of the off flavours molecules it forms CuS, poorly soluble in wine, and in this way it eliminates sulphur compounds. The use of copper needs, however, carefulness: besides the legal limit, it can compromise the shelf-life of the final wine.

5.1.11 Nitrogen requirements in fermentation

Saccharomyces cerevisiae yeast cells require a relatively high level of nitrogen-based nutrients to complete the fermentation of grape must, producing around 12–15% vol. ethanol. Free Assimilable Nitrogen (FAN) represents a key nutrient that in natural grape musts is often at suboptimal doses. The minimal concentration depends on the strains, but Bell and Henschke (2005) found that more than 140 mg/L FAN is often required for the fermentation of filtered musts at low-temperature (<15°C), containing moderated sugar concentration (20%). Nitrogen sources are rapidly up taken by yeast in the early stages of the fermentation, during which they fill the biosynthetic pools of amino acids needed for protein synthesis and growth, while the surplus is stored in the cell vacuole (Vilanova. *et* al., 2007). Grape must contains a variety of nitrogen compounds, but the most important are the free aminoacids, ammonium ion, and small peptides. These nitrogen compounds, except for proline, constitute the Yeast Assimilable Nitrogen (YAN). Nitrogen affects yeast cells in two aspects: biomass production during fermentation and the fermentation rate (Varela *et* al., 2004). Therefore, the nitrogen content regulates both fermentation rate and end. To support this fact, there is the observation that the lack of nitrogen has been pointed as one of the main responsible elements of stuck or sluggish fermentations (Bisson, 1999; Taillandier *et* al., 2007). The nitrogen content affects also other yeast pathways: for example, it acts on the redox status of the cells, which affects the production of ethanol and other metabolites such as glycerol, acetic acid, and succinic acid (Albers *et* al., 1996; Radler, 1993; Camarasa *et* al., 2003).

The quality and the concentration of the nitrogen sources are critical in the formation of some aromatic molecules (Gutiérrez *et* al., 2013), very important for wine sensory quality.

Not all the nitrogen sources equally support the yeast growth: in mixed condition of amino acids and ammonium, such as the grape must is, the wine yeasts prefer some nitrogen sources more than others, and the pattern of the preferential uptake of the nitrogen sources is determined by different molecular mechanisms. In *Saccharomyces cerevisiae* the mechanism is called Nitrogen Catabolite Repression (NCR). The NCR allows cells to detect the presence of the best sources of nitrogen and to limit the use of those which cannot allow an optimal growth. The detection of the nitrogen sources activates a signalling mechanism culminating in a deep regulatory system at gene expression level: the activation of the genes involved in the transport and metabolism of the best nitrogen sources, the suppression of the genes involved in the transport and use of poorer sources. Only when the best nitrogen sources are consumed (ammonium, glutamine, and asparagine) the yeast cells will activate the utilisation of the poorer sources of nitrogen (arginine, glutamate, alanine, etc.) (Mas *et* al., 2014).

5.1.12 Nitrogen uptake

The nitrogen uptake in *Saccharomyces cerevisiae* requires a symport mechanism, so it needs metabolic energy to take place because of the consequent need to throughout protons after the nitrogen income and restore the differential membrane potential.

S. cerevisiae uses two main typologies of nitrogen transport, differentially used basing on the fermentation stage. During the early fermentation stages the ammonium ion is rapidly consumed, much more than free aminoacids. As a matter of fact, all literature agrees to admit how the ammonium ion is the first nitrogen source to be consumed in fermentation and that that its concentration regulates the fermentation process. When ammonium ion decreases the yeast cell activates high-affinity plasma membrane transport proteins, also called Specific Aminoacids Permeases. Such transporters allow the cell to rapidly

assimilate single aminoacids or small groups of aminoacids that cell serves, in particular the aromatic aminoacids (tyrosine and tryptophan) and the branched-chain aminoacids (leucine, isoleucine, valine). The other aminoacids are usually synthetized by the yeast cell using the ammonium ion, if available. This transport mechanism is extremely important in yeast metabolism, as it is on the basis of the transamination reactions, and the aminoacids in this way up taken can be concentrated into the vacuole and used in a later time according to the yeast cell needs.

When ammonium ion is almost depleted, near to the stationary phase, the yeast cell activates another kind of aminoacids transport system: this is the case of the General Aminoacids Permeases (GAP), a non-specific nitrogen uptake system able to transport all the aminoacids which is inhibited by the ammonium ion presence: this is why this transport mechanism is activated only at this stage of the fermentation process and why the ammonium ion is considered a nitrogen uptake regulator.

The ethanol increment in the growth media, due to the fermentation progress, has a toxic effect on the yeast cell plasma membrane: due to its toxicity, ethanol is an inhibitor of all the transport systems which require symport proteins, and the aminoacid uptake is included. As a consequence, the balance between the yeast nitrogen utilization ability and the ethanol toxicity determines the fermentation slowing.

5.1.13 Nitrogen metabolism

Yeast cell manages the nitrogen sources *via* the so-called anaplerotic way of the Krebs's Tricarboxylic Acids Cycle (TCA). Thanks to specific reactions in the mitochondria, which start from pyruvic acid to produce *alpha*-keto acids, intermediates of the TCA cycle, the yeast cell is able to produce the carbon skeletons needed for the aminoacids biosynthesis. In particular the *alpha*-keto glutarate (or 2-oxo glutarate) is the *alpha*-keto acid on the basis of the ammonia nitrogen incorporation in aminoacids. In the main

reaction of this pathway the $–NH_2$ residue of the ammonium ion passes to the *alpha*-keto glutarate: in this way this carbon skeleton is converted in glutamate and the inorganic nitrogen passed in an organic *status*. This step requires the oxidation of a NADPH molecule. When ammonium ion is at high concentration the glutamate is condensed in glutamine, a cell nitrogen reserve. When ammonium ion decreases its concentration the glutamine can be re-converted in glutamate.

The second main aminoacid biosynthesis reaction, involving all other aminoacids, requests another important *alpha*-keto acid: the oxaloacetate. This is a transamination reaction where the oxaloacetate can receive the $–NH_2$ residue from glutamate, so that it returns to the initial *alpha*-keto glutarate form. In this way, the *alpha*-keto glutarate can be re-utilized by the cell to generate another aminoacid: the aspartate.

Besides the already cited, other carbon skeletons are important for nitrogen incorporation in aminoacids: 3-phosphoglicerate, ribose 5-phosphate, pyruvate, phosphoenolpyruvate and erytrose-4-phosphate. The aminoacids generated by these ways can be used by the yeast cell for the synthesis of the proteins needed for all the basic cell functions.

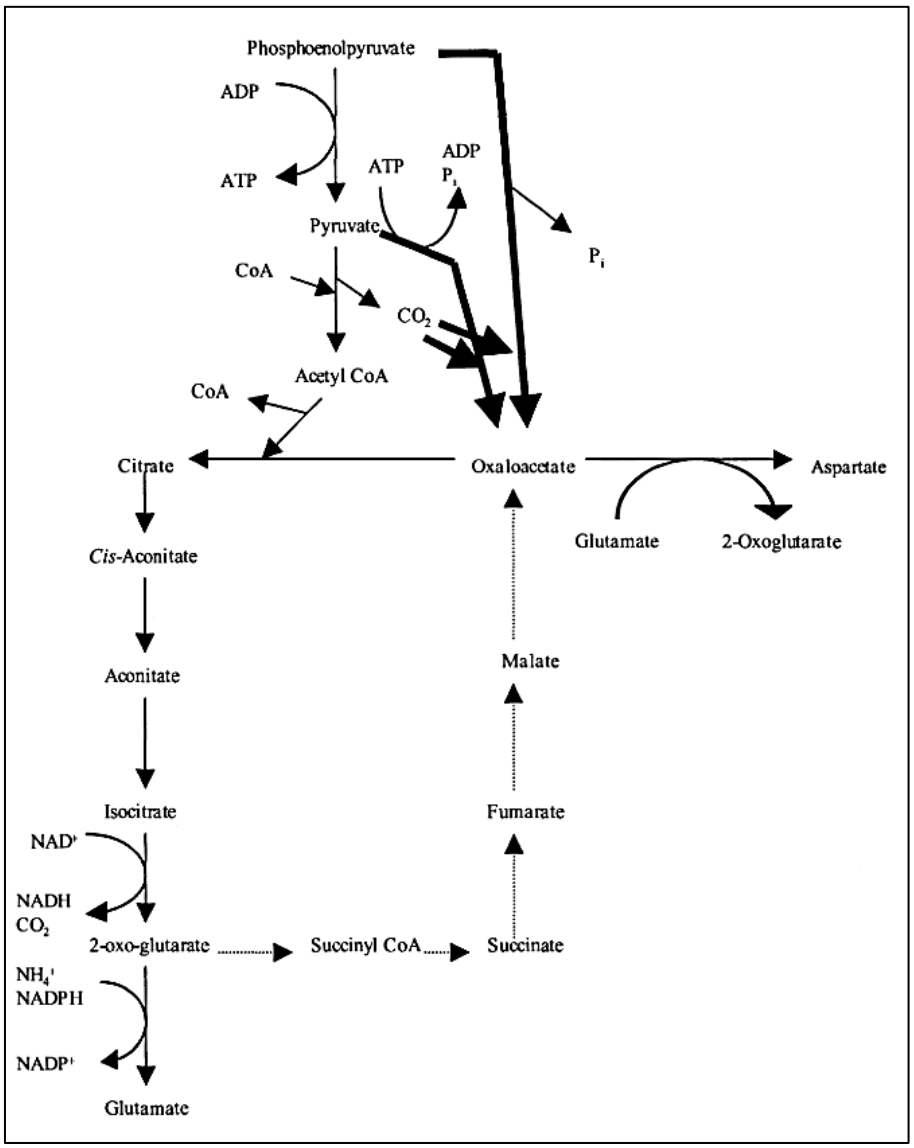


Figure 4.2

TCA cycle and anaplerotic formation of oxaloacetate. The solid arrows show the synthetic routes to aspartate and glutamate from glycolytic intermediates with the heavy arrows indicating the two anaplerotic routes. The dotted arrows show the reactions necessary to complete the TCA cycle. The cycle functions in fully differentiated mitochondria in the presence of oxygen to effect complete oxidation of glucose. (Figure 2.4 in Slaughter, 2003)

The TCA cycle interruption at the oxaloacetate level (**Figure 4.2**), which happens in fermentation conditions, is due to the absence of the FAD enzymatic co-factor. FAD is

the second complex of the respiratory chain and its presence is linked to the respiration. If the respiration metabolism is not active, the FAD is absent and the TCA cycle reaction chain is interrupted at the succinate to fumarate oxidation step: this is the reason why an accumulation of succinate is observed at the end of the fermentation.

5.1.14 The nitrogen supply in the vinification processes

When the nitrogen grape must content is too much low a supply is legally allowed. This operation can be done before or during the fermentation process, depending on the technological goals.

An adequate nitrogen supply is needed to ensure a high cell multiplication, in order to reach a numerous yeast population (Bely *et* al., 1990; Henschke and Jiranek, 1993; Henschke, 1997) and a good CO_2 production kinetic. The maximum allowed nitrogen addition in European Union is up to 30 g/hL (Reg. CE N. 606/2009) of an authorized nitrogen-based commercial product, corresponding to around 60 mg/L of assimilable nitrogen. The addition can be done using DAP (di-ammonium bi-phosphate – $(NH_4)_2HPO_4$) or an aminoacids-rich yeast derivate.

The ammonium addition allows accelerating the first fermentation stages, and so anticipating the yeast population exponential phase and the fermentation closure. The addition of aminoacids has a less evident impact on the fermentation process itself, but has a great effect on the yeast nitrogen handling (see previous paragraphs): by this way, a proper aminoacid supply allows acting on the aromatic characteristics of the final wines, in particular white wines (Ribéreau-Gayon *et* al., 2006).

5.1.15 The nitrogen-dependent wine aromas

The biosynthetic way leading to the yeast aromatic secondary metabolites takes place because of the carbon skeletons obtained after the $–NH_2$ residue removal from the up taken aminoacids (transamination) can undergo to a decarboxylation reaction. In this way the carbon skeletons are converted into aldehydes which can be reduced to higher alcohols, while a NADH molecule is oxidized. This reaction chain is known as the Ehrlich way or aminoacids catabolism. It is active during the stationary phase, when the yeast activates the non-specific transporter proteins. At this fermentation stage a high amount of non-essential aminoacids enters in the cell, where they serve for their $–NH_2$ residue. After the transamination reaction the resulting carbon skeleton is re-utilized by the yeast cell as a NADH oxidation tool in the Ehrlich reaction.

The higher alcohols so produced represent one of the major groups of aromatic compounds in wines, directly contributing to the final aromatic *bouquet*.

Moreover, a relevant increase in the higher alcohols concentration has the collateral effect of esters synthesis induction: the formation of this class of molecules conferring flowered and fruity aromas to the wines is an esterification reaction between an alcohol and an organic acid. Considering the concentrations in wines, is easy to foresee that the most present esters belong to the ethylic esters class (involving ethanol and a short-medium chain fatty acid), while the others belong to the acetic esters class (involving acetyl-CoA and a higher alcohol).

The esterification reaction is one of the possible metabolic pathways that the yeast cell can use to free the “A” coenzyme from the acidic residue: the CoA is fundamental in fatty acids biosynthesis, as they represent the main constituents of the plasma membrane phospholipids.

A proper nitrogen supply allows the yeast cell to efficiently perform the alcoholic fermentation, favouring the desirable aromatic molecules biosynthesis and limiting the sulphur compounds formation (off flavour).

In this work two industrial yeast strains, usually utilized in the Prosecco sparkling wine production in the Conegliano-Valdobbiadene DOC and DOCG areas, have been tested in two second fermentation conditions, adopting the Martinotti's method for sparkling wine production. As the final aim of this work is to determine the reaction of the yeast cells to the wine chilling at the end of the second fermentation, and the cells response to this abiotic stress is likely influenced by the residual sugar concentration, the two second fermentation conditions differed only for the initial sugar content. In this way, two sets of comparisons would have been possible to analyse: between the two strains at the same residual sugars content and between the same strain in two different residual sugars conditions.

5.1.16 Aim of the work

In this work the most critical topics of the yeast utilization in second wine fermentation, the technological process needed for the sparkling wine obtaining, have been investigated in two industrial *Saccharomyces cerevisiae* wine yeast strains: EC1118 and QA23, both used in the production of the Prosecco DOC Sparkling wine (Conegliano-Valdobbiadene). The main topics taken into account were the yeast cells adaptation to the high ethanol concentration (*pied-de-cuve*); cells viability, nitrogen and sugars consumption; cells viability during the autoclave chilling at the end of the second fermentation. During chilling, cells have been harvested for total RNA extraction to be used in transcriptomic analysis to answer how the yeast cells react to this stress, with the

aim of improving the sensory quality of the sparkling wine obtained with the Martinotti's method. Results show how EC1118 has been characterized by lower cells viability than QA23 since the ethanol adaptation procedure and all along the fermentative process. This difference reflected to the pressure evolution: despite a slightly longer *lag* phase, QA23 showed the fastest pressure increase kinetic and cells viability higher than EC1118 from autoclaves inoculum to the end of the process. Data of the total RNA extraction, RNA quantification and the integrity and quality check are also presented

5.2 Material and method

5.2.1 Yeast strains used in this work

Two industrial strains belonging to the *Saccharomyces cerevisiae* species have been used (**Table 4.1**). They have been chosen considering their great use in the Prosecco base wines and Sparkling wine production.

Strain name	Species	Producer
Lalvin EC1118	*Saccharomyces cerevisiae*	Lallemand Wine
Lalvin QA23	*Saccharomyces cerevisiae*	Lallemand Wine

Table 4.1

Yeast strains used in this work.

5.2.2 Base wine

The base wine used in this work have been gently supplied by Borgo Molino Vigne & Vini S.r.l. (Roncadelle, TV) cellar. It was a Prosecco DOC base wine from the 2017 vintage. Chemical parameters of the base wine were: ethanol 10.15±0.02 % vol.; free SO_2 11 mg/L; total SO_2 80 mg/L; volatile acidity 0.53±0.01 g/L; pH 3.24; residual sugars 13.70±0.12 g/L.

5.2.3 *Pied de cuve* preparation

Each yeast strain has been adapted to ethanol using the following *pied-de-cuve* technique protocol, gently provided by a professional in the field.

I) Re-hydration phase

in 300 ml of deionized water at 50°C 6 grams of Go-Ferm® Protect (Lallemand Wine; sterols and minerals rich yeast autolysated) dehydrated active yeast protector have been dissolved, together with 15 grams of sucrose. When water temperature decreased to 40°C, as reported by the Active Dry Yeast (ADY) manufacturer's specifications, 12 grams of ADY have been added and dissolved, for a final dose of 10 g/hl. After 10 minutes stop, the mixture has been gently stirred and let stay 10 minutes long again, in order to ensure a complete cells rehydration.

II) First acclimation phase

While rehydration phase was being running, 750 ml of base wine have been homogenized with 450 ml of deionized water, and then heat to 30°C. Then, in this solution have been added and dissolved 225 g of sucrose, 0.471 g DAP (Fischer Scientific)[1] ($(NH_4)_2HPO_4$) and 4.604 g of yeast extract (Yeast Extract Powder | OXOID™ - Thermo Fischer Scientific). This nutrients-enriched solution of base wine and water has been added to rehydrated yeasts for a final ethanol concentration of 5% vol. and incubated at 21°C for 16 hours. Final concentrations of each component are reported in **Table 4.2**.

III) Second acclimation phase

After 16 hours of incubation time, in 1.5 L of base wine have been added and dissolved: 150 g of sucrose, 0.707 g of DAP and 6.923 g of yeast extract. The enriched solution has been added to the yeast culture after 10 minutes stirring, and

[1] The yeast extract powder has been chosen to substitute the industrial fermentation nutrients suppliers to improve experimental reproducibility. It is a wide used ingredient for microbial growth media preparation. The yeast extract is made from yeast cells suspensions, extracting all the hydro soluble cell constituents after the cell walls crushing by thermal and pressure treatment. After centrifugation and physical separation of the cell wall debris and hydrophobic constituents, the so-enriched supernatant is recovered, sterilized by autoclaving and dehydrated, obtaining a ready-to-use powdery preparation (Zarei et al. 2016).

then incubated at 21°C for 24 hours. Final concentrations of each component are reported in **Table 4.2**.

IV) Third acclimation phase

After 24 hours of incubation time, in 3 L of base wine have been added and dissolved: 300 g of sucrose, 1.414 g of DAP and 13.846 g of yeast extract. The enriched solution has been added to the yeast culture after 10 minutes stirring, and then incubated at 21°C for 24 hours. Final concentrations of each component are reported in **Table 4.2**.

IV) Fourth acclimation phase

After 24 hours of incubation time, in 6 L of base wine have been added and dissolved: 600 g of sucrose, 2.828 g of DAP and 27.692 g of yeast extract. The enriched solution has been added to the yeast culture after 10 minutes stirring, and then incubated at 21°C for 24 hours. Final concentrations of each component are reported in **Table 4.2**.

At the end of the *pied de cuve* preparation procedure 12 L of adapted yeast pre-culture have been obtained, corresponding to the 10% of the final volume of wine to be submitted to the second fermentation for each yeast strain.

Procedure Phase	Sucrose (g/L)	Inorganic YAN (mg/L) DAP supplied	Organic YAN (mg/L) yeast extract supplied
1[a] acclimation	150	67	133
2[a] acclimation	50	50	100
3[a] acclimation	50	50	100
4[a] acclimation	50	50	100

Table 4.2

Nutrients concentration added to each step of the *pied de cuve* preparation.

5.2.4 Second fermentation trial

For the present work, micro-scaled equipment for sparkling wine production has been used, at the CIRVE department (Centro Interdipartimentale per la Ricerca in Viticoltura ed Enologia) of the University of Padova, sited in Conegliano (Treviso, Italy).

The equipment is provided with eight independent pressure-tight tanks (autoclaves) of 30 L capacity each. The temperature settings and the internal pressure and temperature monitoring are controlled by a dedicated software PLC based (Elettra 80 S.r.l. – Mareno di Piave, TV). Thanks to the data sets recorder by this software has been possible obtaining the pressure increase kinetic for each autoclave.

The trial has been performed setting two fermentation conditions for each of the two yeast strains, in biological duplicate. The two experimental conditions were set in order to obtain two Prosecco sparkling wine typologies: the Dry version (with residual sugars between 27 and 32 g/L) and the Brut version (with 12 g/L or less of residual sugars).

Each autoclave has been filled with 24.3 L of base wine, differently sweetened on the basis of the experimental thesis (**Table 4.3**). In the autoclaves dedicated to the Dry version 1050 g of sucrose have been added, and in the autoclaves dedicated to the Brut version 600 g of sucrose have been added.

To avoid the risk of nitrogen starvation in all autoclaves have been added 20 g/hl of yeast extract (correspondent to a YAN supply of 9 mg/L of assimilable amino acidic nitrogen) and 5 g/hl of DAP (correspondent to YAN supply of 11 mg/L of assimilable ammonia nitrogen), for a total YAN supply of 20 mg/l of total YAN.

When all nutrients were added and the base temperature reached the PLC-set value of 17°C, 2.7 L of the prepared *pied de cuve* has been added to the correspondent autoclaves, correspondent to the 10% of the final volume.

Autoclave	Yeast strain	Version	Added sucrose (g/L)	Added yeast extract (g/hL)	Added DAP (g/hL)
A1	Lalvin EC1118	Dry	35	20	5
A2	Lalvin EC1118	Dry	35	20	5
A3	Lalvin EC1118	Brut	20	20	5
A4	Lalvin EC1118	Brut	20	20	5
A5	Lalvin QA23	Dry	35	20	5
A6	Lalvin QA23	Dry	35	20	5
A7	Lalvin QA23	Brut	20	20	5
A8	Lalvin QA23	Brut	20	20	5

Table 4.3

Experimental plan.

The second fermentation has been performed at 17°C, regularly stirring the fermenting wines using the internal autoclaves equipment for ten minutes, two times a day. Chilling at the end of the fermentation has been forced mimicking the temperature decrease of industrial-scaled equipment until the reaching of 6°C for the last samplings (0.5°C each 15 minutes, for a total of 6 hours long chilling), then the wines have been rapidly cooled at -3°C for tartaric stabilization, as industrial protocols requires.

Finale pressure value was set at 4.7 bar, corresponding to 5 bar measured at 20°C.

5.2.5 Cells counts and viability analysis

Cells were sampled for the total counts and for viability evaluation. Sampling points were: at the end of each step of the acclimation protocol for the *pied de cuve* preparation; immediately after the *pied de cuve* inoculation in the autoclaves (Inoculum); when the 0.5 bars of internal pressure value has been reached for the first time per each autoclave (Degassing); at the end of the second fermentation (FF17), corresponding to the reaching of 5 bars of internal pressure (Dry version) or at around 12 g/L of residual sugars (Brut version). Cells viability has also been evaluated during the wine chilling (when the wine temperature reached 6°C (FF6).

Cells total counts and viability evaluation have been performed by flow cytometry (CyFlow® SL – Partec, Japan), using a colouring kit to differentiate viable cells from dead or damaged cells: the kit absorption by the yeast cells membrane depends on theur physiological *status* and, consequently, on their viability (Yeast Control – Viability; Sysmex Partec GmbH, Germany). The kit is composed by two reagents, each containing different molecules that are differentially absorbed by the cell. The flow cytometer lets 200 µl of treated cells sample pass through a chamber, where it is lighted by a blue laser ray at λ 488_{nm}.. The instrument also records the scattered light along the laser ray direction (forward scatter – FSC) and the scattered light across the laser ray direction (side scattered – SSC). Here, the excited molecules of the kit reagents emit green or red fluorescence if the cells are alive or dead, respectively, and the instrument records the green (FL1) or red (FL2) fluorescence emissions. Flow cytometry data have been analysed using the FloMax® (Partec, Japan) software.

5.2.6 Chemical analysis

The samplings for chemical and cells analysis have been performed at the T0 (immediately after the *pied de cuve* inoculation: Inoculum); when the 0.5 bars of internal pressure value has been reached for the first time per each autoclave (Degassing); at the end of the second fermentation (FF17); during the wine chilling (when the wine temperature reached 6°C (FF6). Each sample has been rapidly centrifuged, filtered and immediately analysed for acetaldehyde quantification or freeze at -20°C.

Samples have been analysed for acetaldehyde, ammonia nitrogen, amino nitrogen, residual sugars, ethanol, glycerol, acetic acid and succinic acid.

In the acetaldehyde, ammonia nitrogen, amino nitrogen analysis the automatic Hyperlab Wine Analyzer (Steroglass S.r.l. – Italy, Perugia) instrument has been used.

All the other compounds have been determined by HPLC (High-performance liquid chromatography) analysis. Ten µl for each sample has been run in triplicate. For components separation Waters 1525 binary HPLC pump provided with Aminex HPX_87H 300mm X 7.8mm column has been used. Detectors were: Waters 2487 Dual band Detector, set at 210_{nm} wavelength and Waters 2414 Refractive Index Detector.

5.2.7 Statistical analysis

The student's *t* test or the one-way analysis of variance (ANOVA) followed by the Tukey *post-hoc* test has been performed using the XLSTAT 7.5.2 (Addinsoft SARL, Paris, France) software. The analysis has been carried out comparing the averages of three independent replicates, and differences were considered statistically significant for *p* value lower than 0.05.

5.3 Results

5.3.1 Cellular population and viability in the *pied de cuve*

The aim of the *pied de cuve* preparation practice is to obtain cells populations well-adapted to the base wine, to be used in the second fermentation for sparkling wine production. Adopting this practise winemakers obtain the shortening of cells population *lag* phase after the inoculum and the reduction of the stuck fermentation risk (Benucci *et* al., 2016).

The yeasts adaptation to the base wine was carried out according to a protocol commonly used for the production of the Prosecco sparkling wine with the Martinotti's method. This protocol includes five adaptation phases: a first rehydration phase (A); an initial doubling of volume with base wine (B); three successive volume doublings at regular intervals of 24 hours of distance each (C, D, E). At each doubling in volume, sucrose and nitrogenous nutrients were added, in order to ensure exceeding nutrition to the cells, as Prosecco winemakers usually do in their *pied de cuve* preparation worksheet. At the end of each adaptation phase, samples of *pied de cuve* were taken for each yeast strain, in order to analyse the total number of cells and the percentages of alive and dead cells (**Figure 4.3**).

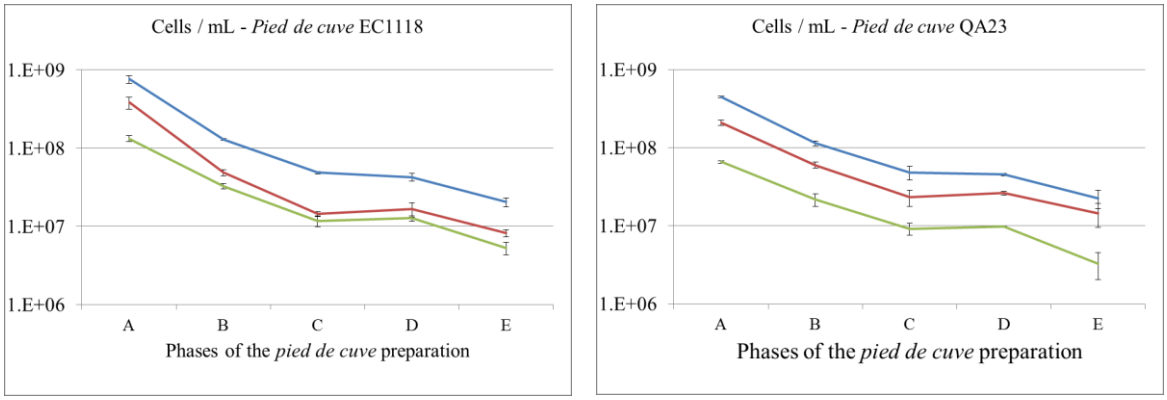


Figure 4.3

Yeast cell population dynamic of the two yeast strains during the ethanol adaptation (*pied de cuve*). A) End of rehydration. B) End of the first acclimation. C) End of the second acclimation. D) End of the third acclimation. E) End of the fourth acclimation. (■) Total Cells/mL (■) Alive Cells/mL (■) Dead Cells/mL.

During the adaptation, a decrease in the concentration of the population was observed, except during the transition between the phase C and the phase D in which the concentration remained almost constant, for both the yeasts. Theese results are probably due to the subsequential dilutions of cell cultures, during which the variations in cell concentration depend on the volume doubling and cell growth rates. The observed growth rates are lower than those observed in the preparation of the yeasts for the grape musts inoculation: this is highly probably due to the profound differences in the medium to which cells need to be adapted. In particular, the most difficult element to manage in the yeast adaptation is the ethanol concentration, which reduces the yeasts growth and viability (Garofalo *et* al., 2016).

At the end of the adaptation process the number of viable cells per mL was $8.2 \cdot 10^6$ for EC1118 and $1.4 \cdot 10^7$ for QA23 (**Figure 4.3**).

Although QA23 concluded the adaptation phase with a number of viable cells on average higher than that of EC1118, this difference was not significant (Student's t test $p = 0.09$).

5.3.2 Acetaldehyde production in the *pied de cuve*

Acetaldehyde is an intermediate product of the alcoholic fermentation, and for this reason it is normally contained in wines. The concentration of this compound is very variable, usually from 10 to 300 mg/L, and is a typical character of the yeasts belonging to the species *Saccharomyces cerevisiae* (Vincenzini *et* al., 2005). The presence of acetaldehyde also depends on other factors, among which the degree of oxidation of the wine: the dosage of its content is used just as an indicator of the degree of oxidation. Wines that contain more than 500 mg/L are usually considered non-tradable. White wines generally have an average value of 80 mg/L.

Acetaldehyde is a very volatile and reactive compound, which tends to react and bond with numerous substances present in wines, such as amino acids, proteins, and especially with sulphur dioxide, with which it establishes irreversible bonds. The irreversible bond with sulphur dioxide generates combined acetaldehyde, and consequently there is a strong reduction of the antiseptic, antioxidant and anti-fermentative effect of sulphur dioxide (Nadai *et* al., 2015): for this reason the winemakers spend lots of efforts in minimize the production of acetaldehyde, by both yeast fermentation control and simultaneously adopting all the possible precautions for minimizing the wine oxidation.

If low concentrations of acetaldehyde contribute to the aromatic complexity of the wine, at high concentrations it gives instead a pungent aroma of senescent fruit, leading to the typical "oxidized" descriptor and therefore compromising the olfactory finesse of the wine, by flattening the olfactory *bouquet*.

It is also a biologically toxic compound, with a potentially mutagenic effect due to its ability to bind nucleic acids (Wang et al., 2000).

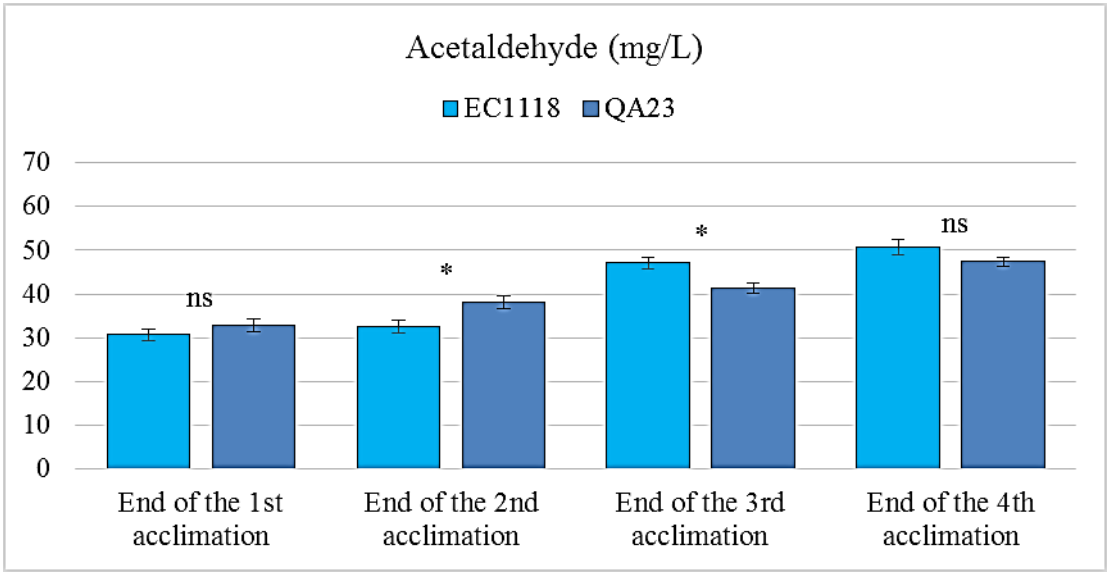


Figure 4.4

Acetaldehyde production during the adaptation phases (*pied de cuve*) of the two yeast strains. (*) significant difference by Student's *t* test. (ns) non-significant difference at *alpha* = 0.05.

During the adaptation phases, a progressive increase in the amount of acetaldehyde in the medium was observed. It was significantly different between the two yeast strains only at the end of the second and third acclimatization, while at the end of the fourth acclimatization, despite the EC1118 higher found acetaldehyde, the difference was not significant (**Figure 4.4**).

At the end of the preparation of the *pied de cuve*, therefore, in the cultures of the two yeasts similar amounts of acetaldehyde have been found: this can means that the oxidation effect of the base wine, which suffered the oxygenation during the cell cultures

volume doubling caused by aeration, had a greater impact than that of the yeast strain in determining the acetaldehyde content at the end of the process.

5.3.3 Nitrogen consumption in the *pied de cuve*

The consumption of amino and ammonia nitrogen was monitored at the end of each step of the preparation of the *pied de cuve*, to evaluate the nitrogen demand of the two yeast strains for the same adaptation conditions. Nitrogenous nutrition at this stage is a parameter to which oenologists pay high attention, in order to avoid in the yeasts a nutritional deficiency that could subsequently affect the production of biomass, the second fermentation kinetics and the production of undesirable sulphur compounds. Furthermore, nitrogen nutrition influences the redox balance of the cell, which in turn affects the production of ethanol and other metabolites such as glycerol, acetic acid and succinic acid. In particular, it has been observed that glutamic acid increases the production of succinic acid, acetic acid, *aplha*-keto glutarate acid and fumaric acid (Albers *et* al., 1996, Camarasa *et* al., 2003).

Although the role of nitrogen is a key factor for the overall balance of the cell, not all sources of nitrogen have the same relevance to yeast. The yeasts in fact show strong preferences for the ammonium ion and some amino acids, and this behaviour has different molecular bases. In *S. cerevisiae* this mechanism is known under the name of Nitrogen Catabolite Repression (NCR). It allows the cell to detect the presence of the most preferred nitrogen sources, and to exploit them first, in this way limiting the use of the so-called "alternative" nitrogen sources. The detection of the most interesting sources of nitrogen for yeast generates a chain of molecular events that leads to gene expression regulation. In particular, it leads to the activation of the transport and the metabolism of the preferred sources, and at the same time a gene repression against the transport and the

metabolism of sources less useful for yeast. When the best sources of nitrogen (ammonium, glutamic acid and asparagine) are depleted, the repression of transport and metabolism of less valuable sources is less important, and at this time the yeast also begins to use alternative sources (arginine, glutamate, alanine, etc.) (Vendramini *et* al., 2017; Mas *et* al., 2014).

Observing the data concerning the nitrogen content at the end of each adaptation phase, it is possible to observe how the consumption of amino nitrogen was relevant in both yeast strains only at the end of the first adaptation phase. On the contrary, the ammonium ion, the source of nitrogen that *S. cerevisiae* uses preferentially, showed a different consumption and it is exhausted both at the end of the first phase and at the end of the second phase of adaptation, and is still strongly used also at the end of the third phase (**Figure 4.5**).

In particular, at the end of the third phase EC1118 uses significantly more ammonia nitrogen than QA23, while the two strains show no significant differences with regard to amino nitrogen. At the end of the fourth phase instead, and therefore at the end of the adaptation, EC1118 has used significantly more amino nitrogen compared to QA23, and on the contrary a lower use of ammonia nitrogen is observed.

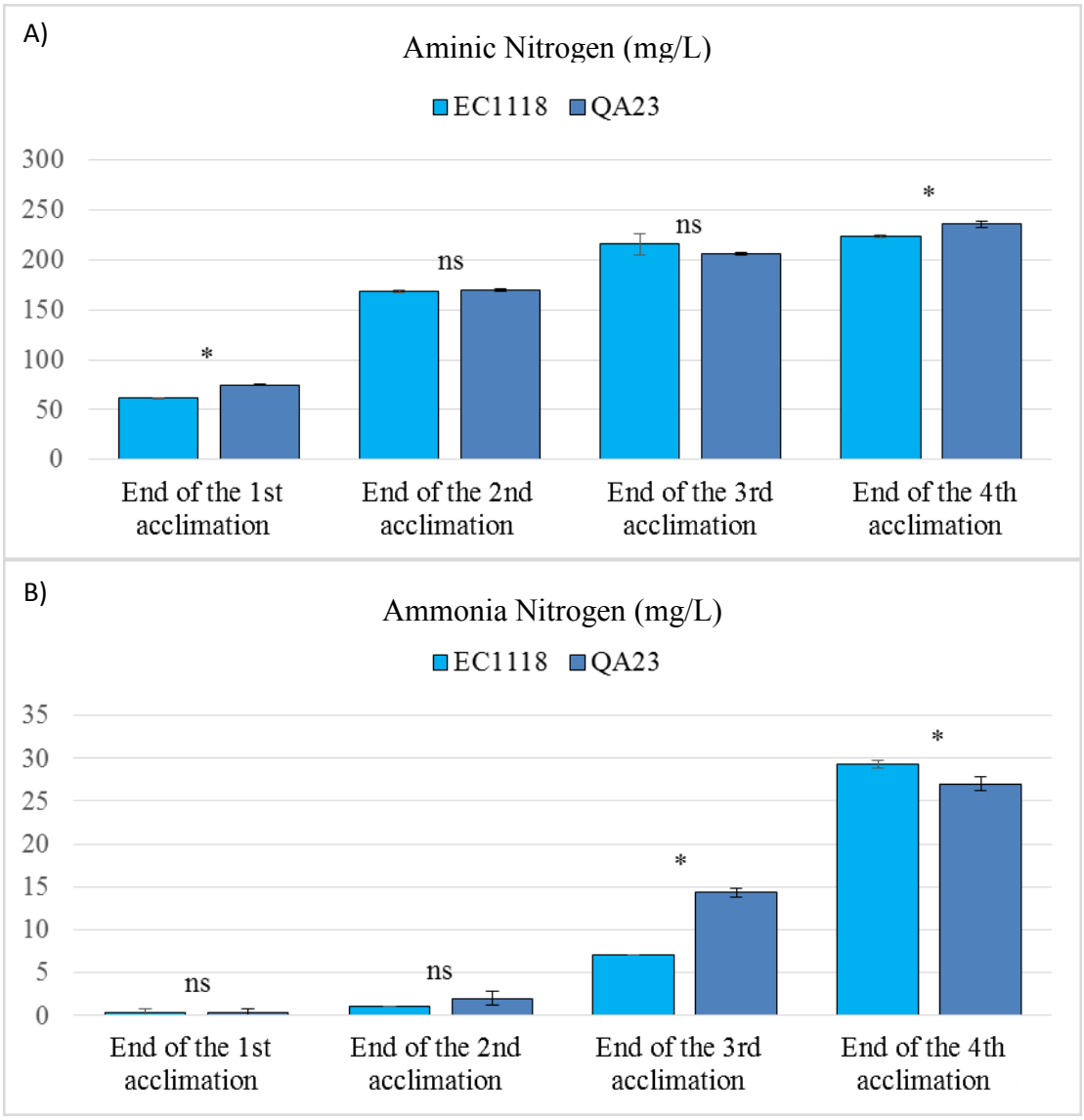


Figure 4.5

Amino nitrogen (A) and ammonia nitrogen (B) residues during the ethanol adaptation phases (*pied de cuve*) of the two yeast strains. (*) significant difference by the Student's *t* test. (ns) non-significant difference at *alpha* = 0.05.

These results confirm the clear preference of the *S. cerevisiae* yeasts for ammonia nitrogen also during the adaptation phase to the second fermentation, while the amino nitrogen seems to have a more limited importance for yeast nutrition in this process.

5.3.5 Second fermentation curves

To monitor the second fermentation process, conducted at a constant temperature of 17°C, the pressure data were recorded, for each strain in the two conditions (Dry and Brut), thanks to the experimental PLC management software (**Figure 4.6**).

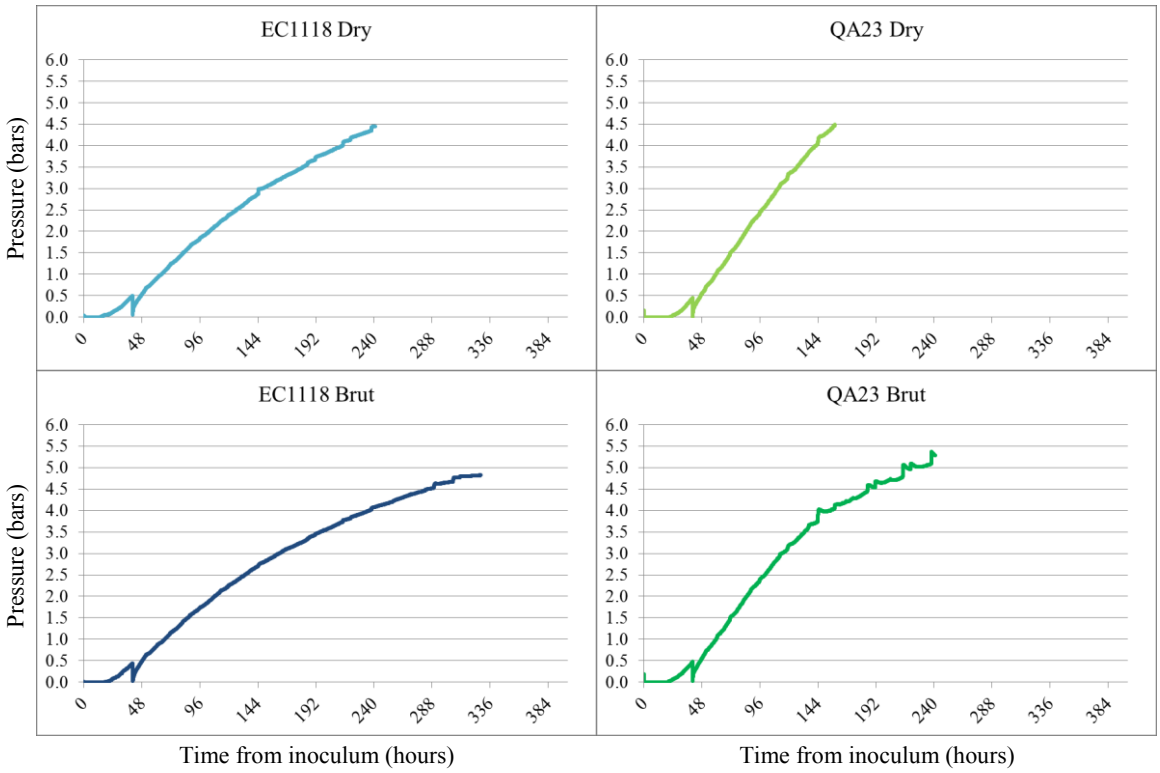


Figure 4.6

Pressure increments during the second fermentation.

The *lag* phase lasted 13 hours in the case of EC1118 and 20 hours in the case of QA23. This *lag* phase does not correspond to the cell growth *lag* phase, but represents the start of recordable pressure increasing in the pressure-tight tanks. However, this parameter indicated that EC1118 exhibited a faster fermentation start with respect to QA23. At the end of this phase, the QA23 strain showed a much stronger pressure increase compared to EC1118 in both conditions. In fact, the pressure of 0.5 bars, at which the autoclaves were

degassed (as indicated by the adopted sparkling wine production protocol), was reached simultaneously by the two yeasts, 46 hours after inoculation. This trend was maintained throughout the fermentation phase: QA23 was faster than EC1118 in completing fermentation in both the Dry and the Brut versions. The fermentation of EC1118 Dry, QA23 Dry, EC1118 Brut and QA23 Brut were in fact completed in 10, 14, 8 and 10 days, respectively.

5.3.6 Cell growth and viability during the second fermentation

The number of inoculated live cells is of fundamental importance for the success of the sparkling wine production process. The ability of the cell population to quickly start the fermentation process depends on this parameter.

At the time of inoculation, the number of live cells was $1 \cdot 10^6$ cells/mL for both yeasts. At the end of fermentation, the QA23 strain increased the number of cells to a greater extent, with a viable population reaching $1 \cdot 10^7$ cells/mL, while the population of the EC1118 strain grew much less, remaining close to $1 \cdot 10^6$ cells/mL in the case of the Brut version and reaching $1.5 \cdot 10^6$ cells/mL in the Dry version (**Figure 4.7**).

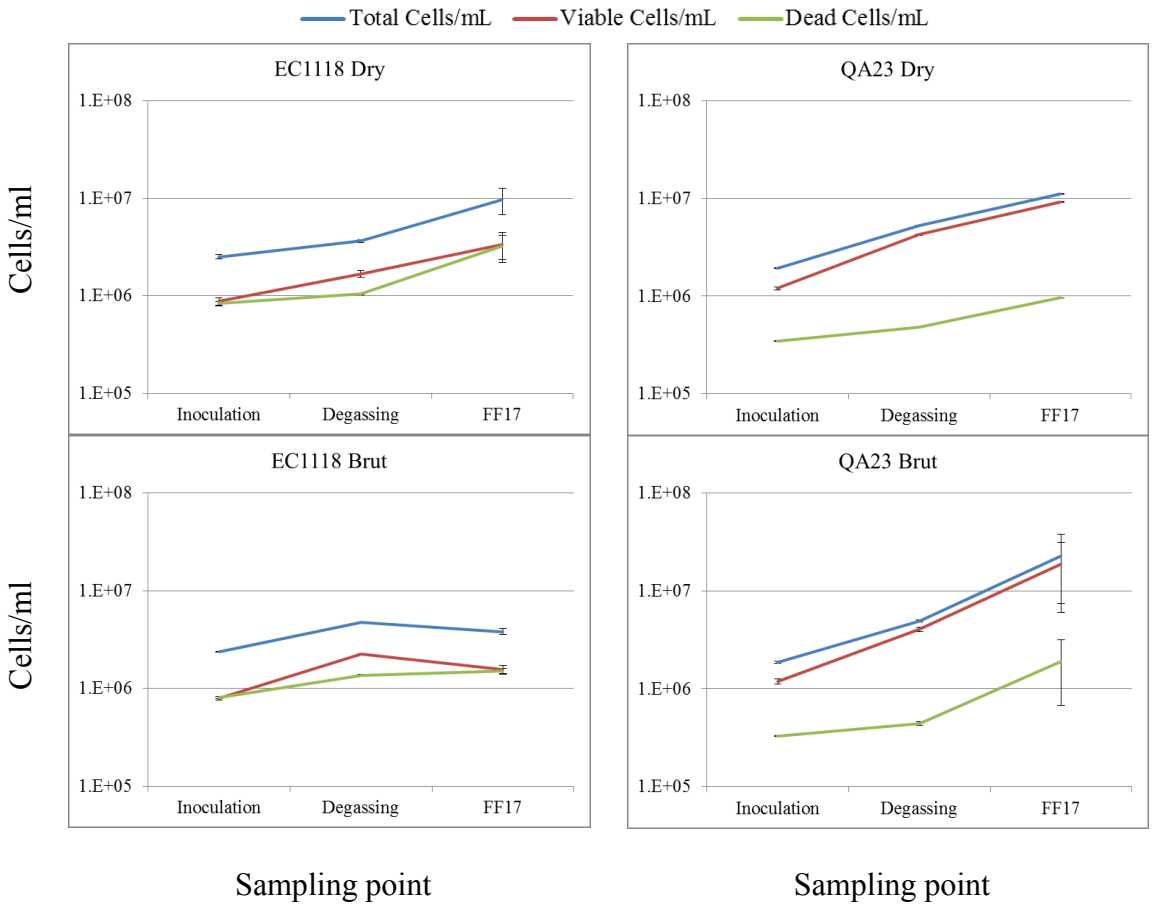


Figure 4.7

Strains yeast cells populations dynamic during the second fermentation.

(FF17) End of the second fermentation.

In both the case of the Dry version and the Brut version, for the EC1118 yeast after an initial increase in the number of cells, the number of live cells at the end of fermentation was equivalent to that of dead cells (**Figure 4.7**). This did not occur for the yeast QA23, which instead carried out all the phases of the second fermentation with a clear prevalence of live cells on the dead ones, demonstrating greater viability in the second fermentation conditions compared to EC1118 in all the samplings that were carried out: Inoculum, Degassing, End of the second fermentation (FF17), End the second

fermentation during cooling (when the temperature of the wine has reached 6°C – FF6) and after a two-hour stop at 6°C (FF6stop) (**Table 4.4**).

Table 4.4

Viable/Total cells/mL ratio (%) at different sampling points: Inoculum, Degassing, End of the second fermentation (FF17), End the second fermentation during cooling (when the temperature of the wine has reached 6°C – FF6) and after a two-hour stop at 6°C (FF6stop).

		Viable cells / Total cells (%)				
Yeast strain	Version	Inoculum	Degassing	FF17	FF6	FF6stop
EC1118	Dry	34.8	45.9	34.2	34.8	34.8
EC1118	Brut	33.8	47.4	41.2	41.4	39.5
QA23	Dry	62.4	81.2	83.0	83.9	84.1
QA23	Brut	64.1	81.9	82.9	82.5	82.4

From the percentages shown in **Table 4.4** appears that the yeast QA23 is clearly more viable, and therefore probably better adapted to the environment, with respect to the EC1118 yeast. The ratio of live cells / total cells in QA23, in fact, never dropped below 60%, and remained above 80% until the end of the fermentation process. EC1118 instead showed a ratio of live cells on total cells close to 34% at the time of inoculation, a value which then rose up to 46-47% at the time at the first reaching of 0.5 bars of internal pressure (Degassing) and then again at 34% at end of fermentation (FF17) and subsequent samples (FF6 and FF6stop).

5.3.7 Chemical analysis

Results from HPLC analysis of the collected samples are reported in **Table 4.5**.

The initial sugar contents are the sum of the sugars content of the base wine, the residual sugar content in the adapted yeasts inoculums and the base wine sweetening addition.

Table 4.5

HPLC analysis results.

Data are the average of three independent instrumental replicates for two biological replicates ± standard deviations.

Sampling point	Yeast Strain	Wine version	Glucose (g/L)	Fructose (g/L)	Ethanol (%)	Glycerol (g/L)	Acetic acid (g/L)	Succinic acid (g/L)
Inoculum	EC1118	Dry	28.76±0.40	28.59±0.50	10.45±0.07	5.29±0.19	0.55±0.01	2.11±0.07
		Brut	21.05±0.12	21.68±0.05	10.34±0.07	5.40±0.02	0.42±0.15	1.82±0.02
	QA23	Dry	26.92±0.23	26.58±0.27	10.42±0.11	5.34±0.01	0.50±0.07	1.98±0.02
		Brut	21.06±0.12	21.52±0.13	10.34±0.06	5.33±0.05	0.81±0.12	1.80±0.05
Degassing	EC1118	Dry	25.72±0.21	27.84±0.48	10.88±0.07	5.70±0.03	0.90±0.02	2.07±0.01
		Brut	17.60±0.43	20.27±0.37	10.77±0.17	5.62±0.13	0.86±0.01	1.76±0.03
	QA23	Dry	24.03±0.23	25.94±0.16	10.91±0.01	5.64±0.13	0.88±0.04	1.99±0.02
		Brut	16.40±0.34	18.86±0.22	10.80±0.10	5.57±0.03	0.85±0.01	1.72±0.02
FF17	EC1118	Dry	14.08±0.08	16.25±0.13	11.65±0.16	5.92±0.04	0.87±0.01	1.87±0.03
		Brut	4.55±0.42	7.62±0.13	11.07±0.12	4.72±0.06	0.89±0.02	1.00±0.12
	QA23	Dry	12.91±0.22	13.37±0.13	11.72±0.06	5.75±0.02	0.86±0.02	1.82±0.03
		Brut	1.75±0.15	3.44±0.20	11.11±0.16	4.74±0.02	0.96±0.13	0.85±0.01

The present experiment was conducted using the same base wine, characterized by an alcohol content of 10.15±0.02 % vol. The results of the HPLC analyses reveal that in the case of the Dry and Brut versions wines similar alcohol contents were reached, around 11.70 and 11.1 % vol., respectively.

Glucose and fructose are the fermentable sugars which are transformed by yeast during alcoholic fermentation. During the second fermentation in autoclave, unlike the primary

alcoholic fermentation of the must, the initial concentration of sugars is much lower, so much so that the yeast is much less affected by the osmotic stress.

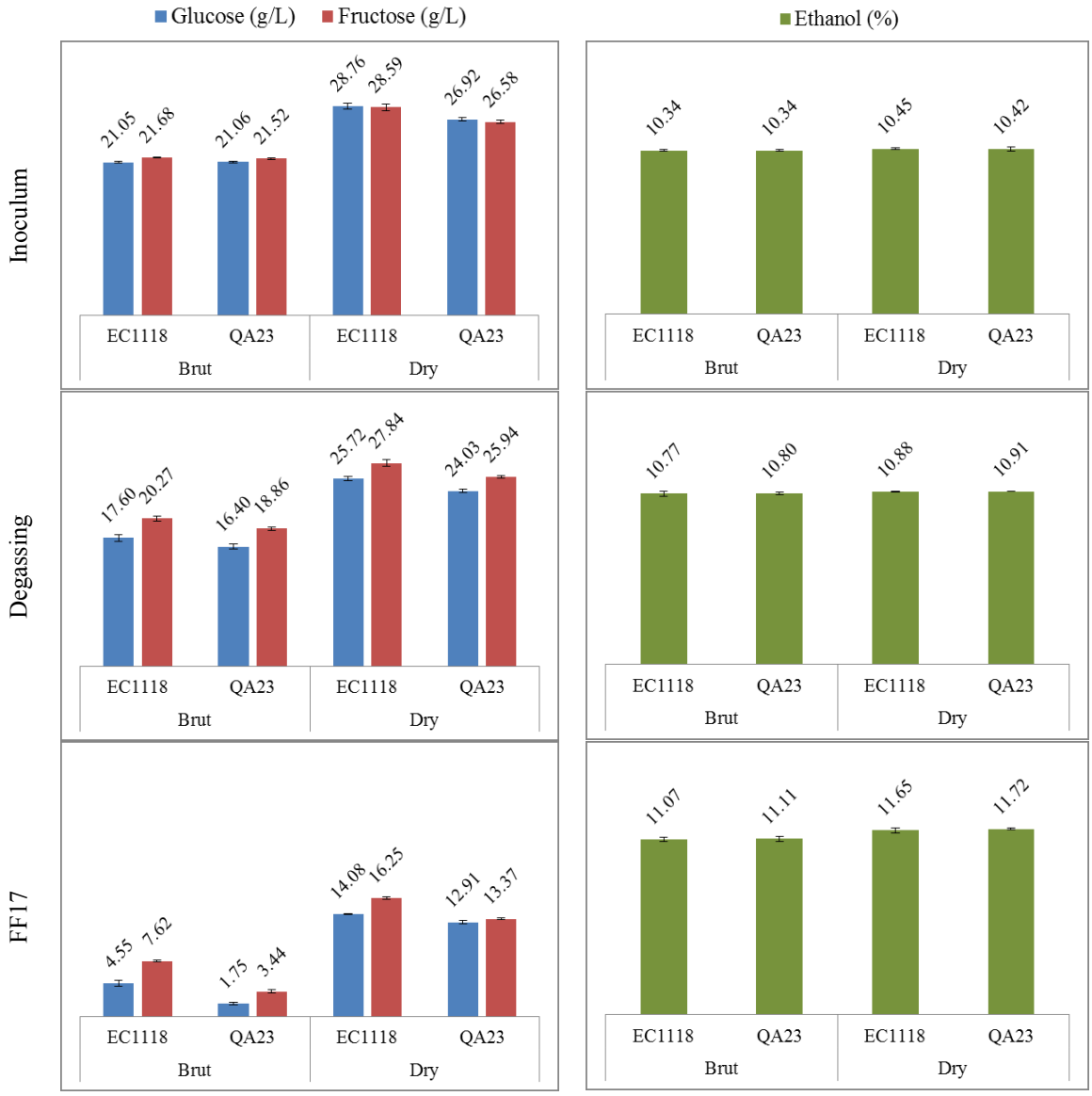


Figure 4.8

Glucose and fructose consumption and ethanol increase during the second fermentation.

The EC1118 Dry version fermentation started with about 2 g/L of glucose and of fructose more than QA23. However, when the final pressure of 5 bars were reached and the

fermentations were stopped (FF17) the two yeast strains in the Dry wine version left similar same amount of sugars (**Table 4.5 and Figure 4.8**).

Despite previous observations, the two yeast strains slightly differ for the real sugars consumption and for the ethanol yield, as is shown in **Figure 4.9**.

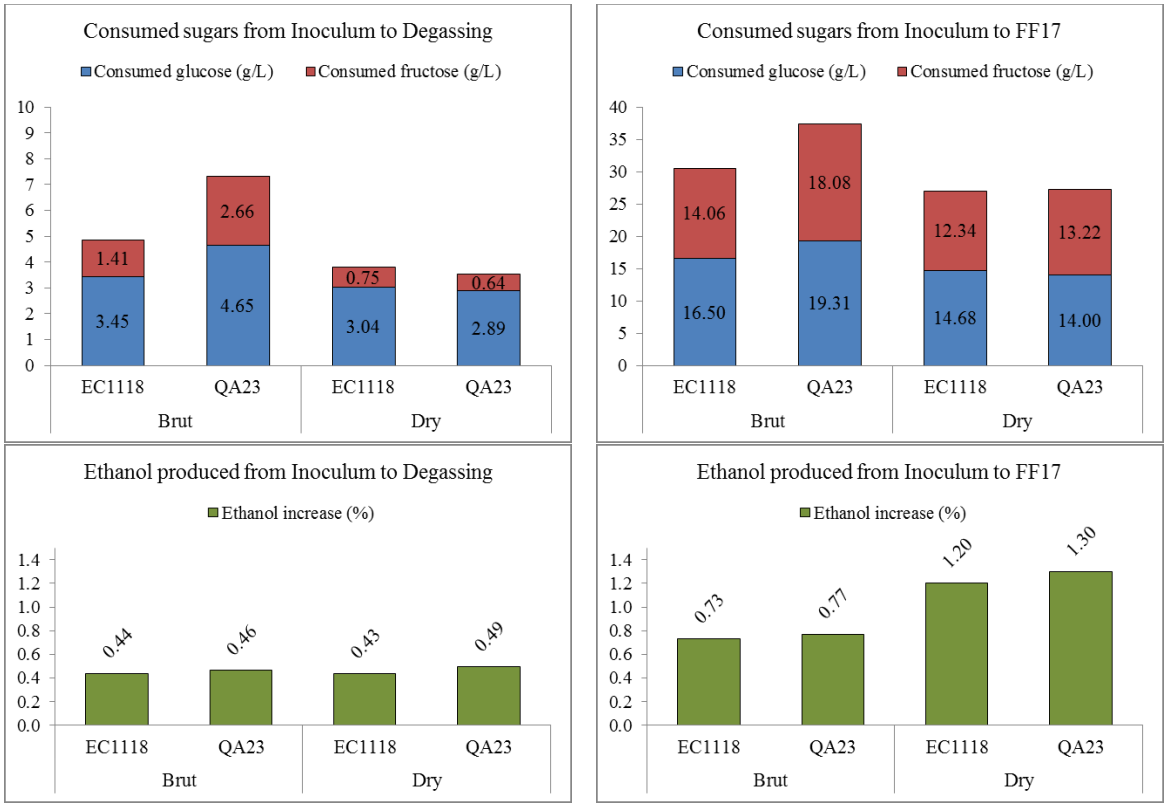


Figure 4.9

Consumed sugars (g/L) and produced ethanol (% vol.) by the two yeast strains during the second fermentation.

5.3.8 Nitrogen utilization during the second fermentation

During the preparation for the second fermentation, winemakers pay great attention to the nitrogenous yeast nutrition, for two essential reasons: the first concerns the purpose of ensure an adequate nutrition to the yeasts, while the second, no less important, concerns the risk of microbiological instability deriving from the residual nitrogen at the end of fermentation. The technological goal is, therefore, to guarantee the necessary nourishment to the yeast to have a good second fermentation performance, without leaving any residual nitrogen in the final wine. In the present work such a technological parameter has not been taken into account, as the main aim was to ensure non-limiting nitrogen nutrition.

Differently from EC1118, QA23 consumed amount amino-nitrogen, even if the residual amino nitrogen is very high in both cases (**Figure 4.10**), showing how during this production process the nitrogen needs of the yeasts cells seem to be much more contained than happens in the fermentation of grape juices, and this is consistent also with the differences in the sugars content of the two matrixes: relatively low in base wines and very high in grape musts.

In the case of the ammonia nitrogen, on the other hand, the results were clearly different from those related to amino nitrogen. From the inoculation onwards, in fact, both yeasts quickly consumed this nitrogenous source, leaving the end of the second fermentation only traces of it. Only at the end of the second fermentation has been observing a difference in the ammonium ion consumption between the two yeast strains: at tis sampling point QA23 left higher amounts of ammonia nitrogen than EC1118, in particular the QA23 Dry wine version (**Figures 4.10 and 4.11**).

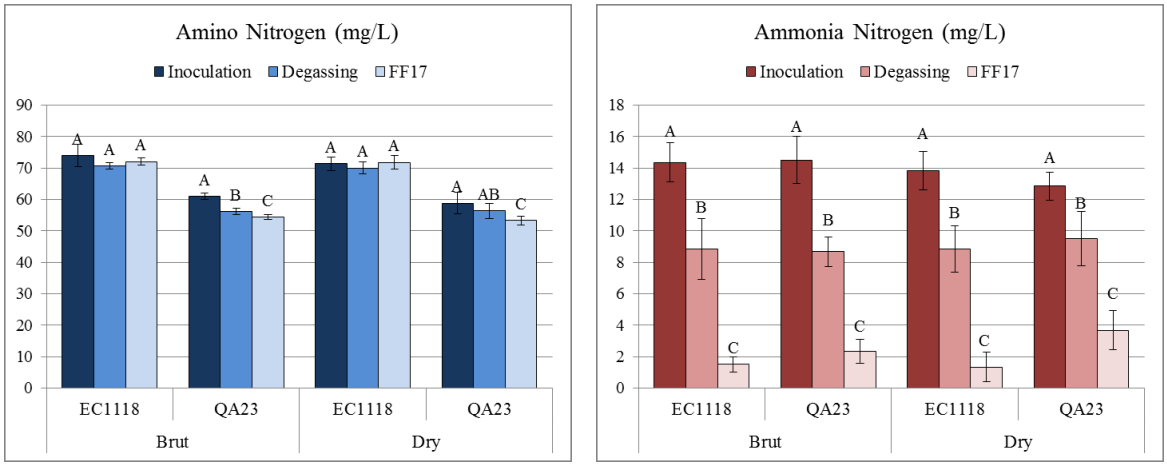


Figure 4.10

Amino nitrogen and ammonia nitrogen at the different sampling points: Inoculum, Degassing and at the end of the second fermentation (FF17). Letters indicate significant differences between the means at different sampling points, for each strain and condition, using one-way ANOVA followed by the Tuckey *post-hoc* test (α=0.05).

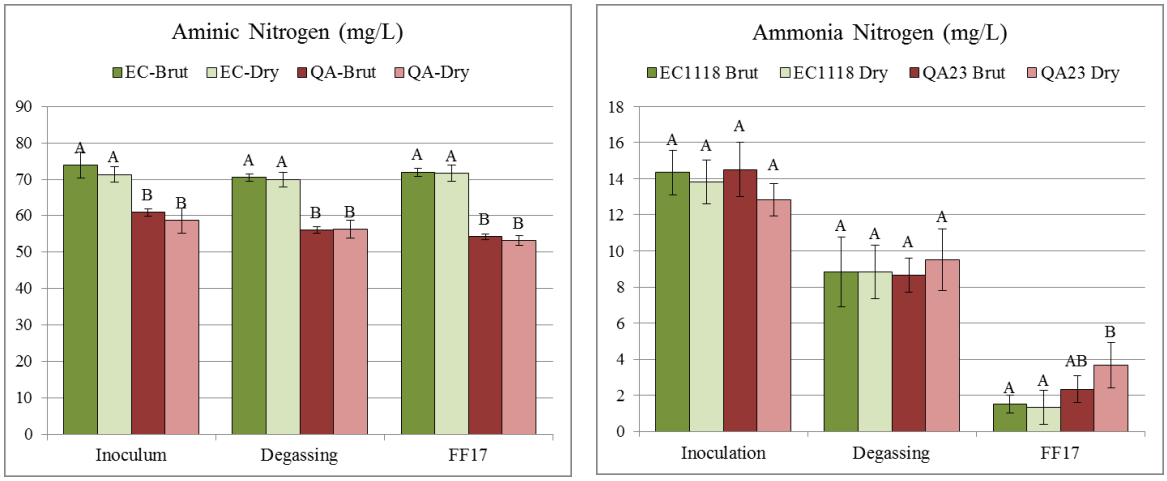


Figure 4.11

Amino nitrogen and ammonia nitrogen at the different sampling points: Inoculum, Degassing and at the end of the second fermentation (FF17). Letters indicate significant differences between the means of each strain and condition, at the different sampling points, using one-way ANOVA followed by the Tuckey *post-hoc* test (α=0.05).

5.3.9 Acetaldehyde production during the second fermentation

The amount of acetaldehyde at the end of the second fermentation is almost equal to the inoculum value in both strains (**Figure 4.12**). The acetaldehyde increase at the degassing time point is probably due to the fermentation process itself. In fact, besides the alcoholic fermentation where the pyruvate is decarboxylated to acetaldehyde, which is then reduced to ethanol, it represents an intermediate of several metabolic pathways: for example, the fatty acids and phospholipids synthesis and the cell redox balancing under fermentation condition, playing a role in the NADH oxidation in the mitochondria.

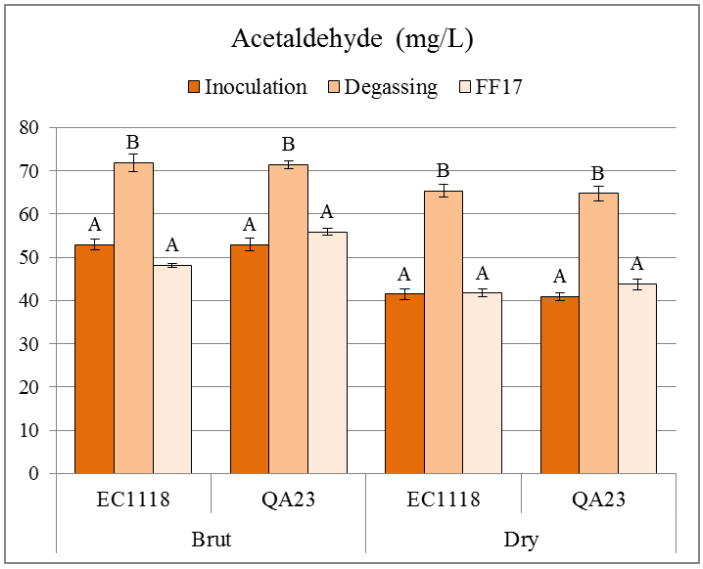


Figure 4.12

Acetaldehyde content at the different sampling points: Inoculum, Degassing and at the end of the second fermentation (FF17). Letters indicate significant differences between the means at different group, using one-way ANOVA followed by the Tuckey *post-hoc* test (α=0.05).

5.3.10 Yeasts cells pellets recovery for RNA extraction

For each strain and wine version cells were collected for transcriptomic analysis at two sampling points: when the internal pressure reached 5 bars and during the wine chilling, when the temperature reached 6°C. Each autoclave represents a separate biological replicate, and in all the procedures regarding cells pellet recovery, RNA extraction, concentration determination and quality check, has been separately processed. The sampling from autoclaves has been performed taking 100 ml specimen of sparkling wine, after 10 minutes of stirring using the autoclave equipment. After sampling, cells were rapidly centrifuged to remove the growth media, washed with water and the pellet was immediately frozen by immersion in liquid nitrogen, and then stored at -80 °C.

5.3.11 RNA extraction

The total RNA has been extracted from each sample using the PureLink® RNA Mini Kit (Ambion) that combines cell disruption, phenol extraction and RNA purification. All water used in the following procedures was treated overnight with diethylpyrocarbonate (DEPC) 0.1% v/v or dimethylpyrocarbonate (DMPC) 0.1% v/v and autoclaved before use to remove RNase. All used plastic equipment was RNase-free guaranteed. Cells were resuspended in 400 µl TRIzol® Reagent (Invitrogen Life Technologies) and crushed by vortexing for 4 min with 300 µl glass beads. The total volume was adjusted to 1 ml with Trizol solution.

Extractions have been performed as explained by the protocol of the kit:

- 5 min incubation at room temperature, the:
- 200µl chloroform addition, in order to separate the aqueous and the organic phase with a brief agitation.

- 3 min incubation at room temperature
- centrifugation at 12000 × g for 15 min
- after centrifugation, recovering of about 600 µl of aqueous phase;
- RNA precipitation adding equal volume of 70% ethanol,
- tube vortexing to homogenize the components
- sample transferring to the spin cartridge
- centrifugation at 12000 × g for 15 sec at room temperature.
- discarding of the flow through
- addition of 700µl wash buffer I
- centrifugation at 12000 × g for 15 sec
- placing the spin cartridge into a new collection tube.
- Addition of 500 µl wash buffer II with ethanol
- centrifugation at 12000 × g for 15 sec
- discarding of the flow through (2 times)
- centrifugation 2 min at 12000 × g to dry the membrane with bound RNA.
- placing of the spin cartridge into a new DNase/RNase-free recovery tube
- addition of 35-50 µl RNase free water to the centre of the spin cartridge
- incubation for 1 min
- centrifugation at 12000 × g for 2 min to elute the RNA from the membrane into the recovery tube. The elution step was repeated twice.

An aliquot of the purified total RNA per each sample was taken for the quality and quantity measuring, and then the remaining samples were stored at -80°C.

5.3.12 RNA integrity evaluation

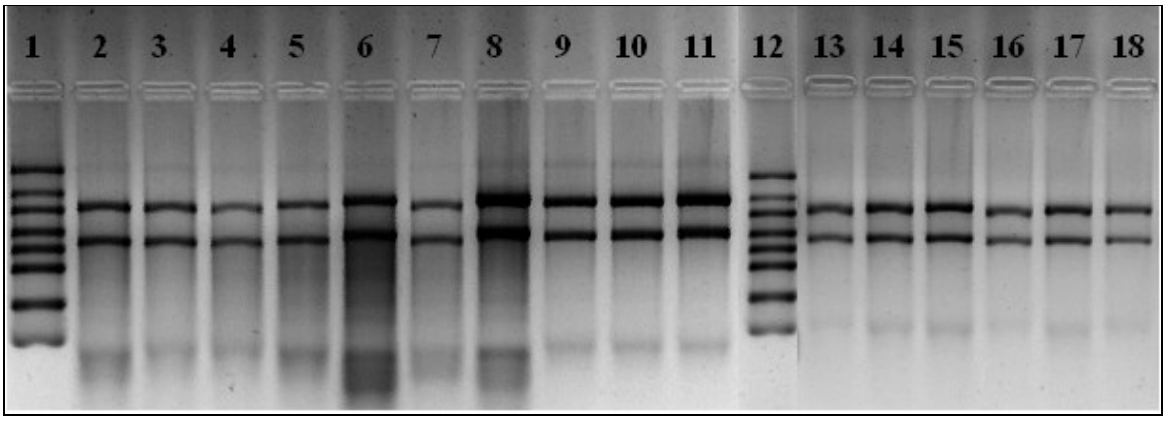
Samples containing 4-5 µg of RNA were resuspended in denaturing loading dye (form amide 30%, formaldehyde 10%, loading dye 15% (Fermentas International Inc.)

containing fycoll, bromophenol blue and xylenecianol blue), heated at 65°C for 10 minutes and then rapidly cooled in ice. RNA integrity was evaluated by electrophoresis on 1.5% agarose gels, under denaturing conditions (2% vol. formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7). For each well 2 µl of treated sample were loaded in the gel. An RNA ladder (0.3–7.4 kb, Fermentas International Inc.) was used as a molecular weight standard and bands were visualized by UV trans-illuminator. Digital images were acquired with an EDAS290 image capturing system (Kodak, Rochester, NY, USA). Electrophoresis was performed running the gels for 5 minutes at 50 V, until the samples exit the wells, then at 90 V for 30 minutes. All the procedure steps were performed using RNase/DNAse free reagents and instruments

The evaluation of the extracted RNA integrity by visualization on agarose gel after denaturing electrophoresis is based on the assumption that the intact total RNA musts hve sharp 28S (around 2kbp) and 18S (around 3.8 kbp) rRNA bands. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band. The RNA ladder run together with the samples allows sizing the bands, and represents an additional parameter to evaluate if the gel has been properly run (**Figure 4.13**).

Figure 4.13

RNA visualization on denaturing agarose gel after electrophoresis. For each enumerated gel position the RNA sample name is reported.



1. *RNA Ladder (0.3-7.4 kb)*				**10.** A1 – EC1118	Dry	FF6
2. A1 – EC1118	Dry	FF17		**11.** A2 – EC1118	Dry	FF6
3. A2 – EC1118	Dry	FF17		**12.** *RNA Ladder (0.3-7.4 kb)*		
4. A3 – EC1118	Brut	FF17		**13.** A3 – EC1118	Brut	FF6
5. A4 – EC1118	Brut	FF17		**14.** A4 – EC1118	Brut	FF6
6. A5 – QA23	Dry	FF17		**15.** A5 – QA23	Dry	FF6
7. A6 – QA23	Dry	FF17		**16.** A6 – QA23	Dry	FF6
8. A7 – QA23	Brut	FF17		**17.** A7 – QA23	Brut	FF6
9. A8 – QA23	Brut	FF17		**18.** A8 – QA23	Brut	FF6

5.3.13 RNA quantification and preparation for sequencing

During the extraction procedures the RNA could be contaminated by salt, proteins, solvents and genomic DNA. These contaminants affect the RNA utilization, leading to difficult reverse transcription and sequencing. Optical density is widely used to assay the RNA concentration and to check for salts, solvents or proteins contamination. The most important RNA spectrophotometric parameters are an OD 260/280 ratio of 1.8-2 and an OD 260/230 ratio of 1.8 or greater. The nucleic acids are detected at 260 nm, whereas protein, salt and solvents are detected at 230 or 280 nm. If the OD 260/280 and OD 260/230 ratios exceed the range limits the RNA should be re-extracted or purified.

The extracted RNA samples have been analysed by spectrophotometry using a NanoDrop® ND-1000 equipment. Spectrophotometric quantification results are reported in **Table 4.6**.

Table 4.6

Quantification and quality parameters of the total RNA samples obtained by spectrophotometry.

Sample Code	Sample Name	Sample Description	A260/280	A260/230	[ng/µl]
CSX	A1-1	EC1118_Dry(A1)_FF17	2.15	2.42	370.31
CSY	A2-1	EC1118_Dry(A2)_FF17	2.20	2.13	354.57
CTA	A3-1	EC1118_Brut(A3)_FF17	2.14	2.26	240.18
CTB	A4-1	EC1118_Brut(A4)_FF17	2.08	2.12	572.69
CTD	A5-1	QA23_Dry(A5)_FF17	2.16	1.90	192.01
CTE	A6-1	QA23_Dry(A6)_FF17	2.17	1.07	138.99
CTG	A7-1	QA23_Brut(A7)_FF17	2.24	2.09	608.21
CTH	A8-1	QA23_Brut(A8)_FF17	2.16	1.29	231.45
CTJ	A1-2	EC1118_Dry(A1)_FF6	2.16	1.45	919.02
CTK	A2-2	EC1118_Dry(A2)_FF6	2.15	2.15	273.02
CTM	A3-2	EC1118_Brut(A5)_FF6	2.15	1.76	330.13
CTO	A4-2	EC1118_Brut(A6)_FF6	2.13	1.37	224.96
CTP	A5-2	QA23_Dry(A5)_FF6	2.13	1.17	232.14
CTQ	A6-2	QA23_Dry(A6)_FF6	2.12	1.77	191.95
CTS	A7-2	QA23_Brut(A7)_FF6	2.14	2.20	233.87
CTT	A8-2	QA23_Brut(A8)_FF6	2.13	2.08	296.55

RNA samples were also submitted to a fluorescent-based quantification, a more sensitive method alternative to UV absorbance to quantify nucleic acid, which is generally preferred for further RNA-sequencing. The method uses fluorescent dyes that bind to dsDNA, RNA and ssDNA. In this method, after a calibration using standards the samples are incubated with the fluorescent dye. The dye binds to the nucleic acid, changes it conformation and the result is an increase of the fluorescence at a specific wavelength

(depending on the dye). The fluorescence is measured, and converted to a nucleic acid concentration using the linear regression equation obtained by the standard curve.

The extracted RNA samples have been analysed by fluorescence emission analysis using Qubit® RNA HS Assay Kit (Thermo-Fisher Scientific) and Qubit® 2.0 Fluorometer (Life Technologies Thermo-Fisher Scientific) equipment. Fluorescent dye-based quantification results are reported in **Table 4.7**.

Table 4.7

Total RNA concentrations obtained by fluorescent dye-based quantification.

Sample Code	Sample Name	Sample Description				[µg/mL]
CSX	A1-1	EC1118	Dry	(A1)	FF17	35.6
CSY	A2-1	EC1118	Dry	(A2)	FF17	60.0
CTA	A3-1	EC1118	Brut	(A3)	FF17	19.7
CTB	A4-1	EC1118	Brut	(A4)	FF17	48.7
CTD	A5-1	QA23	Dry	(A5)	FF17	15.4
CTE	A6-1	QA23	Dry	(A6)	FF17	67.0
CTG	A7-1	QA23	Brut	(A7)	FF17	46.4
CTH	A8-1	QA23	Brut	(A8)	FF17	18.4
CTJ	A1-2	EC1118	Dry	(A1)	FF6	30.3
CTK	A2-2	EC1118	Dry	(A2)	FF6	22.5
CTM	A3-2	EC1118	Brut	(A5)	FF6	28.2
CTO	A4-2	EC1118	Brut	(A6)	FF6	18.4
CTP	A5-2	QA23	Dry	(A5)	FF6	19.4
CTQ	A6-2	QA23	Dry	(A6)	FF6	99.0
CTS	A7-2	QA23	Brut	(A7)	FF6	18.5
CTT	A8-2	QA23	Brut	(A8)	FF6	23.3

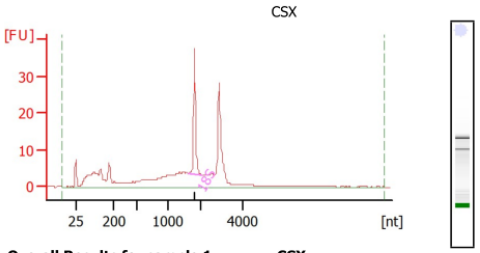
5.3.14 RNA quality assessment by electropherogram

After the previously reported preliminary RNA quantification and quality check, the effective quality of RNA has been determined using the Eukaryote total RNA 6000 Nano LabChip on the Agilent 2100 bioanalyzer. This tool provides a high RNA quality assessment given by the RNA Integrity Number (RIN). This parameter allows to completely removing the user-dependent interpretation in RNA quality control by taking into account the entire electrophoretic trace. The RIN software algorithm classifies the eukaryotic total RNA using a 1 to 10 range, where 1 means that the RNA sample is highly degraded and 10 means that RNA is perfectly intact. RIN values can also be correlated with specific downstream experiment, such as microarray analysis or RT-PCR, and in this way it allows setting a threshold for minimum quality RIN value of the total RNA samples. The RIN value threshold, as a consequence, depends on the laboratory equipment.

In the present work the total RNA samples were evaluated using the Agilent 2100 bioanalyzer for the RIN classification (CRIBI Biotechnology Center, University of Padova – PD, Italy). Electropherogram of the samples are here reported (**Figure 4.14**) and RIN values were summarized in **Table 4.8**

Figure 4.14

Electropherograms and RIN (RNA Integrity Number) of the total RNA samples (CRIBI Biotechnology Center).

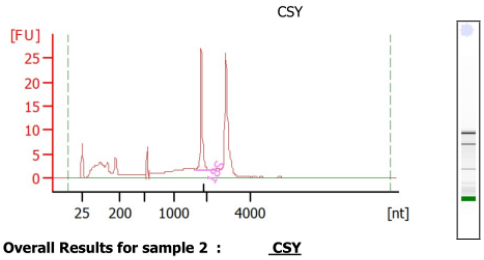


Overall Results for sample 1 : CSX

RNA Area:	249,5
RNA Concentration:	296 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	5.5 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 5.50

Fragment table for sample 1 : CSX

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.655	2.062	31,1	12,5

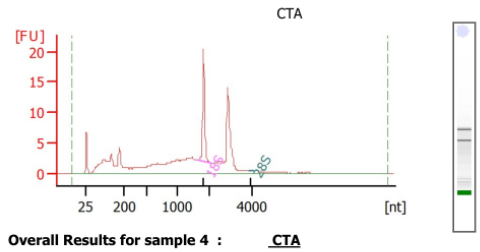


Overall Results for sample 2 : CSY

RNA Area:	168,6
RNA Concentration:	200 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	5.8 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 5.80

Fragment table for sample 2 : CSY

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.667	2.261	22,8	13,5

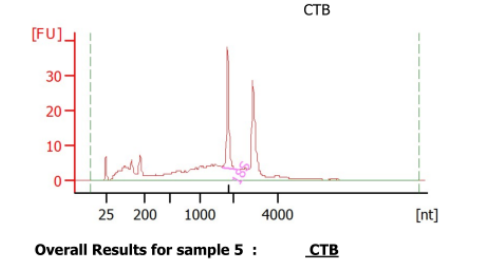


Overall Results for sample 4 : CTA

RNA Area:	157,9
RNA Concentration:	187 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	5.3 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 5.30

Fragment table for sample 4 : CTA

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.641	2.052	16,3	10,3
28S	3.897	4.094	0,1	0,0

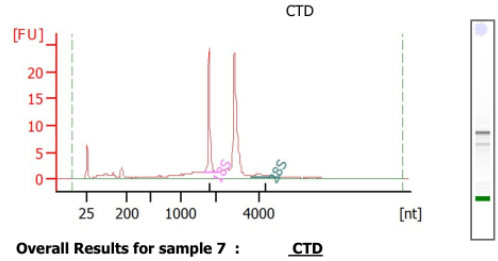


Overall Results for sample 5 : CTB

RNA Area:	276,3
RNA Concentration:	328 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	5.5 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 5.50

Fragment table for sample 5 : CTB

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.663	2.099	31,8	11,5

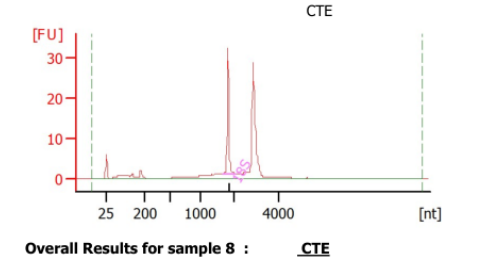


Overall Results for sample 7 : CTD

RNA Area:	114,5
RNA Concentration:	136 ng/µl
rRNA Ratio [28s / 18s]:	0,1
RNA Integrity Number (RIN):	6.6 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.60

Fragment table for sample 7 : CTD

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.649	2.081	19,6	17,1
28S	3.642	4.916	1,8	1,6



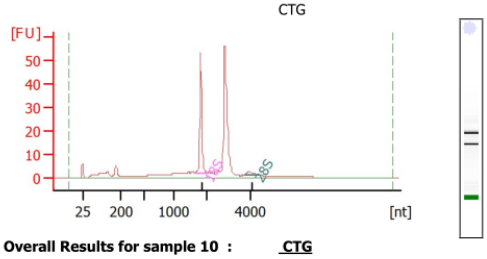
Overall Results for sample 8 : CTE

RNA Area:	117,5
RNA Concentration:	139 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	6.3 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.30

Fragment table for sample 8 : CTE

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.647	2.064	24,2	20,6

CTG



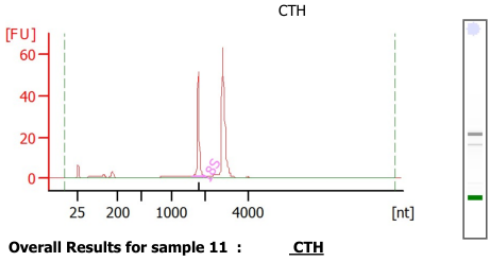
Overall Results for sample 10 : CTG

RNA Area:	246,6
RNA Concentration:	292 ng/µl
rRNA Ratio [28s / 18s]:	0,1
RNA Integrity Number (RIN):	6.2 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.20

Fragment table for sample 10 : CTG

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.633	2.015	44,6	18,1
28S	3.709	4.427	3,7	1,5

CTH



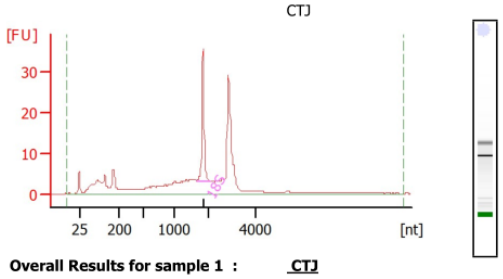
Overall Results for sample 11 : CTH

RNA Area:	176,7
RNA Concentration:	210 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	6.5 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.50

Fragment table for sample 11 : CTH

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.621	2.028	39,7	22,5

CTJ



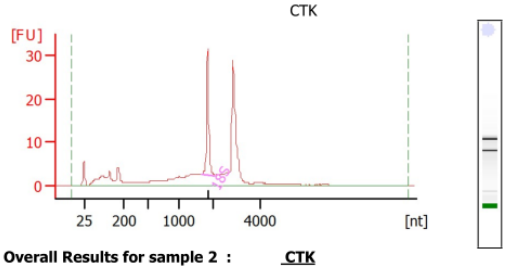
Overall Results for sample 1 : CTJ

RNA Area:	236,0
RNA Concentration:	455 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	5.8 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 5.80

Fragment table for sample 1 : CTJ

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.639	2.073	30,4	12,9

CTK



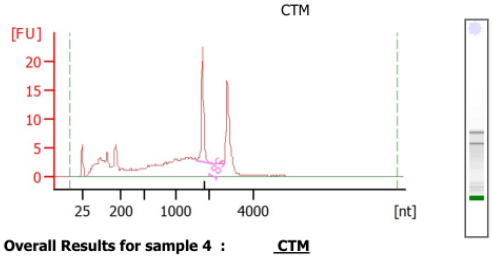
Overall Results for sample 2 : CTK

RNA Area:	196,1
RNA Concentration:	378 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	5.8 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 5.80

Fragment table for sample 2 : CTK

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.650	2.073	27,3	13,9

CTM



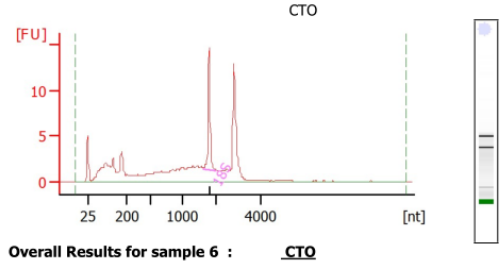
Overall Results for sample 4 : CTM

RNA Area:	195,4
RNA Concentration:	376 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	5 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN:5

Fragment table for sample 4 : CTM

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.641	2.221	19,0	9,7

CTO



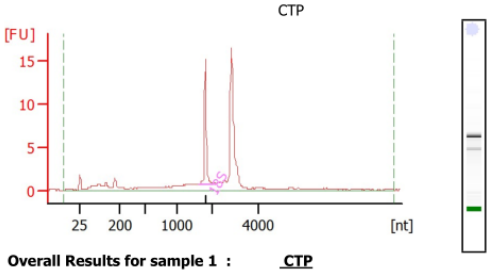
Overall Results for sample 6 : CTO

RNA Area:	111,5
RNA Concentration:	215 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	5.4 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 5.40

Fragment table for sample 6 : CTO

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.631	2.045	12,0	10,7

CTP



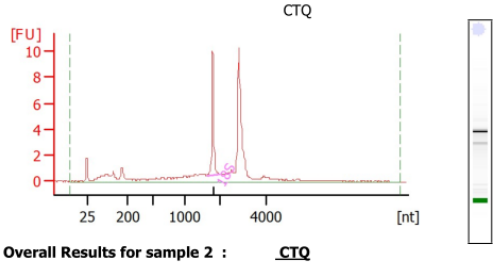
Overall Results for sample 1 : CTP

RNA Area:	70,2
RNA Concentration:	315 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	6.2 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.20

Fragment table for sample 1 : CTP

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.632	2.017	11,8	16,8

CTQ



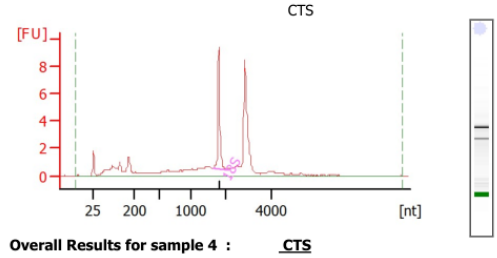
Overall Results for sample 2 : CTQ

RNA Area:	52,7
RNA Concentration:	237 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	6.1 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.10

Fragment table for sample 2 : CTQ

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.635	2.034	8,1	15,3

CTS



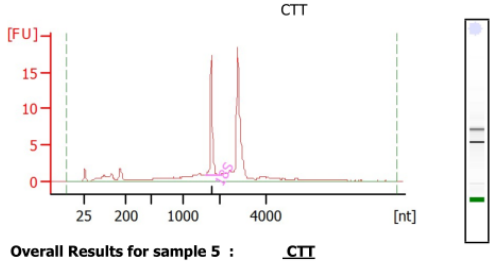
Overall Results for sample 4 : CTS

RNA Area:	53,6
RNA Concentration:	241 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	6.1 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.10

Fragment table for sample 4 : CTS

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.650	2.038	7,7	14,3

CTT



Overall Results for sample 5 : CTT

RNA Area:	83,5
RNA Concentration:	375 ng/µl
rRNA Ratio [28s / 18s]:	0,0
RNA Integrity Number (RIN):	6.1 (B.02.08, Anomaly Threshold(s) manually adapted)
Result Flagging Color:	
Result Flagging Label:	RIN: 6.10

Fragment table for sample 5 : CTT

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1.614	2.011	14,1	16,9

Table 4.8

RIN values of the total RNA samples.

Sample Code	Sample Name	Sample Description				RIN
CSX	A1-1	EC1118	Dry	(A1)	FF17	5.5
CSY	A2-1	EC1118	Dry	(A2)	FF17	5.8
CTA	A3-1	EC1118	Brut	(A3)	FF17	5.3
CTB	A4-1	EC1118	Brut	(A4)	FF17	5.5
CTJ	A1-2	EC1118	Dry	(A1)	FF6	5.8
CTK	A2-2	EC1118	Dry	(A2)	FF6	5.8
CTM	A3-2	EC1118	Brut	(A5)	FF6	5.0
CTO	A4-2	EC1118	Brut	(A6)	FF6	5.4
CTD	A5-1	QA23	Dry	(A5)	FF17	6.2
CTE	A6-1	QA23	Dry	(A6)	FF17	6.1
CTG	A7-1	QA23	Brut	(A7)	FF17	6.1
CTH	A8-1	QA23	Brut	(A8)	FF17	6.5
CTP	A5-2	QA23	Dry	(A5)	FF6	6.6
CTQ	A6-2	QA23	Dry	(A6)	FF6	6.3
CTS	A7-2	QA23	Brut	(A7)	FF6	6.2
CTT	A8-2	QA23	Brut	(A8)	FF6	6.5

The RINs of the total RNA samples ranged from 5 to 6.6 and this range was judged adequate for the coming after RNA sequencing experiment by the sequencing centre, so they have been definitely processed for sequencing.

5.4 Discussion and Conclusions

In this work two commercial yeast strains belonging to the *Saccharomyces cerevisiae* species, both commonly used for the production of Prosecco sparkling wine in autoclave (Martinotti method) were compared. Two experiments have been set up for each strain, with the same adaptation protocol (*pied de cuve*), to obtain two types of wine: the Dry version (with greater residual sugar content) and the Brut version (with less residual sugar content).

The results emerged during the course of this work show how the two strains are very different, especially in terms of second fermentation kinetics and cell viability during the fermentation process and during the cooling of the obtained sparkling wine. It turned out that the yeast QA23, despite a longer initial *lag* phase than EC1118, led to faster fermentation kinetics. This aspect may not be a synonym for the adequacy of the second fermentation kinetics, due to the repercussions that a too rapid fermentation may have on the wine quality parameters of sparkling wines (olfactory finesse, aromatic bouquet, *perlage* ...).

The viable and total population, during the second fermentation, has had a limited increase, especially for EC1118, and this is consistent with the very limited nitrogen consumption. Despite this, the kinetics of pressure increase proved to be regular and consistent with those usually observed in this type of production process.

The poor nitrogen utilization indicates that the nitrogenous nutrition of yeasts during the second fermentation, in particular the nutrition based on amino acids, has a reduced role compared to the fermentation of the must, independently of the yeast strain, as reported also by some authors in the case of the production of wines sparkling wines with the classic method (Martí-Raga *et* al., 2015). On the other hand, the greatest impact seems to

be due to the ammonia nitrogen based nutrition: at the end of fermentation, this nutrient was in fact almost depleted by both yeast strains. This evidence is strongly suggested to be due to the inefficient cell plasma membrane nitrogen uptake. In such conditions, indeed, the ethanol toxic effect is high: the base wine content was 10% vol., a concentration usually reached at the end of the grape musts fermentation. Moreover, it is plausible to suppose that the ethanol toxicity, which acts penetrating into the phospholipidic layer of the plasma membrane, can worse if the environment is under pressure, as is the case of the autoclave. The pressure evolution curve of EC1118 and QA23 reflected the cells behaviour of the two yeasts: QA23 was able to increase its cells population, while EC1118 did not, and consequently the EC1118 pressure curve was slower. This aspect may not be a synonym of adequacy for the sparkling wine production, due to the repercussions that a too rapid fermentation may have on the wine quality parameters: olfactory finesse, aromatic bouquet, *perlage* ... Results, however, suggests that the most suitable yeast strains for the second fermentation under the Martinotti's method is not the most ethanol tolerant but the most able to manage the nitrogen resources, in particular the amino nitrogen.

The wine chilling at the end of the second fermentation didn't affect the cells viability: the values of viable cells percentage on the total after the first step of cooling at 6°C (FF6) were similar to that of the end of the second fermentation at 17°C (FF17). Even after two hours of stop at 6°C cells showed to be not affected by the low temperature. This results demonstrated that even the conditions of a just obtained sparkling wine (ethanol concentration > 10% vol., low pH, high pressure) are very stressful for the yeast cells they are able to resist and also to survive an additional stress, like the chilling from 17 to 6°C. As cells maintained their viability, there are high probabilities that they reacted to such stress modulating their transcriptional profile. The RNA extraction and

purification work here performed has been functional to the next RNA sequencing and the transcriptomic analysis, in order to understand what happens in the cells during the last stage of the second fermentation.

About the RNA recovery, a sub-optimal RIN numbers were obtained. This element can be charged on the difficult cells isolation conditions: the cells recovered from the autoclaves at the end of the second fermentation were suspended in a solution with high ethanol concentration (higher than 11% vol.) and tartaric acid salts, due to the wine composition. As previously reported the liquid samples containing the cells were immediately centrifuged at 4°C, in order to accelerate the precipitation of the solid fraction, using a pre-chilled centrifuge. Then the obtained pellets were rapidly washed with sterile deionized water at 4°C, frozen in liquid nitrogen and stored at -80°C. The whole cells recovering procedure, from autoclave sampling to cell freezing lasts for around 15 minutes, less than the 20 minutes time which is generally considered adequate for the recovery of good quality RNA. In the extraction protocol the first step is the cells crushing with the TRIzol® Reagent: during this step the total RNA faces all the other cellular components and the solids wine components which were not possible to eliminate during the cells recovery, so at this step the RNA could have faced the presence of such degrading contaminants.

Another explication of the sub-optimal obtained RIN values is the high number of dead or damaged yeast cells in the wine samples: this is suggested by observing that the lower RIN numbers were obtained from EC1118 total RNA samples with respect to the ones from QA23 samples.

As reported above, the EC1118 yeast strain showed very low cells viability all along the second fermentation process. At the end of the fermentation (FF17) the EC1118 viability

was 34.2-41.4%, against the 82.9-83.0% of QA23. Similarly, when the 6°C temperature was reached cells viability was of 34.8-41.2% and 82.5-83.9%, respectively. This means that at the sampling time points in the EC1118 samples there was a ratio of about 1:3 between viable (and so intact) and dead or damaged cells (and so under a possible uncontrolled outflow of cells components), while QA23 had a ratio of 4:5 of viable cells on the total. This difference in viable on total cells can have affected the quality of the extracted RNA: the high amount of dead/damaged cells with respect to the viable and intact cells, in fact, could have increased the release of degrading agents before that the purifying steps of the RNA extraction protocol could have been performed.

In conclusion, this work underlined a clear difference among the kinetics of second fermentation and the cells viability of the two strains under the Martinotti's method. By the obtained results this difference seems to be attributable to the amino nitrogen uptake and management. Fermentation was stopped when 5 bar of internal pressure were reached. Then, the wine chilling performed in industrial scale has been mimicked, setting a controlled cooling by PLC equipment. The data of cells viability during these second fermentation final stages indicates that the cells were in a physiological condition compatible with a transcriptional stress-response; despite low amounts, the extracted total RNA was suitable to undergo the sequencing process, so the very next step to reach the aim of the present study will be the interpretation of the RNA sequencing analysis.

6. Conclusions

In conclusion, in this work high genotypic variability was found in the sampled vineyards of the Vale dos Vinhedos area and most of the genotypes revealed to be vine-specific. From the phenotypic traits analysis the high copper tolerance level suggested an environmental adaptation to the strong use of copper-based fungicides. Finally, as 33% of the tested strains showed good fermentation performance, high copper and sulphite tolerance, Vale dos Vinhedos vineyards seem to be an interesting yeast genotype reservoir for the selection of starters that match the need of local winemaking.

In the present work a population of 273 *Saccharomyces cerevisiae* strains has been tested for copper and sulphites tolerance: 153 came from vineyard and 20 were commercial wine starters. Copper tolerance has been analysed with respect to the number of copies of the *CUP1* gene, encoding for a metallothionein involved in copper detoxification. Our results agree with previous literature findings, and indicate that the *CUP1* gene is associated to copper tolerance when the number of copies oversteps a certain threshold, that is our study was 8 copies. The higher influence of *CUP1* gene copies number on copper tolerance has been found among Italian vineyard strains: Brazilian strains, despite they were more tolerant to copper, presented a low *CUP1* gene number of copies, suggesting that they developed a different copper tolerance mechanism. Since the strongest association between copper tolerance and *CUP1* gene copies number has been found in strains from Italian vineyards, which have a longer winemaking tradition than Brazilian ones, the adaptation of autochthonous yeasts to copper could be linked to the exposure duration to this element.

Sulphites tolerance has been analysed with respect to the presence of the XVI-VIII genomic translocation, which causes the overexpression of the sulphites efflux pump Ssu1p. This genetic trait resulted generally associated to yeasts sulphites tolerance.

Despite expectations, among Industrial strains the presence of the translocation is not associated to the tolerant phenotype, while all vineyard strains did.

Finally, an association between copper and sulphites tolerance has been found among the tested wine yeasts, suggesting that the development of tolerance mechanisms against these chemicals could be connected. A possible common point could be the high use of copper sulphate as antifungal pesticide in vine-growing, leading to a multiple resistance strategy: if copper stimulates copper tolerance *via* the *CUP1* gene duplication, sulphate stimulates the sulphites tolerance *via* the occurrence of the XVI-VIII translocation (overexpression of Ssu1p).

The fermentable carbon sources uptake in four *S. cerevisiae* strains has then been investigated. The expression of the hexose transporters genes has been analysed by Real Time PCR during the stationary phase of synthetic must fermentation. This work represents an effort to deep investigate the utilization of the hexoses transporter genes of four *Saccharomyces cerevisiae* strains, under fermentation conditions. Differently from the most of the literature on this theme, the investigated diploid strains were not artificially depleted for any gene. This peculiarity made harder to understand the expression patterns, but on the other hand gave an actual data background.

The study involved the *FSY1* gene, found in EC1118 and encoding for a high affinity fructose/H^+ symporter. *FSY1* was present in the 25% of the vineyard strains. Gene expression analysis evidenced deep differences in the sugar transporters utilization, in particular for the fructose transporter gene: in oenological conditions, the differential expression of *FSY1* enhanced the carbon sources utilization ability of the yeasts strains.

This work can contribute in improving the wine yeasts characterization by giving a tool for their distinction for fitness in the winemaking environment, at transcriptional level.

In this work two commercial yeast strains belonging to the *Saccharomyces cerevisiae* species, both commonly used for the production of Prosecco sparkling wine in autoclave (Martinotti method) were compared. Two experiments have been set up for each strain, with the same adaptation protocol (*pied de cuve*), to obtain two types of wine: the Dry version (with greater residual sugar content) and the Brut version (with less residual sugar content).

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References

Acree TE, Sonoff EP, Splittstoesser DF. (1972) Effect of yeast strain and type of sulfur compound on hydrogen sulfide production. American Journal of Enology and Viticulture. 23(1):6-9.

Adamo GM, Brocca S, Passolunghi S, Salvato B, Lotti M (2012) Laboratory evolution of copper tolerant yeast strains. Microbial Cell Factories 11(1). https://doi.org/10.1186/1475-2859-11-1.

Albers E, Larsson C, Lidén G, Niklasson C, Gustafsson L (1996) Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. Applied and environmental microbiology 62(9):3187-95.

Alexandre H, Ansanay-Galeote V, Dequin S, Blondin B (2001) Global gene expression during short-term ethanol stress in *Saccharomyces cerevisiae*. FEBS Letters 498. http://doi.org/10.1016/S0014-5793(01)02503-0.

Almeida P, Barbosa R, Zalar P, Imanishi Y, Shimizu K, Turchetti B, Legras JL, Serra M, Dequin S, Couloux A, Guy J, Bensasson D, Gonçalves P, Sampaio JP (2015) A population genomics insight into the Mediterranean origins of wine yeast domestication. Molecular ecology 24(21):5412-27. https://doi.org/10.1111/mec.13341.

Alonso-del-Real J, Lairón-Peris M, Barrio E, Querol A (2017) Effect of Temperature on the Prevalence of *Saccharomyces* Non *cerevisiae* Species against a *S. cerevisiae* Wine Strain in Wine Fermentation: Competition. Physiological Fitness and Influence in Final Wine Composition. Front Microbiol 8:150. http://doi.org/10.3389/fmicb.2017.00150.

Álvarez-Pérez JM, Campo E, San-Juan F, Coque JJR, Ferreira V, Hernández-Orte P (2012) Sensory and chemical characterisation of the aroma of Prieto Picudo rosé

wines: The differential role of autochthonous yeast strains on aroma profiles. Food Chemistry 133(2):284-92. https://doi.org/10.1016/j.foodchem.2012.01.024.

Alves Baffi M, dos Santos Bezerra C, Arévalo-Villena M, Briones-Pérez AI, Gomes E, Da Silva R (2011) Isolation and molecular identification of wine yeasts from a Brazilian vineyard. Ann Microbiol 61 (1):75-78. http://doi.org/10.1007/s13213-010-0099-z.

Ambardar S, Gupta R, Trakroo D, Lal R, Vakhlu J (2016) High Throughput Sequencing: An Overview of Sequencing Chemistry. Indian J Microbiol 56(4):394–404. http://doi.org/10.1007/s12088-016-0606-4.

Anjos J, Rodrigues de Sousa H, Roca C, Cássio F, Luttik M, Pronk JT, Salema-Oom M, Gonçalves P (2013) Fsy1, the sole hexose-proton transporter characterized in *Saccharomyces* yeasts, exhibits a variable fructose:H^+ stoichiometry. Biochimica et Biophysica Acta 1828(2):201-7. https://doi.org/10.1016/j.bbamem.2012.08.011.

Antunovics Z, Irinyi L, Sipiczki M (2005) Combined application of methods to taxonomic identification of *Saccharomyces* strains in fermenting botrytized grape must. J Appl Microbiol 98:971-979. http://doi.org/10.1111/j.1365-2672.2005.02543.x.

Argueso JL, Carazzolle MF, Mieczkowski PA, Duarte FM, Netto OVC, Missawa SK, Galzerani F, Costa GGL, Vidal RO, Noronha MF, Dominska M, Andrietta MGS, Andrietta SR, Cunha AF, Gomes LH, Tavares FCA, Alcarde AR, Dietrich FS, McCusker JH, Petes TD, Gonçalo A.G. Pereira GAG (2009) Genome structure of a *Saccharomyces cerevisiae* strain widely used in bioethanol production. Genome Res. http://doi.org/10.1101/gr.091777.109.

Avram Dorina, Bakalinksy Alan T (1997) *SSU1* encodes a plasma membrane protein with a central role in a network of proteins conferring sulfite tolerance in *Saccharomyces cerevisiae*. Journal of Bacteriology 197(18):5971-4. http://doi.org/10.1128/jb.179.18.5971-5974.1997.

Ayres Peter G (2004) Alexis Millardet: France's forgotten mycologist. Mycologist 18(1). http://doi.org/10.1017/S0269915X04001090.

Azenha M, Vasconcelos MT, Moradas-Ferreira P (2000) The Influence of Cu Concentration on Ethanolic Fermentation by *Saccharomyces cerevisiae*. J Biosci Bioeng 90(2):163-167. http://doi.org/10.1016/S1389-1723(00)80104-8.

Aziz A, Trotel-Aziz P, Dhuicq L, Jeandet P, Couderchet M, Vernet G (2006) Chitosan Oligomers and Copper Sulfate Induce Grapevine Defense Reactions and Resistance to Gray Mold and Downy Mildew. Phytopathology 96(11):1188-1194. http://doi.org/10.1094/PHYTO-96-1188.

Bakalinsky AT, Snow R (1990) The chromosomal constitution of wine strains of *Saccharomyces cerevisiae*. Yeast 6:367–82.

Bell SJ, Henschke PA (2005) Implications of nitrogen nutrition for grapes, fermentation and wine. Australian Journal of Grape and Wine Research. 11(3):242-95.

Bely L, Sablayrolles J, Barre P (1990) Description of alcoholic fermentation kinetics: its variability and significance. Am J Enol Viticult 40:319–24.

Benucci I, Liburdi K, Cerreti M, Esti M (2016) Characterization of Active Dry Wine Yeast During Starter Culture (Pied de Cuve) Preparation for Sparkling Wine Production. Journal of Food Science, 81: M2015-M2020. doi:10.1111/1750-3841.13379

Berthels NJ, Cordero Otero RR, Bauer FF, Thevelein JM, Pretorius IS (2004) Discrepancy in glucose and fructose utilisation during fermentation by *Saccharomyces cerevisiae* wine yeast strains. FEMS Yeast Research 4(7):683–9. https://doi.org/10.1016/j.femsyr.2004.02.005.

Bianchin F, Favero C, Gaiotti F, Marcuzzo P, Peressini A, Scienza A, Tomasi D (2016) L'eredità della Serenissima. Vigneti e vini della DOC Venezia. Ed. Veneto Agricoltura.

Bisson LF, Coons DM, Kruckeberg AL, Lewis DA (1993) Yeast Sugar Transporters. Critical Reviews in Biochemistry and Molecular Biology 28(4):259-308. http://doi.org/10.3109/10409239309078437.

Bisson LF (1999) Stuck and sluggish fermentations. American Journal of Enology and Viticulture 50(1):107-19.

Blatayron L, Sablayrolles JM (2001) Stuck and slow fermentations in enology: statistical study of causes and effectiveness of combined additions of oxygen and diammonium phosphate. J. Biosci Bioeng 91:184–9.

Boer VM, de Winde JH, Pronk JT, Piper MDW (2003) The Genome-wide Transcriptional Responses of *Saccharomyces cerevisiae* Grown on Glucose in Aerobic Chemostat Cultures Limited for Carbon, Nitrogen, Phosphorus, or Sulfur. The Journal of Biological Chemistry 278:3265-74. http://doi.org/10.1074/jbc.M209759200.

Bokulich NA, Ohta M, Richardson PM, Mills DA (2013) Monitoring seasonal changes in winery-resident microbiota. PLoS ONE 8(6):e66437. http://doi.org/10.1371/journal.pone.0066437.

Bokulich NA, Thorngate JH, Richardson PM, Mills, DA (2014). Microbial biogeography of wine grapes is conditioned by cultivar, vintage, and climate. Proceedings of the National Academy of Sciences 11(1):139-48. https://doi.org/10.1073/pnas.1317377110.

Boles E, Hollenberg CP (1997) The molecular genetics of hexose transport in yeasts. FEMS Microbiology Reviews 21(1):85-111.https://doi.org/10.1111/j.1574-6976.1997.tb00346.x-.

Bohlscheid JC, Fellman JK, Wang XD, Ansen D, Edwards CG (2007) The influence of nitrogen and biotin interactions on the performance of *Saccharomyces* in alcoholic fermentations. Journal of applied microbiology 102(2):390-400. https://doi.org/10.1111/j.1365-2672.2006.03180.x.

Borneman AR, Forgan AH, Pretorius IS, Chambers PJ (2008) Comparative genome analysis of a *Saccharomyces cerevisiae* wine strain. FEMS Yeast Research, 8(7):1185–95. https://doi.org/10.1111/j.1567-1364.2008.00434.x.

Borneman AR, Desany BA, Riches D, Affourtit JP, Forgan AH, Pretorius IS, Egholm M, Chambers PJ (2011) Whole-Genome Comparison Reveals Novel Genetic Elements That Characterize the Genome of Industrial Strains of *Saccharomyces cerevisiae*. PLoS Genet 7(2):e1001287. https://doi.org/10.1371/journal.pgen.1001287.

Bovo B, Giacomini A, Corich V (2011) Effects of grape marcs acidification treatment on the evolution of indigenous yeast populations during the production of grappa. J Appl Microbiol 111(2):382-388. http://doi.wiley.com/10.1111/j.1365-2672.2011.05060.x.

Bovo B, Nardi T, Fontana F, Carlot M, Giacomini A, Corich V (2012) Acidification of grape marc for alcoholic beverage production: effects on indigenous microflora and aroma profile after distillation. Int J Food Microbiol 152(3):100-6. https://doi.org/10.1016/j.ijfoodmicro.2011.10.006.

Brun LA, Maillet J, Richarte J, Herrmann P, Remy JC (1998) Relationships between extractable copper, soil properties and copper uptake by wild plants in vineyard soils. Environ Pollut 102:151-161. http://doi.org/10.1016/S0269-7491(98)00120-1.

Callejon RM, Clavijo A, Ortigueira P, Troncoso AM, Paneque P, Morales ML (2010) Volatile and sensory profile of organic red wines produced by different selected

autochthonous and commercial *Saccharomyces cerevisiae* strains. Analytica Chimica Acta 660(1–2):68-75. https://doi.org/10.1016/j.aca.2009.09.040.

Camarasa C, Grivet JP, Dequin S (2003) Investigation by 13C-NMR and tricarboxylic acid (TCA) deletion mutant analysis of pathways for succinate formation in Saccharomyces cerevisiae during anaerobic fermentation. Microbiology 149(9):2669-78.

Camarasa C, Sanchez I, Brial P, Bigey F, Dequin S (2011) Phenotypic Landscape of *Saccharomyces cerevisiae* during Wine Fermentation: Evidence for Origin-Dependent Metabolic Traits. PLoS ONE 6(9):e25147. https://doi.org/10.1371/journal.pone.0025147.

Camargo UA, Tonietto J, Hoffmann A (2011) Progressos na viticultura brasileira. Rev Bras Frutic 33(1):144-149.

Cankorur-Cetinkaya A, Dereli E, Eraslan S, Karabekmez E, Dikicioglu D, Kirdar B (2012) A Novel Strategy for Selection and Validation of Reference Genes in Dynamic Multidimensional Experimental Design in Yeast. PLoS ONE 7(6):e38351. http://doi.org/10.1371/journal.pone.0038351.

Capece A, Romaniello R, Siesto G, Pietrafesa R, Massari C, Poeta C, Romano P (2010) Selection of indigenous *Saccharomyces cerevisiae* strains for Nero d'Avola wine and evaluation of selected starter implantation in pilot fermentation. Int J Food Microbiol 144:187-192. http://doi.org/10.1016/j.ijfoodmicro.2010.09.009.

Cappello M, Bleve G, Grieco F, Dellaglio F, Zacheo G (2004) Characterization of *Saccharomyces cerevisiae* strains isolated from must of grape grown in experimental vineyard. J Appl Microbiol 97: 1274-1280. http://doi:10.1111/j.1365-2672.2004.02412.x.

Carreto L, Eiriz MF, Gomes AC, Pereira PM, Schuller D, Santos MAS (2008) Comparative genomics of wild type yeast strains unveils important genome diversity. BMC Genomics 9:524. https://doi.org/10.1186/1471-2164-9-524.

Casalone Enrico, Colella Carlo M, Daly Simona, Gallori Enzo, Moriani Luca, Polsinelli Mario (1992) Mechanism of resistance to sulphite in *Saccharomyces cerevisiae*. Current Genetics 22(6):435-40. https://doi.org/10.1007/BF00326407.

Castellari M, Versari A, Spinabelli U, Galassi S, Amati A (2000) An improved HPLC method far the analysis of organic acids, carbohydrates and alcohols in grape musts and wines. Journal of Chromatography and R.T. 23(13):2047-56.

Chandler M, Stanley GA, Rogers P, Chambers P (2004) A genomic approach to defining the ethanol stress response in the yeast *Saccharomyces cerevisiae*. Annals of Microbiology 54(4):427-54.

Chavan P, Mane S, Kulkarni G, Shaikh S, Ghormade V, Nerkar DP, Shouche Y, Deshpande MV (2009) Natural yeast flora of different varieties of grapes used for wine making in India. Food Microbiol 26(8):801-808. http://doi:10.1016/j.fm.2009.05.005.

Cipollina C, van den Brink J, Daran-Lapujade P, Pronk JT, Porro D, de Winde JH (2008) *Saccharomyces cerevisiae SFP1*: at the crossroads of central metabolism and ribosome biogenesis. Microbiology 154:1686-99. http://doi.org/10.1099/mic.0.2008/017392-0.

Ciriacy M, Reifenberger E (1997) Hexose transport. p. 45–65. In F. K. Zimmermann and K.-D. Entian (ed.), Yeast sugar metabolism: biochemistry, genetics, biotechnology, and applications. Technomic, Lancaster, Pa.

Comi G, Maifreni M, Manzano M, Lagazio C, Cocolin L (2000) Mitochondrial DNA restriction enzyme analysis and evaluation of the enological characteristics of

Saccharomyces cerevisiae strains isolated from grapes of the wine producing area of Collio (Italy). Int J Food Microbiol 58:117-21.

COMMISSION REGULATION (EC) No 606/2009 of 10 July 2009 laying down certain detailed rules for implementing Council Regulation (EC) No 479/2008 as regards the categories of grapevine products, oenological practices and the applicable restrictions. Available from: http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32009R0606&from=IT [accessed 18 09 2017]

Constantí M, Poblet M, Arola L, Mas A and Guillamón JM (1997) Analysis of yeast populations during alcoholic fermentation in a newly established winery. Am J Enol Vitic 48, 339-44.

Cordero-Bueso G, Arroyo T, Serrano A, Valero E (2011) Remanence and survival of commercial yeast in different ecological niches of the vineyard. FEMS Microbiol Ecol 77:429-437. http://doi.org/10.1111/j.1574-6941.2011.01124.x.

Cortés S, Blanco P (2011) Yeast strain effect on the concentration of major volatile compounds and sensory profile of wines from *Vitis vinifera* var. Treixadura. World Journal of Microbiology and Biotechnology 27(4):925–32. https://doi.org/10.1007/s11274-010-0535-z.

Crosato G, Carlot M, De Iseppi A, Garavaglia J, Massochin NPL, Righetto Ziegler D, de Souza Ramos RC, Rossi RC, Nadai C, Giacomini A, Corich V (2018) Genetic variability and physiological traits of *Saccharomyces cerevisiae* strains isolated from "Vale dos Vinhedos" vineyards reflect agricultural practices and history of this Brazilian wet subtropical area. World J Microbiol Biotechnol 34:105. https://doi.org/10.1007/s11274-018-2490-z.

D. M. 22.12.2010 (2010) Riconoscimento della Denominazione di Origine Controllata e Garantita «Piave Malanotte» o «Malanotte del Piave» e l'approvazione del

relativo disciplinare di produzione. Supplemento n°6 alla Gazzetta Ufficiale n°4 Serie Generale – 7.01.2011.

D.M. 17.07.2009 (2009) Riconoscimento della denominazione di origine controllata dei vini «Prosecco», riconoscimento della denominazione di origine controllata e garantita dei vini «Conegliano Valdobbiadene - Prosecco» e riconoscimento della denominazione di origine controllata e garantita dei vini «Colli Asolani - Prosecco» o «Asolo - Prosecco» per le rispettive sottozone e approvazione dei relativi disciplinari di produzione. Gazzetta Ufficiale n°173 Serie Generale – 28.07.2009.

da Silva GA, Agustini BC, De Mello LMR, Tonietto J (2016) Autochthonous yeast populations from different Brazilian geographic indications. BIO Web of Conferences 7:02030. http://doi.org/10.1051/bioconf/20160702030.

De Freitas J, Wintz H, Kim JH, Poynton H, Fox T, Vulpe C (2003) Yeast, a model organism for iron and copper metabolism studies. BioMetals 16(1):185-197. http://doi.org/10.1023/A:1020771000746.

de Sousa HR, Spencer-Martins I, Gonçalves P (2004) Differential regulation by glucose and fructose of a gene encoding a specific fructose/H+ symporter in *Saccharomyces sensu stricto* yeasts. Yeast 21:519-30. http://doi.org/10.1002/yea.1118.

Delfini C, Formica JV (2001) Wine microbiology: science and technology. Marcel Dekker, New York, US.

Demuyter C, Lollier M, Legras JL, Le Jeune C (2004) Predominance of *Saccharomyces uvarum* during spontaneous alcoholic fermentation for three consecutive years in an Alsatian winery. J Appl Microbiol 97:1140-1148. http://doi.org/10.1111/j.1365-2672.2004.02394.x.

Diderich JA, Schuurmans JM, Van Gaalen MC, Kruckeberg AL, Van Dam K (2001) Functional analysis of the hexose transporter homologue *HXT5* in *Saccharomyces cerevisiae*. Yeast 18(16):1515-24. https://doi.org/10.1002/yea.779.

Divol B, du Toit M, Duckitt E (2012) Surviving in the presence of sulphur dioxide: strategies developed by wine yeasts. Appl Microbiol Biotechnol 95:601-613. http://doi.org/10.1007/s00253-012-4186-x.

Doniger SW, Kim HS, Swain D, Corcuera D, Williams M, Yang SP, Fay JC (2008) A Catalog of Neutral and Deleterious Polymorphism in Yeast. PLoS Genetics 4(8): e1000183. https://doi.org/10.1371/journal.pgen.1000183.

Dunn B, Levine RP, Sherlock G (2005) Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. BMC Genom 6:53. https://doi.org/10.1186/1471-2164-6-53.

Echeverrigaray S, Randon M, da Silva K, Zacaria J, Longaray Delamare AP (2013) Identification and characterization of non-*Saccharomyces* spoilage yeasts isolated from Brazilian wines. World J Microbiol Biotechnol 29:1019-1027. http://doi.org/10.1007/s11274-013-1265-9.

Egli CM, Edinger WD, Mitrakul CM, Henick-Kling T (2002) Dynamics of indigenous and inoculated yeast populations and their effect on the sensory character of Riesling and Chardonnay wines. Journal of Applied Microbiology 85:779-89. http://doi.org/10.1046/j.1365-2672.1998.00521.x.

Estruch F (2000) Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS microbiology reviews 24(4):469-86. https://doi.org/10.1111/j.1574-6976.2000.tb00551.x.

European Commission – PLANTS EU Pesticides database – Products to which MRLs apply (Part A of Annex I to Reg. 396/2005). Available from:

http://ec.europa.eu/food/plant/pesticides/ eu-pesticides-database/public/?event=pesticide.residue.CurrentMRL&language=EN&pestResidueId =262 [accessed 18 09 2017]

Fancellu F (2008) Studio proteomico della parete cellulare di *Saccharomyces cerevisiae* in condizioni di stress. University of Sassary

Favaro L, Corich V, Giacomini A, Basaglia M, Casella S (2013) Grape marcs as unexplored source of new yeasts for future biotechnological applications. W J Microbiol Biotechnol 29(9):1551-62. https://doi.org/10.1007/s11274-013-1319-z.

Fay JC, Benavides JA (2005) Evidence for domesticated and wild populations of *Saccharomyces cerevisiae*. PLoS Genet 1(1):e5. http://doi.org/10.1371/journal.pgen.0010005.

Fensterseifer JE (2007) The emerging Brazilian wine industry. Challenges and prospects for the Serra Gaúcha wine cluster. IJWBR 19(3):187-206. http://doi.org/10.1108/17511060710817221.

Fernandes AR, Sá-Correia I (1999) Comparative effects of *Saccharomyces cerevisiae* cultivation under copper stress on the activity and kinetic parameters of plasma-membrane-bound H^+-ATPases *PMA1* and *PMA2*. Arch Microbiol 171:273-8. http://doi.org/10.1007/s002030050710.

Filiputti W (2016) The modern History of Italian wine Ed. Skira. ISBN: 885722623

Fleet GH (2003) Yeast interactions and wine flavour. Int J Food Microbiol 86:11-22. http://doi.org/10.1016/S0168-1605(03)00245-9.

Flick KM, Spielewoy N, Kalashnikova TI, Guaderrama M, Zhu Q, Chang HC, Wittenberg C (2003) Grr1-dependent Inactivation of Mth1 Mediates Glucose-

induced Dissociation of Rgt1 from *HXT* Gene Promoters. Molecular Biology of the Cell 14(8):3230-41. https://doi.org/10.1091/mbc.e03-03-0135.

Flores Carlos A, Sarmento Eliana C, Weber Eliseu J, Hasenack Heinrich, Pötter Reinaldo O (2013) Increase of detail in soil surveys and its contribution for managing wine quality in Vale dos Vinhedos, Brazil. Ciência e Técnica Vitivinícola 28:370-4.

Fugelsang Kenneth C, Edwards Charles G (2007) Wine Microbiology – Practical Applications and Procedures (2nd edition). Springer, Boston MA https://doi.org/10.1007/978-0-387-33349-6.

Fumi MD, Trioli G, Colagrande O (1987) Preliminary assessment on the use of immobilized yeast cells in sodium alginate for sparkling wine processes. Biotechnol Lett 9:339. https://doi.org/10.1007/BF01025800.

Galeote V, Novo M, Salema-Oom M, Brion C, Valerio E, Goncalves P, Dequin S (2010) *FSY1*, a horizontally transferred gene in the *Saccharomyces cerevisiae* EC1118 wine yeast strain, encodes a high-affinity fructose/H^+ symporter. Microbiology 156:3754–61. http://doi.org/10.1099/mic.0.041673-0.

Galtier N, Nabholz B, Glémin S, Hurst GDD (2009) Mitochondrial DNA as a marker of molecular diversity: a reappraisal. Mol Ecol 18:4541-50. http://doi.org/10.1111/j.1365-294X.2009.04380.x.

Gancedo JM (1998) Yeast Carbon Catabolite Repression. Microbiology and Molecular Biology Reviews. 62(2):334-61.

Garofalo C, Tristezza M, Grieco F, Spano G, Capozzi V (2016) From grape berries to wine: population dynamics of cultivable yeasts associated to ‘‘Nero di Troia’’ autochthonous grape cultivar. World J Microbiol Biotechnol 32:59. https://doi.org/10.1007/s11274-016-2017-4.

Garofalo C, Arena MP, Laddomada B, Cappello MS, Bleve G, Grieco F, Beneduce L, Berbegal C, Spano G, Capozzi V (2016) Starter cultures for sparkling wine. Fermentation 2(4), 21. http://doi:10.3390/fermentation2040021

Gayevskiy V, Goddard MR (2012) Geographic delineations of yeast communities and populations associated with vines and wines in New Zealand. ISME J 6(7):1281-1290. http://doi.org/10.1038/ismej.2011.195.

Geladé R, de Velde SD, Van Dijck P, Thevelein JM (2003) Multi-level response of the yeast genome to glucose. Genome Biology 20034:233. https://doi.org/10.1186/gb-2003-4-11-233.

Gibson BR, Boulton CA, Box WG, Graham NS, Lawrence SJ, Linforth RST, Smart KA (2008) Carbohydrate utilization and the lager yeast transcriptome during brewery fermentation. Yeast 25(8):549-62. https://doi.org/10.1002/yea.1609.

Glover JR, Lindquist S (1998) Hsp104, Hsp70, and Hsp40: A Novel Chaperone System that Rescues Previously Aggregated Proteins. Cell 94:73–82. https://doi.org/10.1016/S0092-8674(00)81223-4.

Goto-Yamamoto N, Kitano K, Shiki K, Yoshida Y, Suzuki T, Iwata T, Hara S (1998) *SSU1-R*, a sulfite resistance gene of wine yeast, is an allele of *SSU1* with a different upstream sequence. Journal of Fermentation and Bioengineering 86(5):427-33. https://doi.org/10.1016/S0922-338X(98)80146-3.

Gower JC (1975) Generalized Procrustes Analysis. Psychometrika 40:33-51.

Guillaume C, Delobel P, Sablayrolles JM, Blondin B (2007) Molecular Basis of Fructose Utilization by the Wine Yeast *Saccharomyces cerevisiae*: a Mutated *HXT3* Allele Enhances Fructose Fermentation. Appl Environ Microbiol 73(8):2432-9. http://doi.org/10.1128/AEM.02269-06.

Gutiérrez A, Beltran G, Warringer J, Guillamón JM (2013) Genetic basis of variations in nitrogen source utilization in four wine commercial yeast strains. PLoS One 8(6):e67166.

Henick-Kling T, Edinger W, Daniel P, Monk P (1998) Selective effects of sulfur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. J Appl Microbiol 84:865-876. http://doi.org/10.1046/j.1365-2672.1998.00423.x.

Henschke PA, Jiranek V (1993) Yeast−growth during fermentation. In:Fleet GH (ed) Wine microbiology and biotechnology. Harwood Academic, Chur, Switzerland, pp 27–54.

Henschke PA (1997) Wine yeast. Yeast Sugar Metabolism.:527-60.

Hodgins-Davis A, Adomas AB, Warringer J, Townsend JP (2012) Abundant Gene-by-Environment Interactions in Gene Expression Reaction Norms to Copper within *Saccharomyces cerevisiae*. Genome Biol Evol 4(11):1061-1079. http://doi.org/10.1093/gbe/evs084.

Hohmann S, Mager WH (1997) Yeast stress responses. Austin New York: R.G. Landes Co.;North American distributor Chapman & Hall.

Ibeas JI, Jimenez J (1997) Mitochondrial DNA loss caused by ethanol in *Saccharomyces* flor yeasts. Applied and Environmental Microbiology 63:7-12.

Judet-Correia D, Charpentier C, Bensoussan, Dantigny P (2011) Modelling the inhibitory effect of copper sulfate on the growth of *Penicillium expansum* and *Botrytis cinerea*. Lett Appl Microbiol 53:558-564. http://doi.org/10.1111/j.1472-765X.2011.03149.x.

Kachalkin AV, Abdullabekova DA, Magomedova ES, Magomedov GG, Chernova IY (2015) Yeasts of the Vineyards in Dagestan and Other Regions. Microbiology 84(3):425-432. http://doi.org/10.1134/S002626171503008X.

Kaniak A, Xue Z, Macool D, Kim JH, Johnston M (2004) Regulatory Network Connecting Two Glucose Signal Transduction Pathways in *Saccharomyces cerevisiae*. Eukaryotic cell 3(1):221-31. http://doi.org/10.1128/EC.3.1.221-231.2004.

Khan W, Augustyn OPH, Van der Westhuizen TJ, Lambrechts MG, Pretorius IS (2000) Geographic distribution and evaluation of *saccharomyces cerevisiae* strains isolated from vineyards in the warmer, Inland Regions of the Western Cape in South Africa. Africa. South African Journal of Enology & Viticulture 21(1):17-31. http://doi.org10.21548/21-1-2184.

Knight SJ, Goddard MR (2016) Sporulation in soil as an overwinter survival strategy in *Saccharomyces cerevisiae*. FEMS Yeast Research 16(1):fov102. https://doi.org/10.1093/femsyr/fov102.

Kobayashi K, Yoshimoto A (1982) Studies on yeast sulfite reductase: IV. Structure and steady-state kinetics. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology 705(3):348-56.

Köppen climate classification. Oxford Reference. Ed. Retrieved 10 Aug. 2018, from http://www.oxfordreference.com/view/10.1093/oi/authority.20110803100042580

Kottek Markus, Grieser Jürgen, Beck Christoph, Rudolf Bruno, Rubel Franz (2006) World Map of the Köppen-Geiger climate classification updated. Meteorologische Zeitschrift 15(3):259-63. http://doi.org/10.1127/0941-2948/2006/0130.

Kreitman GY, Danilewicz JC, Jeffery DW, Elias RJ (2016) Reaction mechanisms of metals with hydrogen sulfide and thiols in model wine. Part 1: Copper-catalyzed oxidation. Journal of agricultural and food chemistry 64(20):4095-104.

Kruckeber A (1996) The hexose transporter family of *Saccharomyces cerevisiae*. Arch Microbiol 166:283–92. https://doi.org/10.1007/s002030050385.

Kruckeberg AL, Bisson LF (1990) The *HXT2* gene of *Saccharomyces cerevisiae* is required for high-affinity glucose transport. Molecular and Cellular Biology. 10(11):5903-13.

Kurtzman CP and Robnett CJ. 2003. Phylogenetic relationships among yeasts of the '*Saccharomyces* complex' determined from multigene sequence analyses. FEMS Yeast Res 3, 417-432.

Legras JL, Erny C, Charpentier C (2014) Population Structure and Comparative Genome Hybridization of European Flor Yeast Reveal a Unique Group of *Saccharomyces cerevisiae* Strains with Few Gene Duplications in Their Genome. PLoS ONE 9(10):e108089. https://doi.org/10.1371/journal.pone.0108089.

Legras JL, Galeote V, Bigey F, Camarasa C, Marsit S, Nidelet T, Sanchez I, Couloux A, Guy J, Franco-Duarte R, Marcet-Houben M, Gabaldon T, Schuller D, Sampaio JP, Dequin S (2018) Adaptation of *S. cerevisiae* to Fermented Food Environments Reveals Remarkable Genome Plasticity and the Footprints of Domestication. Mol Biol Evol 35(7): 1712–1727. https://doi.org/10.1093/molbev/msy066.

Legras J, Merdinoglu D, Cornuet J, Karts F (2007) Bread, beer and wine: *Saccharomyces cerevisiae* diversity reflects human history. Molecular Ecology, 16: 2091-2102. http://doi.org/10.1111/j.1365-294X.2007.03266.x.

Legras JL, Karst F (2003) Optimisation of interdelta analysis for *Saccharomyces cerevisiae* strain characterisation. FEMS Microbiol Lett 221(2):249–255. https://doi.org/10.1016/S0378-1097(03)00205-2.

Liang H, Gaber RF (1996) A novel signal transduction pathway in *Saccharomyces cerevisiae* defined by Snf3-regulated expression of *HXT6*. Mol Biol Cell 7(12):1953–66. https://doi.org/10.1091/mbc.7.12.1953.

Liang HY, Chen JY, Reeves M, Han BZ (2013) Aromatic and sensorial profiles of young Cabernet Sauvignon wines fermented by different Chinese autochthonous *Saccharomyces cerevisiae* strains. Food Res Int 51(2):855-865. http://doi.org/10.1016/j.foodres.2013.01.056.

Lilly M, Lambrechts MG, Pretorius IS (2000) Effect of Increased Yeast Alcohol Acetyltransferase Activity on Flavor Profiles of Wine and Distillates. Appl Environ Microbiol 66(2) :744-53. http://doi.org/10.1128/AEM.66.2.744-753.2000.

Liti G, Carter DM, Moses AM, Warringer J, Parts L, James SA, Davey RP, Roberts IN, Burt A, Koufopanou V, Tsai IJ, Bergman CM, Bensasson D, O'Kelly MJT, van Oudenaarden A, Barton DBH, Bailes E, Nguyen AN, Jones M, Quail MA, Goodhead I, Sims S, Smith F, Blomberg A, Durbin R (2009) Population genomics of domestic and wild yeasts. Nature 458:337–41. https://doi.org/10.1038/nature07743.

Liu Shao-Quan, Pilone Gordon J (2000) An overview of formation and roles of acetaldehyde in winemaking with emphasis on microbiological implications. International Journal of Food Science and Technology 35:49–61. https://doi.org/10.1046/j.1365-2621.2000.00341.x.

Lopes FJP, Vanario-Alonso CE, Spirov AV, Bisch PM, Vieira FMC (2006) Characterizing the mechanism of Bicoid gradient reading by early regulatory elements of the gap gene hunchback: positioning, sharpening and robustness. A Dros Res Conf 47:410A.

Lopez V, Querol A, Ramon D, Fernandez-Espinar MT (2001) A simplified procedure to analyse mitochondrial DNA from industrial yeasts. Int J Food Microbiol 68:75-81. http://doi.org/10.1016/S0168-1605(01)00483-4.

Luyten K, Riou C, Blondin B (2002) The hexose transporters of *Saccharomyces cerevisiae* play different roles during enological fermentation. Yeast 19(8):713-26. https://doi.org/10.1002/yea.869.

MacIsaac KD, Wang T, Gordon DB, Gifford DK, Stormo GD, Fraenkel E (2006) An improved map of conserved regulatory sites for *Saccharomyces cerevisiae*. BMC Bioinformatics. 2006;7:113. http://doi.org/10.1186/1471-2105-7-113.

Maier A, Völker B, Boles E, Fuhrmann GF (2002) Characterisation of glucose transport in *Saccharomyces cerevisiae* with plasma membrane vesicles (countertransport) and intact cells (initial uptake) with single Hxt1, Hxt2, Hxt3, Hxt4, Hxt6, Hxt7 or Gal2 transporters. FEMS Yeast Research 2(4):539–50. https://doi.org/10.1111/j.1567-1364.2002.tb00121.x.

Martini A, Ciani M, Scorzetti G (1996) Direct enumeration and isolation of wine yeasts from grapes surfaces. Am J Enol Vitic 47:435–440. http://doi.org/10.1101/gr.185538.114.

Martiniuk JT, Pacheco B, Russell G, Tong S, Backstrom I, Measday V (2016) Impact of Commercial Strain Use on *Saccharomyces cerevisiae* Population Structure and Dynamics in Pinot Noir Vineyards and Spontaneous Fermentations of a Canadian Winery. PLoS ONE 11(8):e0160259. http://doi.org/10.1371/journal.,pone.0160259.

Martí-Raga M, Sancho M, Guillamón JM, Mas A, Beltran G (2015) The effect of nitrogen addition on the fermentative performance during sparkling wine production. Food Res Int 67:126-135. https://doi.org/10.1016/j.foodres.2014.10.033.

Mas A, Guillamon JM, Torija MJ, Beltran G, Cerezo AB, Troncoso AM, Garcia-Parrilla MC (2014) Bioactive compounds derived from the yeast metabolism of aromatic amino acids during alcoholic fermentation. BioMed research international .

Mello LMR (2015) *Vitivinicultura brasileira: panorama 2014. Comunicado Tecnico*. Brasília: Embrapa Uva e Vinho, 1-6. ISSN: 1808-6802. http://www.infoteca.cnptia.embrapa.br/infoteca/handle/doc/1025934.

Mendes-Ferreira A, Barbosa V, Falco C, Leão C, Mendes-Faia A (2009) The production of hydrogen sulphide and other aroma compounds by wine strains of *Saccharomyces cerevisiae* in synthetic media with different nitrogen concentrations. J Ind Microbiol Biotechnol 36:571-583. http://doi.org/10.1007/s10295-009-0527-x.

Mestres M, Busto O, Guasch J (2000) Analysis of organic sulfur compounds in wine aroma. Journal of Chromatography A 881:569–81.

Mirlean N, Baisch P, Medeanic S (2009) Copper Bioavailability and Fractionation in Copper-Contaminated Sandy Soils in the Wet Subtropics (Southern Brazil). Bull Environ Contam Toxicol 82(3):373-377. http://doi.org/10.1007/s00128-008-9620-5.

Mirlean N, Roisenberg A, Chies JO (2007) Metal contamination of vineyard soils in wet subtropics (southern Brazil). Environ Pollut 149(1):10-17. http://doi:10.1016/j.envpol.2006.12.024.

Mosley AL, Lakshmanan J, Aryal BK, Özcan S (2003) Glucose-mediated Phosphorylation Converts the Transcription Factor Rgt1 from a Repressor to an Activator. The Journal of Biological Chemistry 278:10322-7. http://doi.org/10.1074/jbc.M212802200.

Mortimer R, Polsinelli M (1999) On the origins of wine yeast. Res Microbiol 150:199−204. https://doi.org/10.1016/S0923-2508(99)80036-9.

Mortimer RK, Romano P, Suzzi G, Polsinelli M (2000) Genome renewal: A new phenomenon revealed from a genetic study of 43 strains of *Saccharomyces cerevisiae* derived from natural fermentation of grape musts. Yeast 10(12):1543-52. https://doi.org/10.1002/yea.320101203.

Mueller O, Lightfoot S, Schroeder A (2004) RNA integrity number (RIN)–standardization of RNA quality control. Agilent application note, publication 1-8.

Nadai C, Campanaro S, Giacomini A, Corich V (2015) Selection and validation of reference genes for quantitative real-time PCR studies during *Saccharomyces cerevisiae* alcoholic fermentation in the presence of sulfite. International Journal of Food Microbiology 215:49-56. http://dx.doi.org/10.1016/j.ijfoodmicro.2015.08.012.

Nadai C, Treu L, Campanaro S, Giacomini A, Corich V (2016) Different mechanisms of resistance modulate sulfite tolerance in wine yeasts. Appl Microbiol Biotechnol 100(2):797-813. http://doi.org/10.1007/s00253-015-7169-x.

Nadai C, Bovo B, Giacomini A, Corich V (2018) New rapid PCR protocol based on high-resolution melting analysis to identify *Saccharomyces cerevisiae* and other species within its genus. J Appl Microbiol 124(5):1232-1242. http://doi:10.1111/jam.13709.

Nadal D, Colomer B, Piña B (1996) Molecular polymorphism distribution in phenotypically distinct populations of wine yeast strains. Appl Environ Microbiol 62(6):1944-50.

Nardi T, Carlot M, De Bortoli E, Corich V, Giacomini A (2006) A rapid method for differentiating *Saccharomyces sensu strictu* strains from other yeast species in an

enological environment. FEMS Microbiol Lett 264(2):168-173. http://doi.org/10.1111/j.1574-6968.2006.00450.x.

Nardi T, Corich V, Giacomini A, Blondin B (2010) A sulphite-inducible form of the sulphite efflux gene *SSU1* in a *Saccharomyces cerevisiae* wine yeast. Microbiology 156:1686-96. http://doi.org/10.1099/mic.0.036723-0.

Naumov G, Masneuf IS, Naumova E, Aigle M, Dubourdieu D (2000) Association of *Saccharomyces bayanus* var. uvarum with some french wines: genetic analysis of yeast populations. Res Microbiol 151(8):683-691. http://doi.org/10.1016/S0923-2508(00)90131-1.

Naumov GI, Naumova ES, Antunovics Z, Sipiczki M (2002) *Saccharomyces bayanus* var. uvarum in tokaj wine-making of slovakia and hungary. Appl Microbiol Biotechnol 59:727-730. http://doi.org/10.1007/s00253-002-1077-6.

Naumov GI, Nikonenko TA (1987) Genomic divergence in cultivated and wild strains of the yeast. *Saccharomyces sensu stricto*: four twin species. Dokl Biol Sci 294:330-332.

Nehlin JO, Carlberg M, Ronne H (1989) Yeast galactose permease is related to yeast and mammalian glucose transporters. Gene 85(2):313–9. https://doi.org/10.1016/0378-1119(89)90423-X.

Özcan S, Dover J, Johnston M (1998) Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. EMBO J, 17:2566-2573.

Özcan S, Dover J, Rosenwald AG, Wolfl S, Johnston M (1996) Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proc Natl Acad Sci USA, 93:12428-12432.

Özcan S, Johnston M (1999) Function and Regulation of Yeast Hexose Transporters. Microbiology and Molecular Biology Reviews. 63(3):554-69.

P283 Assembly GCA_000568295.1 (2014) European Nucleotide Archive (ENA). Retrieved 10 Aug. 2018, from https://www.ebi.ac.uk/ena/data/view/GCA_000568295.1.

Park Hoon, Bakalinsky Alan T (2000) *SSU1* mediates sulphite efflux in *Saccharomyces cerevisiae*. Yeast 16(10):881-8. https://doi.org/10.1002/1097-0061(200007)16:10<881::AID-YEA576>3.0.CO;2-3.

Pérez-Coello MS, Briones Pérez AI, Ubeda Iranzo JF, Martin Alvarez PJ (1999) Characteristics of wines fermented with different *Saccharomyces cerevisiae* strains isolated from the La Mancha region. Food Microbiol 16(6):563-573. https://doi.org/10.1006/fmic.1999.0272.

Pérez-Ortín José E., Querol Amparo, Puig Sergi, Barrio Eladio (2002) Molecular characterization of a chromosomal rearrangement involved in the adaptive evolution of yeast strains. Genome Res 12(10):1533-9. http://doi.org/10.1101/gr.436602.

Pickering G, Spink M, Kotseridis Y, Inglis D, Brindle I, Sears M, Beh A (2008) Yeast strain affects 3-isopropyl-2-methoxypyrazine concentration and sensory profile in Cabernet Sauvignon wine. Australian Journal of Grape and Wine Research 14:230-7. http://doi.org/10.1111/j.1755-0238.2008.00026.x.

Pilkington Bridget J, Rose Anthony H (1988) Reactions of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* to Sulphite. Journal of General Microbiology 134:2823-30. http://doi.org/10.1099/00221287-134-10-2823.

Piper PW (1995) The heat shock and ethanol stress responses of yeast exhibit extensive similarity and functional overlap. FEMS Microbiology Letters 134(2-3):121–7. https://doi.org/10.1111/j.1574-6968.1995.tb07925.x.

Pires E, Brányik T (2015) Main Glucose Repression Pathway - Carbohydrate Transport and Metabolism. in Biochemistry of Beer Fermentation. Springer International Publishing AG Switzerland 17-20. http://doi.org/10.1007/978-3-319-15189-2.

Pretorius IS (2000) Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast 16:675-729. https://doi.org/10.1002/1097-0061(20000615)16:8<675::AID-YEA585>3.0.CO;2-B.

Pretorius IS, van der Westhuizen TJ, Augustyn OPH (1999) Yeast Biodiversity in Vineyards and Wineries and Its Importance to the South African Wine Industry. A Review. South African Journal of Enology and Viticulture 20(2):61-70. ISSN 2224-7904. http://dx.doi.org/10.21548/20-2-2234.

Querol A, Barrio E, Ramón D (1992) A comparative study of different methods of yeast strain characterization. Syst Appl Microbiol 15:439-446. http://doi.org/10.1016/S0723-2020(11)80219-5.

Querol A, Belloch C, Fernandez-Espinar MT and Barrio E (2003) Molecular evolution in yeast of biotechnological interest. Int Microbiol 6:201-5. https://doi.org/10.1007/s10123-003-0134-z.

Querol A, Ramon D (1996) The application of molecular techniques in wine microbiology. Trends Food Sci Technol 7(3):73-78. http://doi.org/10.1016/0924-2244(96)81300-8.

Radler, F (1993) Yeast: Metabolism of organic acids. In Wine Microbiology and Biotechnology. Ed. G.H. Fleet (Harwood Academic Publishers: Chur, Switzerland) 165–182.

Rainieri S, Pretorius IS (2000) Selection and improvement of wine yeasts. Annals of Microbiology 50(1):15-31.

Ramírez-Castrillón M, Camargo Mendes SD, Valente P (2017) South Brazilian wines: culturable yeasts associated to bottled wines produced in Rio Grande do Sul and Santa Catarina. World J Microbiol Biotechnol 33:77. http://doi.org/10.1007/s11274-017-2244-3.

Redžepović S, Orlić S, Sikora S, Majdak A, Pretorius I (2002) Identification and characterization of *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* strains isolated from Croatian vineyards. Letters in Applied Microbiology 35: 305-310. http://doi:10.1046/j.1472-765X.2002.01181.x.

Registro de Indicação Geográfica N°IG201008 (INPI) – Lista das Denominações de Origem concedidas. http://www.inpi.gov.br/menu-servicos/indicacao-geografica/pedidos-de-indicacao-geografica-no-brasil (accessed August, 10th 2018).

Regódon JA, Peréz F, Valdés ME, De Miguel C, Ramírez M (1997) A simple and effective procedure for selection of wine yeast strains. Food Microbiol 14(3):247-54. https://doi.org/10.1006/fmic.1996.0091.

Reifenberger E, E Boles, Ciriacy M (1997) Kinetic characterization of individual hexose transporters of *Saccharomyces cerevisiae* and their relation to the triggering mechanisms of glucose repression. Eur J Biochem 245(2):324–33. https://doi.org/10.1111/j.1432-1033.1997.00324.x.

Reifenberger E, Freidel K, Ciriacy M (1995) Identification of novel *HXT* genes in *Saccharomyces cerevisiae* reveals the impact of individual hexose transporters on qlycolytic flux. Molecular Biology 16(1):157-67. https://doi.org/10.1111/j.1365-2958.1995.tb02400.x.

Rementeria A, Rodriguez JA, Cadaval A, Amenabar R, Muguruza JR, Hernando FL, Sevilla MJ (2003) Yeast associated with spontaneous fermentations of white wines from the "Txakoli de Bizkaia" region (Basque Country. North Spain). Int J Food Microbiol 86(1-2):201-207. http://doi.org/10.1016/S0168-1605(03)00289-7.

Ribéreau-Gayon P, Dubourdieu D, Doneche B, Lonvaud A (2006) Handbook of Enology: The Microbiology of Wine and Vinifications, Volume 1, 2nd Edition. John Wiley and Sons, New York, US. http://doi.org/10.1002/0470010363.ch1.

Rodicio R, Heinisch JJ (2017) Chapter 8: Carbohydrate Metabolism in Wine Yeasts. 189-213. In H. König et al. (eds.), Biology of Microorganisms on Grapes, in Must and in Wine. Springer International Publishing AG. http://doi.org/10.1007/978-3-319-60021-5_8.

Rodríguez-Navarro S, Fischer T, Luo MJ, Antúnez O, Brettschneider S, Lechner J, Pérez-Ortín JE, Reed R, Hurt E (2004) Sus1, a Functional Component of the SAGA Histone Acetylase Complex and the Nuclear Pore-Associated mRNA Export Machinery. 116(1):75-86. https://doi.org/10.1016/S0092-8674(03)01025-0.

Roman H (1981) Development of yeast as an experimental organism, in The Molecular and Cellular Biology of the Yeast *Saccharomyces* (eds Strathern JN, Jones EW, Broach JR), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1–9.

Romano P, Capece A, Serafino V, Romaniello R, Poeta C (2008) Biodiversity of wild strains of *Saccharomyces cerevisiae* as tool to complement and optimize wine quality. World J Microbiol Biotechnol 24:1797-1802. http://doi.org/10.1007/s11274-008-9672-z.

Romano P, Fiore C, Paraggio M, Caruso M, Capece A (2003) Function of yeast species and strains in wine flavour. International journal of food microbiology 86(1):169-180. https://doi.org/10.1016/S0168-1605(03)00290-3.

Ruyters S, Salaets P, Oorts K, Smolders E (2013) Copper toxicity in soils under established vineyards in Europe: A survey. Sci Total Environ 443:470-477. http://doi.org/10.1016/j.scitotenv.2012.11.001.

Sabate J, Cano J, Esteve-Zarzoso B, Guillamón JM (2002) Isolation and identification of yeasts associated with vineyard and winery by RFLP analysis of ribosomal genes and mitochondrial DNA. Microbiol Res 157:267-274. http://doi.org/10.1078/0944-5013-00163.

Salata F, Golasi I, Proietti R, de Lieto Vollaro A (2017) Implications of climate and outdoor thermal comfort on tourism: the case of Italy. Int J Biometeorol 61:2229–44. http://doi.org/10.1007/s00484-017-1430-1.

Sales K, Brandt W, Rumbak E, Lindsey G (2000) The LEA-like protein HSP 12 in Saccharomyces cerevisiae has a plasma membrane location and protects membranes against desiccation and ethanol-induced stress. Biochimica et Biophysica Acta (BBA) – Biomembranes 1463(2):267-78. https://doi.org/10.1016/S0005-2736(99)00215-1.

Schacherer J, Shapiro JA, Ruderfer DM, Kruglyak L (2009) Comprehensive polymorphism survey elucidates population structure of *Saccharomyces cerevisiae*. Nature 458:342–5. https://doi.org/10.1038/nature07670.

Schuck MR, Moreira FM, Guerra MP, Voltolini JA, Grando MS, Silva ALD (2009) Molecular characterization of grapevine from Santa Catarina. Brazil. using microsatellite markers. Braz J Agric Res 44(5):487-495. http://doi.org/10.1590/S0100-204X2009000500008.

Schuller D, Alves H, Dequin S, Casal M (2005) Ecological survey of *Saccharomyces cerevisiae* strains from vineyards in the Vinho Verde Region of Portugal. FEMS Microbiol Ecol 51(2):167–177. https://doi.org/10.1016/j.femsec.2004.08.003.

Schuller D, Cardoso F, Sousa S, Gomes P, Gomes AC (2012) Genetic Diversity and Population Structure of *Saccharomyces cerevisiae* Strains Isolated from Different Grape Varieties and Winemaking Regions. PLoS ONE 7(2):e32507. http://doi.org/10.1371/journal.,pone.0032507.

Schuller D, Valero E, Dequin S, Casal M (2004) Survey of molecular methods for the typing of wine yeast strains. FEMS Microbiol Lett 231:19–26.

Shinohara T, Furuya H, Yanagida F, Takeo M (2003) Ecological Distribution and Phenotypic Diversity of *Saccharomyces cerevisiae* Strains from the Wine-Producing Area in Yamanashi, Japan. Microbiol Cult Collect 19(2):69-80. http://agris.fao.org/agris-search/search.do?recordID=JP2004005726.

Siderius MH, Mager WH (1997) General stress response: In search of a common denominator. In S. Hohmann & WH Mager (Eds.) Yeast stress and responses. 213-30. Austin, Texas: Mol. Biol. Intelligence Units, RG Landes Company.Hood A (1983) Inhibition of growth of wine lactic-acid bacteria by acetaldehyde-bound sulphur dioxide. Aust NZ Grapegrow Winemak 232:34-43.

Siebert TE, Solomon MR, Pollnitz AP, Jeffery DW (2010) Selective Determination of Volatile Sulfur Compounds in Wine by Gas Chromatography with Sulfur Chemiluminescence Detection. J Agric Food Chem58:9454–62. http://doi.org/10.1021/jf102008r.

Schütz M, Kunkee RE (1977) Formation of hydrogen sulfide from elemental sulfur during fermentation by wine yeast. American Journal of Enology and Viticulture. 1977 Jan 1;28(3):137-44.

Souciet JL (2011) Ten years of the Genolevures Consortium: A brief history. C R Biol 334(8-9):580-584.

Stanley D, Bandara A, Fraser S, Chambers PJ, Stanley GA (2010) The ethanol stress response and ethanol tolerance of *Saccharomyces cerevisiae*. Journal of Applied Microbiology 109:13-24. http://doi.org/10.1111/j.1365-2672.2009.04657.x.

Stratford M, Rose AH (1985) Hydrogen Sulphhide Production from Sulphite by Saccharomyces cerevisiae. Microbiology. 131(6):1417-24.

Stratford M, Morgans P, Rose AH (1987) Sulphur Dioxide Resistance in *Saccharomyces cerevisiae* and *Saccharomycodes ludwigii*. Journal of' General Microbiology 133:2173-9.

Strope PK, Skelly DA, Kozmin SG, Mahadevan G, Stone EA, Magwene PM, Dietrich FS, McCusker JH (2015) The 100-genomes strains, an *S. cerevisiae* resource that illuminates its natural phenotypic and genotypic variation and emergence as an opportunistic pathogen. Genome Res 25:762–774. http://www.genome.org/cgi/doi/10.1101/gr.185538.114.

Symoneaux R, Galmarini MV, Mehinagic E (2012) Comment analysis of consumer's likes and dislikes as an alternative tool to preference mapping. A case study on apples. Food Quality and Preference 24(1):59-66. https://doi.org/10.1016/j.foodqual.2011.08.013.

Taillandier P, Portugal FR, Fuster A, Strehaiano P (2007) Effect of ammonium concentration on alcoholic fermentation kinetics by wine yeasts for high sugar content. Food microbiology 24(1):95-100.

Teste MA, Duquenne M, François JM, Parrou JL (2009) Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*. BMC Molecular Biology 10:99. https://doi.org/10.1186/1471-2199-10-99.

Thomas D, Surdin-Kerjan Y (1987) SAM1, the structural gene for one of the S-adenosylmethionine synthetases in *Saccharomyces cerevisiae*. Sequence and expression. The Journal of Biological Chemistry 262:16704-9.

Thomas CS, Boulton RB, Silacci MW, Gubler WD (1993) The effect of elemental sulfur, yeast strain, and fermentation medium on hydrogen sulfide production during fermentation. American Journal of Enology and Viticulture. 1993 Jan 1;44(2):211-6.

Thomas D, Surdin-Kerjan Y (1997) Metabolism of Sulfur Amino Acids in *Saccharomyces cerevisiae*. Microbiology and Molecular Biology Reviews 61(4):503-32.

Tini V, Zambonelli C, Benevelli M, Castellari L (1995) Sparkling Wines: The autolysogenic *Saccharomyces cerevisiae* strains for the sparkling wines production. INDUSTRIE DELLE BEVANDE. 1995;24:113.

Torrens J, Pilar U, Riu-Aumatell M, Vichi S, López-Tamames E, Buxaderas S (2008) Different commercial yeast strains affecting the volatile and sensory profile of cava base wine. International Journal of Food Microbiology 124(1):48-57. https://doi.org/10.1016/j.ijfoodmicro.2008.02.023.

Torriani S, Zapparoli G, Suzzi G (1999) Genetic and phenotypic diversity of *Saccharomyces sensu stricto* strains isolated from Amarone wine. Antonie van Leeuwenhoek 75:207. https://doi.org/10.1023/A:1001773916407.

Townsend Jeffrey P, Cavalieri D, Hartl DL (2003) Population Genetic Variation in Genome-Wide Gene Expression. Mol Biol Evol 20(6):955-963. http://doi.org/10.1093/molbev/msg106.

Treu L, Toniolo C, Nadai C, Sardu A, Giacomini A, Corich V, Campanaro S (2014) The impact of genomic variability on gene expression in environmental

Saccharomyces cerevisiae strains. Environ Microbiol 16(5):1378-1397. http://doi.org/10.1111/1462-2920.12327.

Valero E, Schuller D, Cambon B, Casal M, Dequin S (2007) Biodiversity of *Saccharomyces* yeast strains from grape berries of wine-producing areas using starter commercial yeasts. FEMS Yeast Res 7:317-329. http://doi.org/10.1111/j.1567-1364.2006.00161.x.

Valero E, Shuller D, Cambon B, Casal M, Dequin S (2005) Dissemination and survival of commercial wine yeast in the vineyard: A large-scale, three-year study. FEMS Yeast Res 5:959-969. http://doi.org/10.1016/j.femsyr.2005.04.007.

Van Bakel H, Strengman E, Wijmenga C, Holstege FCP (2005) Gene expression profiling and phenotype analyses of *S. cerevisiae* in response to changing copper reveals six genes with new roles in copper and iron metabolism. Physiol Genomics 22:356 -367. http://doi.org/10.1152/physiolgenomics.00055.2005.

van der Westhuizen T, Augustyn O, Pretorius I (2017) Geographical Distribution of Indigenous *Saccharomyces cerevisiae* Strains Isolated from Vineyards in the Coastal Regions of the Western Cape in South Africa. S Afr J Enol Vitic 21(1):3-9. http://doi:http://dx.doi.org/10.21548/21-1-2179.

Van Leeuwen C, Friant P, Choné X, Tregoat O, Koundouras S, Dubourdieu D (2004) Influence of Climate, Soil, and Cultivar on *Terroir*. Am J Enol Vitic 55:207-217.

Varela C, Pizarro F, Agosin E (2004) Biomass content governs fermentation rate in nitrogen-deficient wine musts. Applied and environmental microbiology 70(6):3392-400.

Vendramini C, Beltran G, Nadai C, Giacomini A, Mas A, Corich V (2017) The role of nitrogen uptake on the competition ability of three vineyard Saccharomyces

cerevisiae strains. Int J Food Microbiol 258:2-11. https://doi.org/10.1016/j.ijfoodmicro.2017.07.006.

Versavaud A, Courcoux P, Roulland C, Dulau L, Hallet J (1995) Genetic Diversity and Geographical Distribution of Wild *Saccharomyces cerevisiae* Strains from the Wine-Producing Area of Charentes, France. Appl Environ Microbiol 61(10):3521-3529.

Verwaal R, Paalman JWG, Hogenkamp A, Verkleij AJ, Verrips CT, Boonstra J (2002) *HXT5* expression is determined by growth rates in *Saccharomyces cerevisiae*. Yeast 19(12):1029-38. https://doi.org/10.1002/yea.895.

Vezinhet F, Blondin B, Hallet JN (1990) Chromosomal DNA patterns and mitochondrial DNA polymorphism as tools for identification of enological strains of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **32(5)**:568-71. http://doi.org/10.1007/BF00173729.

Vezinhet F, Hallet JN, Valade M, Poulard A (1992) Ecological Survey of Wine Yeast Strains by Molecular Methods of Identification. Am J Enol Vitic 43:83-86.

Viel A, Legras JL, Nadai C, Carlot M, Lombardi A, Crespan M, Migliaro D, Giacomini A, Corich V (2017) The Geographic Distribution of *Saccharomyces cerevisiae* Isolates within three Italian Neighboring Winemaking Regions Reveals Strong Differences in Yeast Abundance, Genetic Diversity and Industrial Strain Dissemination. Front Microbiol 8:1595. http://doi.org/10.3389/fmicb.2017.01595.

Vilanova M, Ugliano M, Varela C, Siebert T, Pretorius IS, Henschke PA (2007) Assimilable nitrogen utilisation and production of volatile and non-volatile compounds in chemically defined medium by *Saccharomyces cerevisiae* wine yeasts. Applied microbiology and biotechnology. 77(1):145-57.

Vincenzini M, Romano P, Farris GA (2005) Microbiologia del vino. Edizioni Ambrosiana

Warringer J, Zörgö E, Cubillos FA, Zia A, Gjuvsland A, Simpson JT, Forsmark A, Durbin R, Omholt SW, Louis EJL, Liti G, Moses A, Blomberg A (2011) Trait Variation in Yeast Is Defined by Population History. PLoS Genet 7(6): e1002111. https://doi.org/10.1371/journal.pgen.1002111.

Westholm JO, Nordberg N, Muren E, Ameur A, Komorowski J, Ronne H (2008) Combinatorial control of gene expression by the three yeast repressors Mig1, Mig2and Mig3. BMC Genomics 9:601. https://doi.org/10.1186/1471-2164-9-601.

Winzeler EA, Castillo-Davis CI, Oshiro G, Liang D, Richards DR, Zhou Y, Hartl DL (2003) Genetic diversity in yeast assessed with whole-genome oligonucleotide arrays. Genetics 163:79–89.

Jian Wu, Nianshu Zhang, Andrew Hayes, Kalliope Panoutsopoulou, Stephen G. Oliver (2004) Global analysis of nutrient control of gene expression in Saccharomyces cerevisiae during growth and starvation. Proceedings of the National Academy of Sciences 101(9):3148-53. http://doi.org/10.1073/pnas.0308321100.

Wurz DA, Pereira de Bem B, Allebrandt R, Bonin B, Dalmolin LG, Canossa AT, Rufato L, Kretzschmar AA (2017) New wine-growing regions of Brazil and their importance in the evolution of Brazilian wine. BIO Web of Conferences 9, 01025 (2017) 40th World Congress of Vine and Wine http://doi.org/10.1051/bioconf/20170901025.

Yasokawa D, Murata S, Kitagawa E, Iwahashi Y, Nakagawa R, Hashido T, Iwahashi H (2008) Mechanisms of copper toxicity in *Saccharomyces cerevisiae* determined by microarray analysis. Environ toxicol 23(5):599-606. http://doi.org/10.1002/tox.20406.

Yoshimoto A, Sato R (1968) Studies on yeast sulfite reductase I. Purification and characterization. Biochimica et Biophysica Acta (BBA)-Bioenergetics 153(3):555-75.

Yuasa N, Nakagawa Y, Hayakawa M, Iimura Y (2004) Distribution of the sulfite resistance gene *SSU1-R* and the variation in its promoter region in wine yeasts. J Biosci Bioeng 98:394-7. https://doi.org/10.1016/S1389-1723(04)00303-2.

Zambonelli C (2003) Microbiologia e Biotecnologia dei Vini. Edagricole, Bologna

Zarei O, Dastmalchi S, Hamzeh-Mivehroud M (2016) A Simple and Rapid Protocol for Producing Yeast Extract from Saccharomyces cerevisiae Suitable for Preparing Bacterial Culture Media. Iranian Journal of Pharmaceutical Research 15(4):907-13.

Zeyl C (2000) Budding yeast as a model organism for population genetics. Yeast 16:773-784. 10.1002/1097-0061(20000615)16:8<773::AID-YEA599>3.0.CO;2-1.

Zhou H, Miller AW, Sosic Z, Buchholz B, Barron AE, Kotler L, Barry, L. Karger BL (2000) DNA Sequencing up to 1300 Bases in Two Hours by Capillary Electrophoresis with Mixed Replaceable Linear Polyacrylamide Solutions. Analytical Chemistry 72(5):1045-52. http://doi.org/10.1021/ac991117c.

Zimmer Adrien, Durand Cécile, Loira Nicolás, Durrens Pascal, Sherman David James, Marullo Philippe (2014) QTL Dissection of Lag Phase in Wine Fermentation Reveals a New Translocation Responsible for *Saccharomyces cerevisiae* Adaptation to Sulfite. PLOS ONE 9(1):e86298. https://doi.org/10.1371/journal.pone.0086298.